UNIVERSITAT DE BARCELONA

Nutrition and gut microbiota modulation as tools for regulating fat accumulation in aquaculture fish

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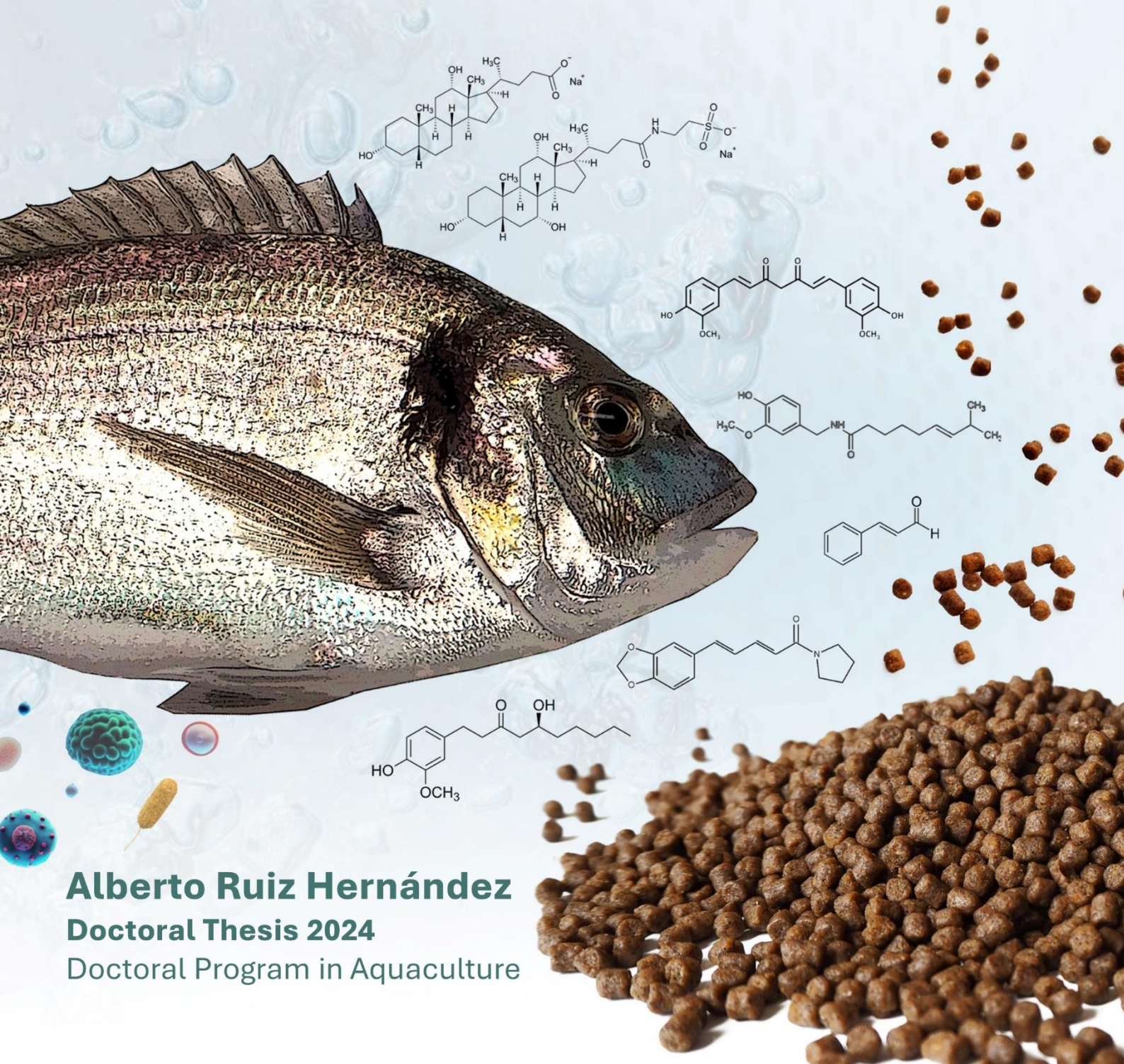
NUTRITION AND GUT MICROBIOTA MODULATION AS TOOLS FOR REGULATING FAT ACCUMULATION IN AQUACULTURE FISH



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NUTRITION AND GUT MICROBIOTA MODULATION AS TOOLS FOR REGULATING FAT ACCUMULATION IN AQUACULTURE FISH

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"The scientific man does not aim at an immediate result.
He does not expect that his advanced ideas will be readily taken up.
His work is like that of a planter – for the future.
His duty is to lay the foundation of those who are to come and point the way."

Nikola Tesla

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Abstract

The sustainability concerns, increasing cost, and insufficient availability of fish oil associated to aquaculture growth, have led to the search of sustainable alternatives in aquafeeds. However, fish oil replacement increases the levels of body fat deposition. An excessive fat accumulation in digestive tissues may cause deregulations in nutrient digestion and absorption, reduced feed efficiency and growth, as well as negatively affect the organoleptic qualities of the fillet. Hence, it is imperative to explore complementary strategies to balance fat accumulation when diminishing fish oil content in aquafeeds. This thesis proposes two strategies to reduce the levels of fat accumulation in fish: the supplementation of diets with additives with digestive and hypolipidemic properties, and the modulation of the gut microbial communities through an intestinal microbiota transplant (IMT). To test these strategies, gilthead seabream (*Sparus aurata*), the most important farmed species in the Mediterranean, was used as a biological model.

Under this context, the effect on fat accumulation, and fish health and condition, of three different additives was evaluated: i) a blend of bile salts containing sodium cholate, sodium deoxycholate, and sodium taurocholate at dietary inclusion levels of 0.06% and 0.12%; ii) a combination of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde (SPICY additive) at 0.1% and 0.15% in the diet; and iii) a combination of turmeric, capsicum, black pepper, and ginger oleoresins (SO additive) at 0.2% in the diet. Results revealed that the tested blend of bile salts at 0.06% in the diet and the SPICY additive at 0.1% and 0.15% promoted somatic growth. Additionally, the SPICY additive reduced the values of feed conversion ratio at both inclusion levels. Furthermore, the blend of bile salts at 0.12% increased lipid apparent digestibility, which was attributed to the higher activity of the bile salt-activate lipase and increased secretion of bile salts into the intestine. A higher bile salt-activate lipase activity was also observed for the SPICY and SO additives at all the inclusion levels tested. Dietary supplementation with the tested additives demonstrated a promising reduction in the levels of fat accumulation in the visceral cavity and digestive organs (liver and intestine) without compromising the proximate composition of the fillet. Moreover, the three additives modulated the local immune response in the intestine, as well as the gut bacterial composition in gilthead seabream without affecting the diversity and structure.

Regarding the IMT, two marine carnivorous fish species that thrive in different environmental conditions were selected, Atlantic salmon (*Salmo salar*) as microbiota's donor, and gilthead seabream as recipient. This approach was designed to develop an IMT protocol and to study the dynamics of the gut bacterial communities after the IMT and under the influence of different dietary treatments. The purpose was further applying this protocol in the gilthead seabreams submitted to the above-mentioned nutritional assays to test the reduction in fat accumulation levels. However, this assay was not conducted since the microbial modulation induced by the tested additives was not robust enough for expecting it to have a determinant role in fat accumulation. Nonetheless, the results of the inter-specific IMT provided insight to

the paramount role of the diet in shaping the gut microbial communities after an IMT, modulating richness, diversity, structure, and composition over time. Unfortunately, many experimental factors, such as the high number of individuals typically managed in aquaculture, suggest that implementation of IMTs as a reliable strategy in the sector are yet remote.

Results from the current thesis indicated that feed additives are a safe strategy to improve the health and condition in farmed fish as well as modulate body fat accumulation without affecting the nutritional quality of the fillet.

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INTRODUCTION

Introduction

1. Overview of the state and importance of aquaculture

In the last century, the world has witnessed its fastest population increase ever, growing from two billion people in 1930 to eight billion people in 2022 (Taagepera and Nemčok, 2023). Despite the slowing growth rate in the last four decades, the world population is expected to reach 9.7 billion people by 2050, which would imply an increase in food production of at least 70% to satisfy the public demand (Galanakis, 2024). Under this framework, a new paradigm has emerged: fulfilling the growing demand for food in a sustainable way by decreasing the environmental footprint of food production systems. In addition, there was a need to maintain and advance socio-economics objectives, including employment opportunities for local communities, support of local cultures, equitable profit distribution, quality of life, attention to animal welfare, and product quality. Under these concerns, aquaculture has become the fastest growing food production sector (Thomas et al., 2021; FAO, 2022).

According to the last statistics from the Food and Agriculture Organization of the United Nations (FAO), in the period of 1990–2020, total world aquaculture expanded by 609% in annual output with an average growth rate of 6.7% per year (FAO, 2022). Nowadays, aquaculture represents nearly half of the overall production of aquatic animals, reaching an estimated value of 88 million tonnes out of 178 million tonnes of total production of aquatic animals in 2020. In that year, the total first sale value of the global aquaculture production was estimated at USD 265 billion (FAO, 2022). According to FAO and APROMAR estimates, in the year 2021, aquaculture production increased to 1,142.5 thousand tonnes in the European Union (EU), and Spain was the country with the highest aquaculture production from the EU, farming 271,060 tonnes of aquatic organisms (23% of total aquaculture production) (APROMAR, 2023).

Coupled to aquaculture growth, consumption of aquatic animals has significantly increased, reaching an amount of 20.5 kg per capita consumption in 2019, with respect to 9.0 kg in 1961. In 2020, 89% of total produced aquatic animals (more than 157 million tonnes) were intended for human consumption, whereas the remaining 20 million tonnes were mainly used to produce fish meal and fish oil (FAO, 2022). The increasing demand and consumption of aquatic foods are in line with the well-documented benefits of fish in human nutrition and health (Thilsted et al., 2016). In this sense, fish are a very valuable source of bioavailable animal protein, essential fatty acids, minerals and vitamins, which are critical for multiple functions, and necessary to prevent malnutrition and diseases (Fiorella et al., 2021). Although the pace of aquatic food consumption is expected to slow down for the next years due to the deceleration of population growth, rising aquatic food prices, and saturated demand in some high-income countries, it is projected that by 2030 the amount of aquatic food intended for

human consumption will increase by 24 million tonnes with respect to 2020, reaching 182 million tonnes (FAO, 2022). Under these premises, the development of aquaculture must be prioritized during the following years as one of the most sustainable food-producing systems, while facing several upcoming challenges, such as disease outbreaks, climatic change consequences, or the search for sustainable intensive aquaculture production systems, among others (Boyd et al., 2020; FAO, 2022).

2. The challenge of fish oil reduction in aquafeeds

In 2030, the total production of fish oil is expected to increase by 13% with respect to 2020 (FAO, 2022), mainly as a result of aquaculture growth and to the growing popularity of fish oil as a nutraceutical supplement for human consumption (Shepherd and Bachis, 2014). Consequently, the insufficient production and availability of fish oil, its subsequent increasing costs, and the sustainability concerns related to its origin, have brought to the forefront the search for other functional and reliable lipid sources that do not compromise the fish and consumer requirements of omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs) and the sustainability of this sector (Naylor et al., 2021). In this sense, n-3 LC-PUFAs, especially eicosapentaenoic acid (EPA; C20:5 n-3) and docosahexaenoic acid (DHA; C22:6 n-3), are necessary to ensure an optimal growth, development, health, and reproduction of fish (Ibeas et al., 1994; Jaya-Ram et al., 2008; Peng et al., 2014) and to maintain the nutritional quality of the fish fillet to human consumers (Tocher, 2009). In addition, EPA and DHA also have many health benefits for the human consumer, reducing inflammatory, neural, and heart diseases (Horrocks and Yeo, 1999; Campoy et al., 2012), as well as lipid metabolism-related disorders (Flachs et al., 2009; Lorente-Cebrián et al., 2013).

Plant-based oils have undoubtedly been the most widely used alternative to fish oils, with palm, soybean, canola/rapeseed, and sunflower oils being the ones most used in aquafeeds during the last decades (Turchini et al., 2009; Gunstone, 2011; Mozanzadeh et al., 2021). However, it is important to notice that the fatty acid profile varies to a large extent among different plant-based oils. In this sense, palm oil is rich in saturated fatty acids (SFAs), with concentrations of almost 50% of total fatty acids with respect to a range of 20-30% SFAs in fish oil (Turchini et al., 2009; Mozanzadeh et al., 2021). Otherwise, canola/rapeseed oil is rich in monounsaturated fatty acids (MUFAs), which account for 62% of total fatty acids, while the fish oil levels of MUFAs vary depending on the species of origin, from 25% for anchovy oil and menhaden oil to 62% for capelin oil (Turchini and Mailer, 2011; Mozanzadeh et al., 2021). On the other hand, soybean and sunflower oils are rich in omega 6 (n-6) PUFAs, with concentrations of 51% and 66% respectively, compared to a range of 1.3-5% in fish oils (Brown and Hart, 2011; Mozanzadeh et al., 2021). The content of n-3 PUFAs varies between plant-based oils (7% for soybean oil, 12% for canola/rapeseed oil, and very close to 0% for sunflower and palm oils) but it is typically lower than the n-3 PUFA levels of fish oil (12-31%). This coupled to the high content of n-6 PUFAs (51% for soybean oil, 20% for canola/rapeseed oil, 66% for sunflower oil, and 9% for palm oil), leads to lower values for the n-3/n-6 (n-3 PUFAs/n-6 PUFAs) ratio (0-0.6% for the mentioned plant-based oils *vs*. 5-24% for fish oil; Mozanzadeh et al., 2021). The n-3/n-6 ratio serves as an indicator of health and immune status in both fish and humans (Oliva-Teles, 2012; Carr, 2023). In addition, a pronounced decrease of the n-3/n-6 ratio in aquafeeds has been associated to a pro-inflammatory response in the fish (Holen et al., 2018).

Other plant-based oils, which are produced at lower levels and have higher production costs, are richer in n-3 PUFAs, such as linseed oil. However, the problem of plant-based oils, including linseed oil, is that their content of PUFAs is mainly composed of C18 (and some shorter main chain) PUFAs, while the content of LC-PUFAs (including EPA and DHA) is very

poor (Turchini et al., 2009). In the particular case of linseed oil, it contains high levels of alpha-linolenic acid (C18:3 n-3), which is a well-known precursor of EPA and DHA in some groups of higher vertebrates (Kartikasari et al., 2012; Stark et al., 2016). Nonetheless, in fish, the transcription rate and the activity of the enzymes involved in the metabolism of alpha-linolenic acid into EPA and DHA are apparently not sufficient to compensate for the n-3 LC-PUFA deficiencies in the fish fatty acid profile (Turchini et al., 2009).

Despite the differences in the fatty acid profile of plant-based oils with respect to the above-mentioned standard values for fish oil, the partial, or even total, substitution of fish oil by plant-based oils does not generally compromise the growth and feed performance of the fish (Ng et al., 2004; Fonseca-Madrigal et al., 2005; Mourente and Bell, 2006; Kenari et al., 2011; Reis et al., 2014). Nonetheless, the composition of the diet is largely reflected in the fish, resulting in imbalances in the fatty acid profile of the whole-body of the fish and edible parts (Piedecausa et al., 2007; Fountoulaki et al., 2009; Kenari et al., 2011). In certain cases, it has also been reported that the incorporation of plant-based oils in fish diets can entail problems in lipid digestibility (Francis et al., 2007; Piedecausa et al., 2007). In addition, the replacement of fish oil by plant-based oils often results in physiological disorders, such as dysregulations in energy and lipid metabolism, inducing lipogenesis (Panserat et al., 2009; Morais et al., 2012; Xu et al., 2022a), accumulation of fat deposits in digestive organs, like the liver (Ruyter et al., 2006; Fountoulaki et al., 2009; Wassef et al., 2015) and the intestine (Caballero et al., 2002; Torrecillas et al., 2017). In terms of immune competence, the incorporation of plant-based oils into aquafeeds has also been associated with reduced antioxidant enzyme activities, bactericidal activity, and disease resistance against infections, as well as with an induction of the pro-inflammatory response (Montero et al., 2010; Tan et al., 2016; Tan et al., 2017). Such inflammatory response may be partly induced by the reduction of n-3 LC-PUFAs, which have anti-inflammatory effects through regulation of the expression of genes involved in multiple signalling pathways, such as the family of peroxisome proliferator-activated receptors (*ppar's*), sterol regulatory element-binding protein-1c (*srebp-1c*), farnesoid X receptor (*fxr*), liver X receptor (*lxr*), hepatocyte nuclear factor 4 alpha (*hnf4a*), and nuclear factor-κB (*nfκb*) (Jump, 2004; Zhao et al., 2004). In addition, EPA and DHA can be metabolized into "lipid mediators" (or eicosanoids), with anti-inflammatory, vasodilatory, and anti-aggregative effects, while n-6 LC-PUFAs can generate eicosanoids with pro-inflammatory, vasoconstrictory, and pro-aggregative effects (Saini and Keum, 2018). Moreover, n-3 LC-PUFAs have well-known hypolipidemic and hypocholesterolemic effects (Flachs et al., 2009; Lorente-Cebrián et al., 2013).

Another alternative to complement the reduction of dietary fish oil in the aquaculture industry are animal-rendered fats, which are widely available and affordable by-products of the meat and leather industry, that are attracting a growing interest in the context of circular bioeconomy and a much lower carbon footprint than other ingredients specifically produced for animal feed manufacturing (EFPRA, 2021). Beef tallow, pork lard, and poultry fat are among the most common animal-rendered fats incorporated in the fish diets. The chemical properties of animal fats depend on the history (*i.e.*, diet, farming conditions), age, and species of the animals used to obtain them (Turchini et al., 2009). Regarding fish health and quality, there are some main advantages of using animal-rendered fats in aquafeeds. First, their content of n-6 PUFAs is significantly lower than in plant-based oils (3% in beef tallow, 10% in

pork lard, 20% in poultry fat). Second, they are overall rich in SFAs and MUFAs (48 and 41% in beef tallow, 39 and 44% in pork lard, 29 and 43% in poultry fat, respectively; Mozanzadeh et al., 2021). In this sense, SFAs and MUFAs are preferentially catabolized for energy production via β-oxidation, which has been shown to spare n-3 LC-PUFAs from catabolism, for deposition in the tissues (Henderson, 1996; Trushenski and Lochmann, 2009). Furthermore, animal fats have reduced levels of linoleic acid (C18:2 n-6) compared to plant-based oils, which is an advantage because C18 PUFAs and specifically linoleic acid, compete with LC-PUFAs for tissue deposition (Rombenso et al., 2021). In addition, several studies have shown that grow-out feeds with high levels of SFAs and lower levels of C18 PUFA yield fillets with greater LC-PUFA content or greater amenability to LC-PUFA restoration (Trushenski et al., 2008, 2011; Trushenski, 2009). Nonetheless, the n-3 PUFAs' content of animal-rendered fats is very scarce (0.6-1%), resulting in low n-3/n-6 ratios (0-0.2%) (Mozanzadeh et al., 2021). As a result, the substitution of fish oil by animal-rendered fats has negative consequences similar to those observed when using plant-based oils as a main lipid source, including modifications in the fatty acid profile of the fillet (Yun et al., 2013; Xue et al., 2006; Mozanzadeh et al. 2016; Monteiro et al., 2018; Campos et al., 2019a), and fat accumulation in the fish fillet and digestive organs (Monteiro et al., 2018; Campos et al., 2019a). However, the changes in energy and lipid metabolism caused by animal-rendered fats are much less studied than in the case of plant-based oils. Another problem associated to the use of animal fats is the low digestibility of saturated fats (Trushenski et al., 2009). In this sense, when using animal-rendered fats very rich in SFAs, such as mammalian fats, at high dietary levels, the lipid digestibility of the diet can be compromised (Caballero et al., 2002; Monteiro et al., 2018), but moderate dietary levels of such fats (Mozanzadeh et al. 2016; Monteiro et al., 2018) and/or the use of other animal-rendered fats with lower levels of SFAs, such as poultry fat, do not necessarily affect lipid digestibility (Campos et al., 2019a, 2019b).

Considering the side-effects of the above-mentioned alternatives to fish oil, the blue food sector is now at a stage of searching for new nutritional strategies to ensure a more judicious use of fish oil while meeting the nutritional requirements of the animal and the consumer. Consequently, during the last years, the overall animal and fish feed industries have paid attention to the potential development of promising alternative oils rich in n-3-LC-PUFAs derived from microalgae (Yaakob et al., 2014), mesopelagic fish (Olsen et al., 2011), zooplankton (Vang et al., 2013), single cell microorganisms (Orozco Colonia et al., 2020) and genetically modified oilseed crops (Ruiz-Lopez et al., 2014), among others. However, the low supply volumes of these oils and their high production costs, make it difficult to scale up such alternatives without expecting prohibitive prices, even higher than those of fish oil (Rombenso et al., 2021). Additionally, during the last decades there has existed a widespread public rejection of genetically modified organisms, since some people consider them as potential threats against the environment and human health (Turchini et al., 2009). A worthwhile endeavor needs to be made to optimize the industrial development of some of these alternatives at low expenses and to change the public opinion, but nowadays it still seems that there is a long way to go to have these novel ingredients widely available to feed formulators. Thus, in the meantime, complementary strategies need to be explored to mitigate the negative impacts of more conventional fish oil alternatives (plant-based oils and animal-rendered fats) while guaranteeing the fish health and nutritional quality (Turchini et al., 2009; Tocher, 2015).

3. The problem of fat accumulation in fish

Teleost fish accumulate lipids, mainly as triacylglycerides, in multiple tissues and organs, including muscle, adipose tissue, liver, pancreas, esophagus, intestine, and brain. The preferential storage sites depend on the species, life stage, and on the nutritional and physiological condition, but are generally the fillet, liver, and perivisceral and subcutaneous adipose tissues (Salmerón, 2018). In this sense, the adipose tissues and fat deposits are reservoirs of metabolic energy that contribute to the early development, growth, swimming activity, and reproduction of fish (Tocher, 2003). The degree of fat deposition in the tissues depends on different mechanisms. In brief, these are mainly: the incorporation into the cells of fatty acids which are converted to triacylglycerides (lipogenesis) and stored in the tissues; the incorporation of non-lipid substrates that are converted into fatty acids, and then triacylglycerides for storage (known as "de novo" lipogenesis); and the metabolization of triacylglycerides into fatty acids and glycerol (lipolysis), which can be released into the blood or used to obtain energy through β-oxidation (Salmerón, 2018). Such mechanisms underlying fat accumulation in fish are regulated by intrinsic (age, genetic background, hormonal factors, reproductive cycle) and extrinsic factors (temperature, salinity, water quality, photoperiod, dietary composition; Weil et al., 2013).

The two main reasons of the interest of studying the regulation of fat accumulation in fish are 1) to study human disorders associated to obesity in zebrafish, as it shares key conserved organs and regulatory pathways with humans, and 2) to learn how to control excessive fat storage in aquaculture species in order to improve the fish health and production (Salmerón, 2018). Regarding the second point, although fat deposits can be used for the benefit of the animals, the problem comes up when the modulation of the mentioned mechanisms leads to a high or an excessive lipid accumulation, which can be detrimental for the fish health and welfare (Salmerón, 2018). Indeed, fish condition has been strongly correlated with the levels of fat in and around the peritoneal cavity (perivisceral and peritoneal fat, respectively), and total fat deposits' content (Grigorakis and Alexis, 2005). Some common frequent examples of drivers of high or excessive lipid accumulation are a low level of swimming activity and feeding with a diet which does not fulfill the fish nutritional requirements (Gisbert et al., 2008; Salmerón, 2018). In this sense, as mentioned in the section "*2. The challenge of fish oil reduction in aquafeeds*", a typical consequence of the replacement of fish oil by plant-based oils and animal-rendered fats in aquafeeds is a high accumulation of fat in fish tissues (Weil et al., 2013).

The excess of fat accumulation in fish is normally reflected in the liver, since this organ plays a major role in lipid metabolism and storage. Some of the morphological disorders attributed to an excess of fat in the fish liver are lipid infiltration into the hepatocytes, increased degree of vacuolization, hepatocyte swelling, displacement of nuclei and cell organelles towards the periphery, nuclei pyknosis, and in some cases even necrosis, which are symptoms usually associated to hepatic steatosis or lipoid liver (Gisbert et al., 2008; Fountoulaki et al., 2009; Wassef et al., 2015; Monteiro et al., 2018; Van Vo et al., 2020; Figure 1). Such disorders can lead to a potential impairment in the liver functions, which, considering its role in lipid, protein and carbohydrate metabolism, immunity, digestion, detoxification, elimination of waste

products, and vitellogenesis, can have a severe impact on the animal health (Bruslé and Gonzàlez i Anadon, 1996). In this regard, some of the reported side-effects of lipoid liver and steatosis in fish are anaemia and immunosuppression, with increased susceptibility to infections (Weisman and Miller, 2006; Hardy, 2012).

An excessive amount of fat storage can also be detected in the intestine by changes in the size of enterocytes' supranuclear vacuoles (either an excessive enlargement or reduction), accumulation of lipid droplets in supranuclear position, apical nuclear displacement, shortening of the height of the mucosal folds, and leucocytic infiltration by an engrossment of the *lamina propria* and submucosa, which are signs of fish enteritis or intestinal steatosis (Caballero et al., 2002; Kraugerud et al., 2007; Torrecillas et al., 2017; Figure 1). These symptoms may also have some negative consequences for the health of the animal, such as variations in the gut microbial profile, deregulations in digestive enzyme activities, modifications of the immune response, and induction of a pro-inflammatory response, among others (Gu et al., 2016; Torrecillas et al., 2017; Fuentes-Quesada et al., 2018). In addition, intestinal steatosis may result in pathological damages, producing epithelial abrasion, cellular necrosis, and also contributing to the inflammatory responses (Gisbert et al., 2008). Besides that, intestinal inflammation can deregulate intestinal motility and impair nutrient digestion and absorption, resulting in reduced feed efficiency, lower growth, and other physiological disorders (Serna-Duque and Esteban, 2020).

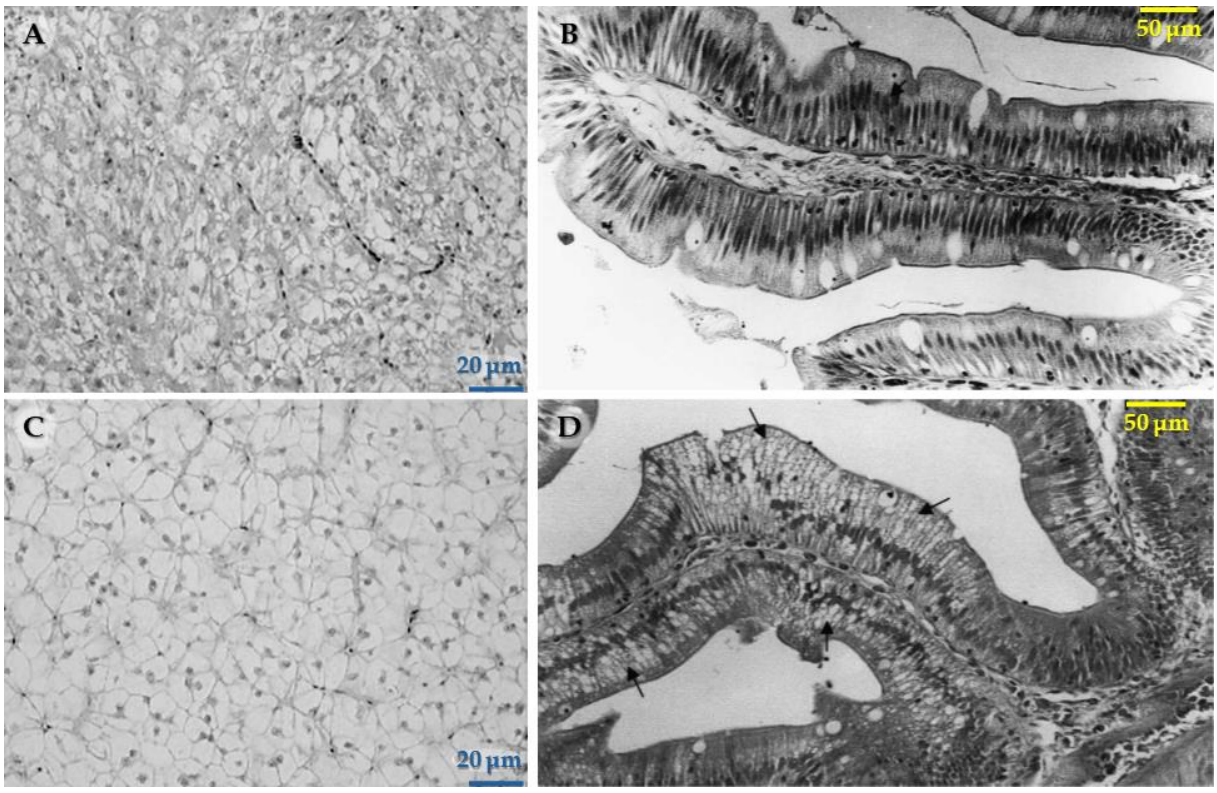

Figure 1. Histological morphology with hematoxylin eosin staining of cross sections of: A) liver of gilthead seabream (*Sparus aurata*), and B) intestine of rainbow trout (*Oncorhynchus mykiss*), both fed with a fish oil-based diet; C) liver of gilthead seabream with high levels of fat accumulation, induced by replacement of fish oil by poultry oil; D) intestine of rainbow trout with high levels of fat accumulation, induced by fish oil substitution by a blend of plant-based oils. The arrows signal the enlarged supranuclear vacuoles and lipid deposits in the supranuclear position of the enterocytes. Adapted from Carvalho et al. (2021) and Caballero et al. (2002), respectively.

In addition to the impact in fish health and welfare, the degree of fat accumulation also affects the quality of the edible product by modifying its organoleptic properties, as well as its yield and shelf-life considering the higher propensity to rancidity derived from rapid fatty acid oxidation (Hsieh and Kinsella, 1989; Salmerón, 2018). Regarding organoleptic properties, the peritoneal fat, which remains as part of the edible part of gutted fish, contributes to the general taste of the fish (even though it is unknown to what extent). In this sense, lipids themselves have a slight taste, but unsaturated fatty acids are autoxidized producing volatile compounds which characterize the fish flavour (Grigorakis, 2007). The visual aspect is another key factor for the consumer's perception of the product, and it has been demonstrated that an excess of perivisceral fat can affect it negatively (Grigorakis, 2007). The colour of the fillet is also related to the fat content, resulting in whiter fillets when the levels of lipids are high (Grigorakis et al., 2003). Regarding odour, the perivisceral fat usually releases a characteristic, strong and unpleasant smell (Grigorakis, 2007). Likewise, the texture of the cooked fillets in the mouth is also highly dependent on the fat content. While fillets rich in fat tend to be more succulent ("juicy"), less fatty fillets are usually drier and more fibrous (Grigorakis, 2007).

In summary, it is necessary to maintain the fish lipid levels within an optimal range to meet the nutritional requirements and quality expectations of consumers, without exceeding these limits, since they can have a negative impact on the public perception and reduce the product demand and marketability (Salmerón, 2018).

4. Potential strategies to minimize fat accumulation

Under the above-mentioned context, there is a wide range of different strategies aimed at regulating fat accumulation in aquaculture fish species, ranging from selective breeding programs (Weil et al., 2013) to feeding and dietary management practices (Kaushik, 2013; Naiel et al., 2022). An overview of the two strategies proposed in the present thesis to modulate fat accumulation in fish and their state-of-the-art is provided below.

4.1 Feed additives

The supplementation of fish diets with feed additives with emulsifying and digestive stimulant properties, and/or which can promote lipid catabolism and inhibit or reduce lipogenesis can be a good potential strategy to mobilize and reduce fat deposits, and to improve the overall health and condition status of the fish (Hoseinifar et al., 2017).

4.1.1 Bile salts

Bile acids are amphipathic molecules (with a hydrophilic and a hydrophobic side) that are synthesized in the liver from cholesterol in a multi-enzymatic pathway. Bile acids have a steroid nucleus (C_{19}) with side chains ending in a carboxylic acid, or a hydroxyl group, which determines the "bile acid" type (C_{27} bile alcohols (nonacidic), C_{27} bile acids or C_{24} bile acids) (Hagey et al., 2010; Romano et al., 2020; Figure 2). The C_{24} bile acids are the most abundant in aquaculture fish, excluding Cypriniformes which have mostly C_{27} bile alcohols (Hagey et al., 2010).

There are two main pathways of bile acid synthesis in the liver. The neutral or "classic" pathway occurs mainly in the endoplasmic reticulum of the hepatocytes and is the preferential pathway of bile acid synthesis. On the other hand, the acidic or "alternative" pathway is initiated in the inner membrane of the mitochondria, where the cholesterol content is very low, with the cholesterol transport into the mitochondria being a rate limiting step and generating low bile acid content (Zhou and Hylemon, 2014). The bile acids synthesized in the liver are known as primary bile acids, and these are mainly chenodeoxycholic acid (CDCA) and cholic acid (CA). Then, bile alcohols are conjugated with esterified sulphate and bile acids are conjugated with taurine or glycine within hepatocytes. The conjugation of bile acids with these amino acids results in the formation of membrane-impermeable molecules, which allows them to further concentrate in the bile and to reach high concentrations in the lumen of the biliary tract and small intestine (Hofmann et al., 2010). Such sulphates of bile alcohols and conjugated bile acids are commonly referred to as "bile salts". According to the available literature, it seems that in fish, bile acids are not well conjugated with glycine, so bile salts are normally conjugated with taurine in fish species (Vessey et al., 1990; Kim et al., 2015; Kortner et al., 2016).

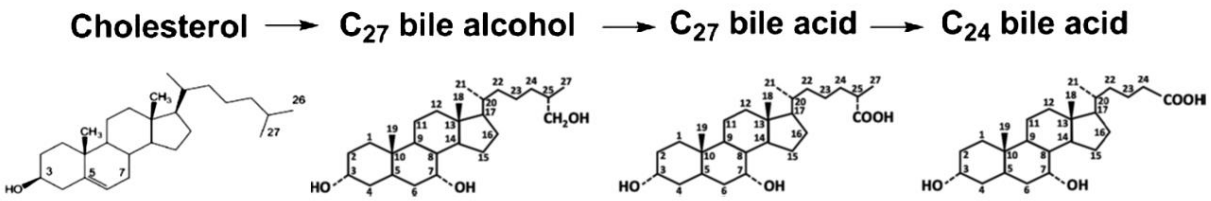

Figure 2. Schematic representation of bile acid synthesis from cholesterol, and their molecular structure. Adapted from Hagey et al. (2010) and Romano et al. (2020).

After conjugation, bile salts are transported and stored in the gallbladder until feed intake, when a decrease in pH and the presence of fatty acids and/or amino acids induce the secretion of the digestive hormone cholecystokinin (CCK), which triggers gallbladder contraction and subsequent secretion of bile salts into the intestine (Le et al., 2019; Romano et al., 2020). Throughout the intestinal tract, primary bile salts undergo further chemical modifications by host bacteria to generate compounds known as secondary bile acids, which are mainly deoxycholic acid (DCA) and lithocholic acid (LCA). This process consists of two types of enzymatic reactions: deconjugation and dehydroxylation. Bile salt hydrolases (BSHs), distributed across most intestinal bacterial phyla, can deconjugate taurine groups (and/or glycine depending on the animal species) from bile salts (Joyce and Gahan, 2017). Afterwards, deconjugated primary bile acids are metabolized (oxidized and/or epimerized) by hydroxysteroid dehydrogenases from colonic bacteria, particularly from members of the genera *Eubacterium* and *Clostridium* clusters XIVa and XI, forming the secondary bile acids (Hofmann et al., 2010; Joyce and Gahan, 2017). Such transformation of primary bile salts by the gut microbiota increases the molecule hydrophobicity, which is associated to a higher toxicity, but simultaneously counteracts the toxicity that may be generated by accumulation of primary bile salts in the intestinal lumen (Schubert et al., 2017). Bile acids are then reabsorbed, mainly through the brush borders of distal enterocytes, and transported back through the portal vein to the liver in a process known as enterohepatic circulation, to be conjugated again and stored in the gallbladder until the next secretion (Hagey et al. 2010; Figure 3). Only a very small portion of bile acids is not reabsorbed and is lost through fecal excretion, which is replaced by new bile acid synthesis in the liver (Zhou and Hylemon, 2014).There is some evidence that suggest that in fish a very similar enterohepatic circulation occurs as in mammals, such as the presence of transporters involved in bile salt absorption and secretion (Ferreira et al., 2014; Murashita et al., 2014; Ellis et al., 2018), the decreasing bile acid content along the intestine (Romarheim et al., 2008; Staessen et al., 2022), and the presence of glycine-conjugated bile salts in the fish gallbladder after their dietary supplementation (Yamamoto et al., 2007; Kortner et al., 2016), among others.

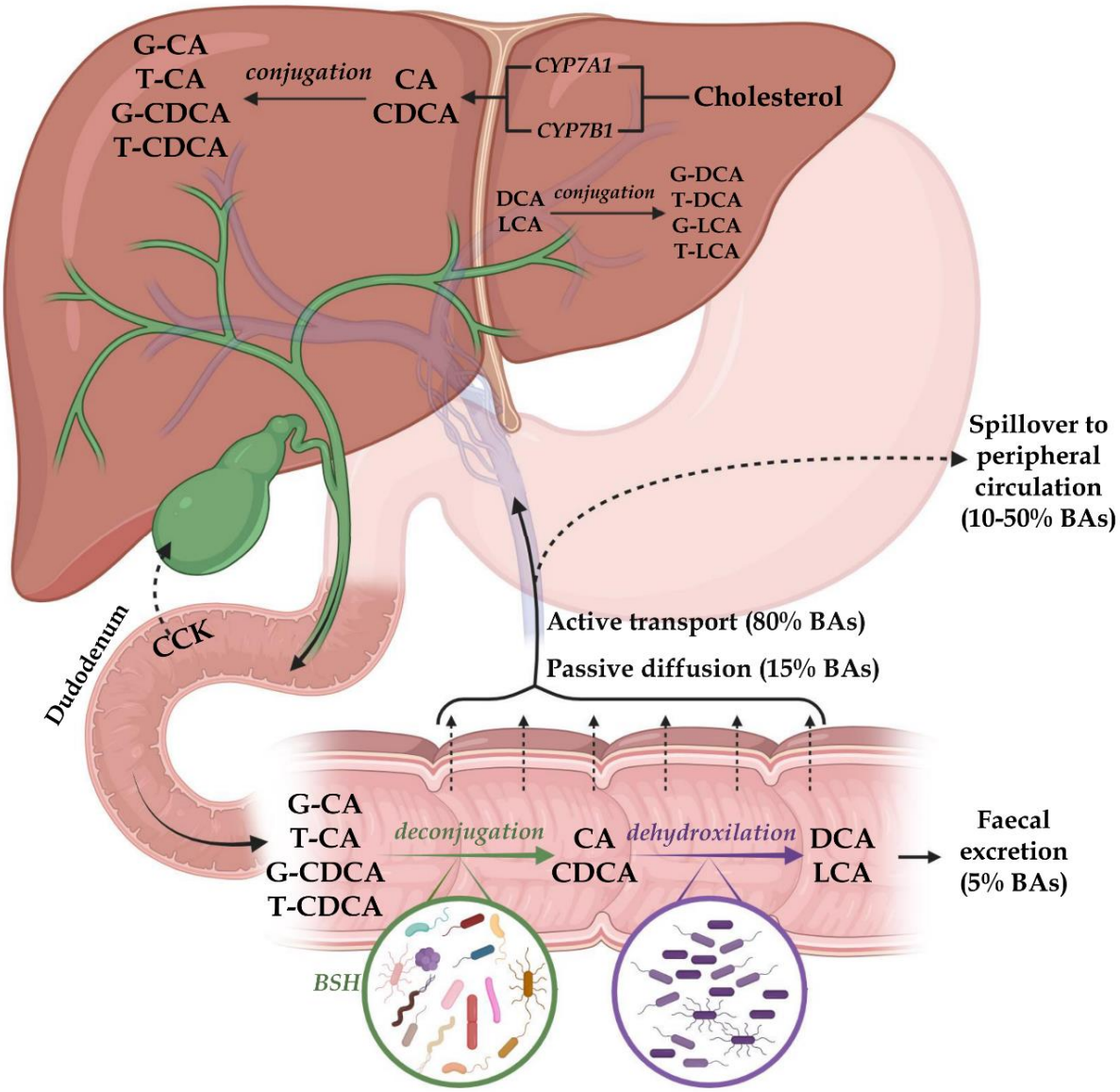

Figure 3. Schematic representation of the synthesis and enterohepatic circulation of bile acids (BAs) in mammals. Primary BAs (CA: cholic acid, and CA: chenodeoxycholic acid) are synthesized in the hepatocytes mainly through the classic pathway, whose first and rate-limiting enzyme is cholesterol 7α-hydroxylase (CYP7A1), but also to a much lesser extent through the alternative pathway, whose rate-limiting enzyme is considered oxysterol 7α-hydroxylase (CYP7B1). Then primary BAs are conjugated with glycine or taurine (forming the "bile salts" G-CA: glycocholic acid, G-CDCA: glycochenodeoxycholic acid, T-CA: taurocholic acid, T-CDCA: taurochenodeoxycholic acid). The bile, containing the bile salts, together with cholesterol, bilirubin, phospholipids, water and electrolytes, are stored in the gallbladder, until the hormone cholecystokinin (CCK) stimulates gallbladder contraction. Then, bile is released into the small intestine via the cystic and the common bile ducts. In the terminal ileum and proximal part of the colon, primary bile salts are deconjugated by different bacterial phyla containing bile salt hydrolases (BSHs). Throughout the colon, such deconjugated bile acids can be dehydroxylated by members of the genera *Eubacterium* and *Clostridium* clusters XIVa and XI, forming the secondary bile acids. While CA is primarily transformed to deoxycholic acid (DCA), CDCA is mainly transformed to lithocholic acid (LCA). The majority of BAs (95%) are reabsorbed, mainly in the terminal ileum, by active transport or passive diffusion, and return to the liver through the hepatic portal vein, and only 5% BAs are excreted in the feces. A portion of BAs undergo systemic circulation to peripheral organs and tissues (*i.e.*, skeletal muscle, adipose tissue) where they can regulate lipid metabolism. In the liver, secondary bile acids can also be conjugated into glycodeoxycholic acid (G-DCA), glycolithocholic acid (G-LCA), taurodeoxycholic acid (T-DCA), taurolithocholic acid (T-LCA).

The importance of bile salts lies in the fact that these molecules can enhance lipid digestion through different mechanisms. First, the water-soluble nature of (conjugated) bile salts allows them to bind to the interface of lipid aggregates, breaking them down into smaller droplets (emulsification) with which they form micelles. In addition, emulsification provides a larger surface area on which lipases can act hydrolyzing the lipid ester bonds (Romano et al., 2020). Furthermore, the binding of bile salts to the interface of lipid droplets causes an orogenic displacement of other compounds which may inhibit lipase activity by accumulation on the interface (*i.e.*, surfactants, proteins, free fatty acids released by the action of the lipase) (Maldonado-Valderrama et al. 2011; Romano et al., 2020). Moreover, bile salts are necessary for the activation of the bile salt-activated lipase, which is the most dominant lipase of marine fish and exhibits a broad substrate specificity (wax esters, mono-, di- and triacylglycerides, phospholipids, ceramides, fat-soluble vitamin esters and cholesteryl esters). Subsequently, bile salts play an essential role in the solubilization and absorption of cholesterol, lipids, and fat-soluble nutrients, such as vitamins A, D, E, K, carotenoids and astaxanthin (Romano et al., 2020), as well as contributing to the elimination of excess cholesterol and to the excretion of lipophilic waste products (*i.e.*, bilirubin, heavy metals, and drug metabolites) (Frisch and Alstrup, 2018).

In addition to their digestive properties, bile salts are also involved in the modulation of the gut microbial communities through the antimicrobial properties that they have in some microbial species (Ridlon et al., 2014). Furthermore, bile salts can be considered as nutrient signalling hormones, by acting as ligands for many cell membrane and nuclear receptors in the enterohepatic system, termed as "bile acid-activated receptors", such as FXR and G protein-coupled bile acid receptor 1 (GPBAR1/TGR5) (Zhou and Hylemon, 2014; Fiorucci et al., 2021). Through bile acid-activated receptors, bile salts can regulate lipid, cholesterol, lipoprotein, glucose, energy metabolism and transport, gut microbial profile, intestinal integrity, immune and inflammatory responses, as well as their own biosynthesis, transport, and metabolism (Schonewille et al., 2016; Frisch and Alstrup, 2018; Fiorucci et al., 2021; Li et al., 2021a). For instance, it has been shown in mammals that the synthesis of primary bile salts is regulated by the levels of bile salts in the liver by LXR and intestinal FXR (Romano et al., 2020; Figure 4). In this sense, LXR is activated in response to high levels of oxysterols, which are products of cholesterol metabolism, and acts as a heterodimer complex with retinoid X receptor (RXR) to activate the transcription of cytochrome P450 cholesterol 7α-hydroxylase (*cyp7a1*) (Frisch and Alstrup, 2018). Also known as cholesterol 7α-monooxygenase, CYP7A1 is the first and rate-limiting enzyme in the classic bile acid synthesis pathway, exclusively expressed in the endoplasmic reticulum of hepatocytes (Chiang and Ferrel, 2020). On the other hand, FXR is activated by high levels of bile salts, and results in the transcription of a small heterodimer partner (*shp*), which interacts with the transcription factors α-fetoprotein transcription factor (FTF) and HNF4α to inhibit the expression of *cyp7a1*. Similarly, high levels of bile acids in the enterocytes activate intestinal FXR, which stimulates the fibroblast growth factor 15/19 (FGF15/19). Then, FGF15/19 reaches the liver though portal vein circulation and binds to a specific receptor inducing signalling pathways, such as mitogen-activated protein kinase (MAPK) signalling, which in the last term inhibit the trans-activation of *cyp7a1* (Chiang and Ferrel, 2020; Romano et al., 2020; Figure 4). Very conserved mechanisms of bile acid synthesis can be expected in fish with respect to those from mammals since recent studies in

fish have shown many similarities on the regulation and functionality of some of the components involved in the above-mentioned pathways (Kortner et al., 2013; Kortner et al., 2014; Wen et al., 2021).

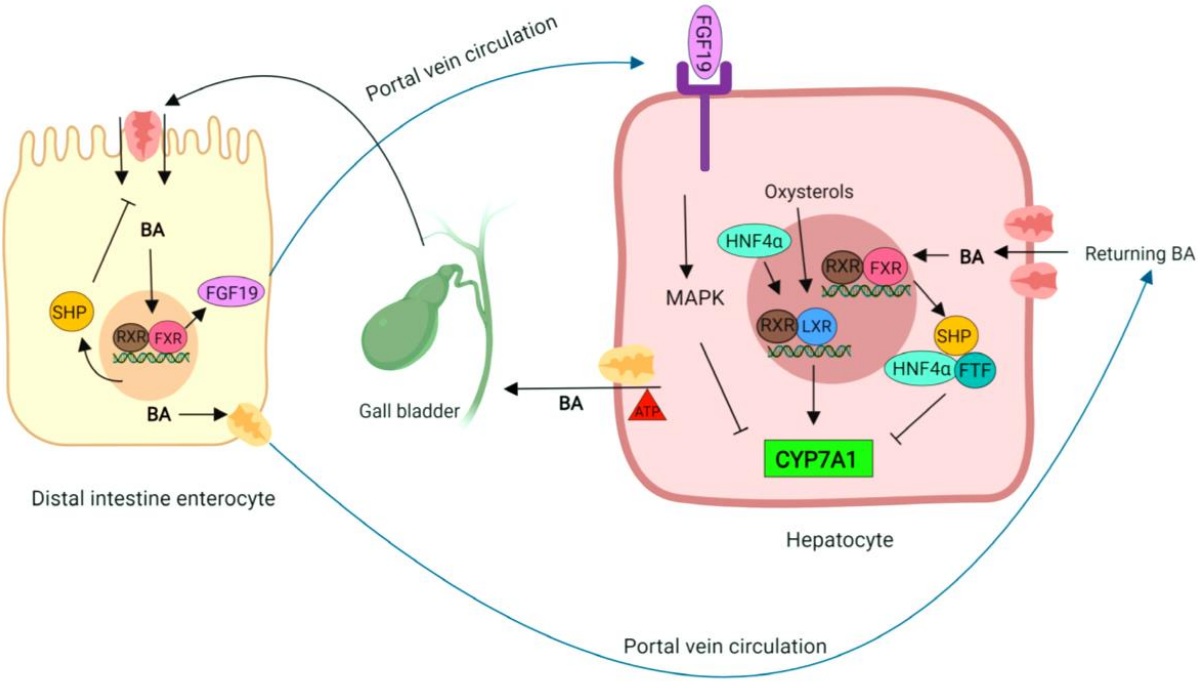

Figure 4. Enterohepatic regulation of primary bile acid synthesis through regulation of cytochrome P450 cholesterol 7α-hydroxylase (*cyp7a1*) trans-activation. Abbreviations: BA: bile acids; CYP7A1: cholesterol 7α-hydroxylase; FGF19: fibroblast growth factor 19 (in humans; FGF15 is the analogue in other mammals); FTF: fetoprotein transcription factor; FXR: farnesoid X receptor; HNF4α: hepatocyte nuclear factor 4α; LXR: liver X receptor; MAPK: mitogen-activated protein kinase; RXR: retinoid X receptor; SHP: small heterodimer partner. Adapted from Romano et al. (2020).

In the poultry industry, the supplementation of the diet with bile acids has been used as a reliable strategy to counteract fat digestibility problems in broiler chicks (Arshad et al., 2021). In this sense, young birds have a very poor capacity to produce and secrete bile salts and lipases until maturation of their gastrointestinal tract at 10-14 days of age, which usually leads to low lipid digestion and absorption, decreased feed utilization and growth performance (Siyal et al., 2017). The supplementation of broiler diets with bile acids enhances their growth and feed utilization, increases lipid digestibility, up-regulates genes involved in lipolysis and down-regulates lipogenesis, decreases the levels of triglycerides, cholesterol and free fatty acids in serum, reduces the accumulation of hepatic and abdominal fat, and improves the carcass quality (Alzawqari et al., 2016; Lai et al., 2018; Ge et al., 2019; Geng et al., 2022; Hu et al., 2024). In laying hens, besides having similar effects to the above-mentioned ones, dietary bile acid supplementation can reduce their mortality, and improve egg production and quality (Yang et al., 2022; Sun et al., 2023). Many studies in the swine industry have also demonstrated the potential of bile acids as feed supplements in improving the growth and feed performance, lipid digestibility and metabolism, and antioxidant capacity in pigs (Cao et al., 2021; Song et al., 2021; Liu et al., 2022a; Qin et al., 2023). In addition, bile acid supplementation in piglet diets

can also modify their gut microbial profile, promoting the growth of bacteria beneficial to health and reducing the abundance of potential pathogens, and can ameliorate the intestinal barrier function and immune response, alleviating the symptoms of disorders such as intrauterine growth retardation and reducing incidence of diarrhea (Song et al., 2021; Liu et al., 2022a; Qin et al., 2023). Similarly, some works have reported that bile acid supplementation induces a higher antioxidant capacity and lipoprotein lipase activity in dairy cows (Chen et al., 2024), and modifies the gut microbial communities and improves the milk's yield and fatty acid composition in dairy goats (Yin et al., 2024).

In the aquaculture sector, several growth and health performance benefits have also been attributed to bile salt supplementation. For instance, an improved growth performance and feed efficiency has been demonstrated in many species when supplementing bile salts in their diets, including turbot (*Scophthalmus maximus*; Gu et al., 2017), rainbow trout (*Oncorhynchus mykiss*; Iwashita et al., 2008), striped catfish (*Pangasianodon hypophthalmus*; Adam et al., 2023), yellow catfish (*Pelteobagrus fulvidraco*; Yao et al., 2022), large yellow croaker (*Larimichthys crocea*; Ding et al., 2020), Chinese perch (*Siniperca chuatsi*; Zhang et al., 2022a), among many other species (Appendix 1). In addition, it is well-demonstrated that supplementation of bile salts in fish promotes lipid metabolism (Zhou et al., 2018a; Jin et al., 2019; Ding et al., 2020; Xu et al., 2022b; Gao et al., 2023) and enhances lipid digestibility (Gu et al., 2017; Jiang et al., 2018; Wang et al., 2022; Xu et al., 2022b; Gao et al., 2023). Consequently, bile salts can reduce the lipid content of the whole-body (Jiang et al., 2018; Zhou et al., 2018a) and liver (Ding et al., 2020; Xu et al., 2022b; Zhang et al., 2022a), decreasing accumulation of lipid droplets (Yin et al., 2021; Xu et al., 2022b; Zhang et al., 2022a), and prevent or reduce inflammation in the gut (Iwashita et al., 2009; Kortner et al., 2016). Furthermore, bile salts can enhance the fish antioxidant status and improve their immune response (Jin et al., 2019; Li et al., 2021b; Wang et al., 2022; Zhang et al., 2022a). Some studies have also shown evidence of a microbial modulation towards an improved health status by bile salts (Zhou et al., 2018a; Li et al., 2021b; Zhang et al., 2022a).

4.1.2 Spices and their active principles

Spices are dried seeds, fruits, roots, barks or flowers of a plant or an herb which have been traditionally used in the culinary sector to give a special flavour, taste, aroma, and colour to the food. These food adjuncts have also preservative properties, being initially used to retain the freshness of the cooked food (Sachan et al., 2018). In addition, spices have been effectively used in the indigenous systems of medicine of India and many other countries due to their well-recognized medicinal properties, including their tonic, carminative, stomachic, diuretic, and antispasmodic effects. Due to their wide range of therapeutic and prophylactic applications, nowadays spices have a worldwide application as nutraceuticals, to prevent and/or reduce obesity, diabetes and even carcinogenesis among other uses (Platel and Srinivasan, 2004; Srinivasan, 2005). During the past four decades, several studies have also shown beneficial physiological effects of spices in animals, such as stimulation of lipid metabolism, digestive, anti-diabetic, antioxidant, and anti-inflammatory potential (Srinivasan, 2005). In this sense, spices are a good source of phytogenics, also termed phytobiotics, defined as environmentally friendly plant-derived bioactive compounds used as functional feed

additives that show positive effects on animal growth and health (Firmino et al., 2021a). Such bioactive compounds (or active principles) are plant secondary metabolites usually present as mixtures, mainly containing phenolic compounds and terpenes that are chemically characterized by their aromatic rings (Christaki et al., 2020). Hence, the growth- and health-promoting benefits of spices on the animal depend on the variability and complexity of the mixture of bioactive compounds, their source, level of dietary incorporation, pharmacokinetics, and their synergistic effects (Firmino et al., 2021a).

Plant bioactive compounds can be isolated and concentrated through many plant extraction methods, which determines the type of plant extract (*i.e.*, dried powder, essential oil, or oleoresin; Gupta et al., 2012). Regarding their mechanism of action in lipid metabolism, many studies in mammals have suggested that the active principles of spices may activate the Transient Receptor Potential Vanilloid 1 (TRPV1). In turn, this receptor may affect mitochondrial functions, such as the inhibition of oxidative phosphorylation, accumulation and retention of calcium ions, and stimulation of ATPase activity, resulting in thermogenesis, fat oxidation, and energy expenditure (Westerterp-Plantenga et al., 2006; Nilius and Appendino, 2013). Similarly, it has been proposed a similar activation of Transient Receptor Potential (TRP) cation channels by bioactive phytogenic compounds in fish (Firmino et al., 2021a). However, some of the mechanisms underlying the effects of spices in fish may be different due to their physiological and metabolic differences with mammals (*i.e.*, the vast majority of fish species are ectothermic, so their energy expenditure is not regulated by thermogenesis; Van de Pol et al., 2017) and still needs to be studied in depth in fish.

Under the wide range of spices which have to date been tested in humans and in the livestock industry, this thesis is focused on the evaluation of the following combinations of spices and active principles (whose importance is individually described below):

1) capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde.

2) turmeric, capsicum, black pepper, and ginger oleoresins.

The term **capsicum** is employed to designate the fruit of *Capsicum* spp., also known as "chilli pepper", "hot red pepper", "red chile", "paprika", "tabasco" or "cayenne" depending on the species and variety of pepper. *Capsicum* spp. are typically annual flowering plants of the Solanaceae family, even though in tropical and subtropical areas some species from this genus may be grown as perennials. It was originated in Central and South America, and nowadays is cultivated worldwide, with the mayor producers being India, China, Pakistan, South Korea, Mexico, and Bangladesh, among many others (Thampi et al., 2003). Capsicum has been extensively used for medicinal purposes due to its wide range of health benefits from the Mayan civilization to the present day, when it is still used by the pharmaceutical industry as a counter-irritant balm (Zachariah and Gobinath, 2008). It has been used to treat sore throat, toothache, cough, stomach pain, rheumatism, parasitic infections, and wound healing (Singletary, 2011). Capsaicinoids are the main active principles of capsicum (0.2-2%) and they give to the spice its bite and pungent taste. The predominant capsaicinoids are the alkaloid capsaicin (50-70% of total capsaicinoids; Figure 5) and dihydrocapsaicin (20-25%).

Capsaicin, and consequently capsicum, has antioxidant, anti-inflammatory, antiplatelet, and antimicrobial effects, as well as hypolipidemic, hypoglycemic, and hypocholesterolemic activities (Jiang, 2019). In humans, capsaicinoid supplementation has been shown to decrease the levels of body fat (Rogers et al., 2018). Similarly, in obese mice fed with a high-fat diet, capsicum has been reported to inhibit adipogenesis, to reduce the size of lipid droplets, and to attenuate hepatic steatosis by suppressing lipogenesis, fatty acid oxidation, and gluconeogenesis (Kim et al., 2017a). On the other hand, under normal conditions,, capsicum often promotes fatty acid oxidation in mammals (Westerterp-Plantenga et al., 2006). Regarding the livestock industry, capsicum supplementation in pig diets enhances the growth performance, digestive enzyme activities, antioxidant capacity, anti-inflammatory response, and gut microbial modulation (Long et al., 2021). An improved growth performance, feed utilization, antioxidant capacity, and immune response was also observed in rabbits when supplementing their diets with capsicum (Elwan et al., 2020). Similarly, capsicum has also positive effects in broilers, in terms of growth performance, antioxidant status, immune function, and meat quality (Liu et al., 2021a). A similar enhanced growth performance and feed efficiency has also been reported in a few fish species using dietary inclusion of capsicum, particularly in Nile tilapia (*Oreochromis niloticus*; Ibrahim et al., 2024) and rainbow trout (Yılmaz et al., 2024) (Appendix 2). The majority of fish studies testing this spice have successfully been devoted to showcasing its potential to improve the coloration of fillets (Yılmaz and Ergün, 2011; Talebi et al., 2013; Yılmaz et al., 2013a; Yanar et al., 2016; Yigit et al., 2021) and sensory characteristics, such as taste, flavour and appearance, from the consumer's point of view (Yanar et al., 2016). Furthermore, an improved digestive capacity, antioxidant, and immune response has been demonstrated in Nile tilapia (Ibrahim et al., 2024), and an ameliorated immune resistance has also been suggested in rainbow trout (Talebi et al., 2013) when supplementing their diets with capsicum.

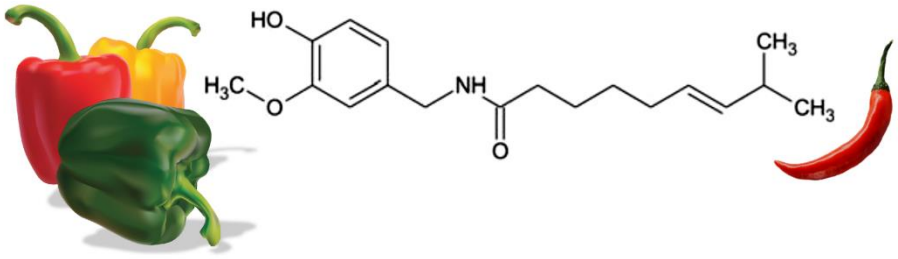

Figure 5. Molecular structure of capsaicin, the main bioactive compound of capsicum (*Capsicum* spp.).

Black pepper (*Piper nigrum*) is a perennial evergreen flowering vine belonging to the Piperaceae family which is usually cultivated for its fruit, known as the peppercorn, that is then dried and used as a spice. This plant is native to South India and to date is extensively cultivated in tropical regions of the Asia-Pacific region, being the most popular and the most widely used spice in the world, collectively termed as the "Black Gold" and the "King of Spices" (Ravindran and Kallupurackal, 2012). This spice is used as a traditional medicine in India, Sri Lanka, and other parts of South and Southeast Asia, to help mitigate the symptoms of asthma, bronchitis, cough, flu, colds, chills, fever, rheumatism, muscular aches and

digestive disorders (Chopra and Nayar, 1956; Ravindran and Kallupurackal, 2012). Black pepper oleoresin has two main components: the volatile oil (0.6-2.6%), which provides its characteristic aromatic flavour (Parthasarathy et al., 2007), and the piperine (5-9%), its major bioactive constituent and the main pungency principle (Jiang, 2019; Figure 6). It contains also other minor bioactive components, such as alkamides, piptigrine, wisanine, and dipiperamide.

Piperine has been demonstrated to have antioxidant, anti-inflammatory, antiallergic, and analgesic activities in mammals (Jiang, 2019). Some works have demonstrated that black pepper supplementation can improve animal growth, feed utilization, and meat quality in some mammals, including swine (Sampath et al., 2020), and poultry (Al-Kassie et al., 2011; Sugiharto et al., 2020). On the other hand, the effect of black pepper and its main active principle in mammalian digestion is not very clear because while some studies have shown that black pepper can enhance gastric acid and bile acid secretion in mammals, reducing their feed transit time (Srinivasan, 2007), other works have shown no such effects when supplementing rat diets with piperine (Platel and Srinivasan, 2004; Srinivasan, 2005). Concerning the aquaculture sector, growth promoting effects of black pepper and piperine have also been observed in some fish species, such as rohu (*Labeo rohita*; Ullah et al., 2021), common carp (*Cyprinus carpio*; Giri et al., 2023), and olive flounder (*Paralichthys olivaceus*; Malintha et al., 2023). Moreover, black pepper and piperine can improve the feed efficiency of common carp (Giri et al., 2023), olive flounder (Malintha et al., 2023), and rainbow trout (Stoev and Zhelyazkov, 2021). Some fish studies have shown improved digestive activities, including lipase activity (Giri et al., 2023), and a reduced whole-body lipid content (El-Houseiny et al., 2019). Many of these studies agreed that black pepper and its main active principle can improve the fish immune status and disease resistance (El-Houseiny et al., 2019; Wojno et al. 2021; Giri et al., 2023; Malintha et al., 2023; Ullah et al., 2021).

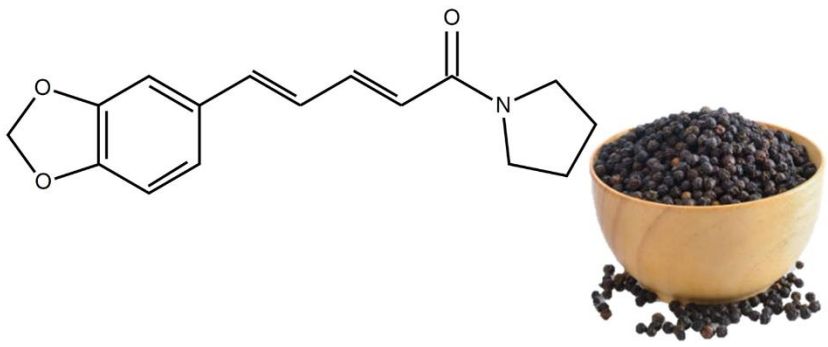

Figure 6. Molecular structure of piperine, the main bioactive compound of black pepper (*Piper nigrum*).

Ginger (*Zingiber officinale*) is an herbaceous perennial deciduous flowering plant from the Zingiberaceae family. The whole plant is refreshingly aromatic, but the consumed part of the plant is the rhizome, which is the horizontal stem of the plant that sends out the roots. Ginger was originated in South-East Asia, probably in India, and nowadays is extensively cultivated in tropical areas, from Asia to Africa and the Caribbean (Mohammad and Hamed, 2012; Vasala, 2012). The ginger rhizome (colloquially known as "ginger root" or just "ginger") is not

only widely used for culinary purposes as a spice, but also has had a prominent role in Chinese, Indian, and Japanese medicine since ancient times (Grant and Lutz, 2000). It is used to prevent and treat stomachache, nausea, vomiting, diarrhea, toothaches, rheumatism, cholera, motion sickness, and hemorrhage (Adewale et al., 2021). The nutritional and nutraceutical advantages of ginger are due to the diverse range of bioactive compounds it contains, which can be classified into three classes: volatile oils, and the non-volatile compounds, gingerol, and diarylheptanoids. Ginger volatile oils are mainly composed of terpenoids, especially monoterpenes and sesquiterpene hydrocarbons, which provide its unique aromatic smell (Shaukat et al., 2023). Gingerols (1-3%) are non-volatile molecules containing a 3-methoxy-4-hydroxyphenyl group connected to fatty acids, and depending on the fatty acids to which they connect, gingerols can be subsequently classified into gingerol, shogaol, zingerone, paradol, gingerdione, and gingerdiol (Liu et al., 2019; Figure 7). These active compounds are the responsible of the distinctive pungent odours and flavours of ginger spice. There are other minor bioactive compounds which can also be found in ginger extracts, known as diarylheptanoids. They contain 1,7-disubstituted phenyl groups and heptane skeletons, they can be divided into linear and cyclic diphenyl heptane compounds and are characterized by its antioxidant and anti-inflammatory activities (Liu et al., 2019).

Ginger and its bioactive compounds have anti-inflammatory, antioxidant, antimicrobial, antiplatelet, antihypertensive, antiglycation, antidiabetic, hypoglycemic, hypolipidemic and hypocholesterolemic effects in mammals (Jiang, 2019; Shaukat et al., 2023). Some of such effects have also been found in poultry, including their antioxidant and antimicrobial activities, as well as an enhancement in the laying rate and performance, and egg quality (Abd El-Hack et al., 2020). Additionally, decreased meat lipid levels, and increased tenderness and pH in the broiler meat have been observed under ginger supplementation (Abd El-Hack et al., 2020). Similarly, in rabbits, improved meat quality, in terms of pH, colour, and decreased lipid oxidation, have been reported when supplementing their diets with ginger (Mancini et al., 2018). Furthermore, several studies have demonstrated the efficiency of ginger in promoting weight loss in obese humans (Jiang, 2019). In this sense, reduced levels of adipose tissue in Japanese quails have also been observed under supplementation of ginger (*Coturnix japonica*) (Herve et al., 2019), and in rats by inclusion of the active principle zingerone (Han et al., 2008). In several fish species, an improved growth and feed performance have been reported when supplementing the fish diets with ginger, such as Asian sea bass (*Lates calcarifer*; Talpur et al., 2013), rohu (Sukumaran et al., 2016), common carp (Fazelan et al., 2020; Mohammadi et al., 2020), striped catfish (Ashry et al., 2023), and rainbow trout (Aqmasjed et al., 2023) (Appendix 2). In addition, as in higher vertebrates, a few works in fish have hinted hypolipidemic effects of ginger, such as reduced levels of lipids, triglycerides, and cholesterol in Asian sea bass (Talpur et al., 2013), increased lipase activity in striped catfish (Ashry et al., 2023), and decreased carcass lipid content in rainbow trout (Mohammadi et al., 2020). Ginger supplementation in fish usually results in an improved immune response, coupled to an increased bactericidal activity (Talpur et al., 2013; Fazelan et al., 2020; Sukumaran et al., 2016; Ashry et al., 2023). Indeed, Ashry et al. (2023) reported a reduction in the abundance of *Vibrio* spp. and fecal coliforms in striped catfish intestine after ginger dietary supplementation.

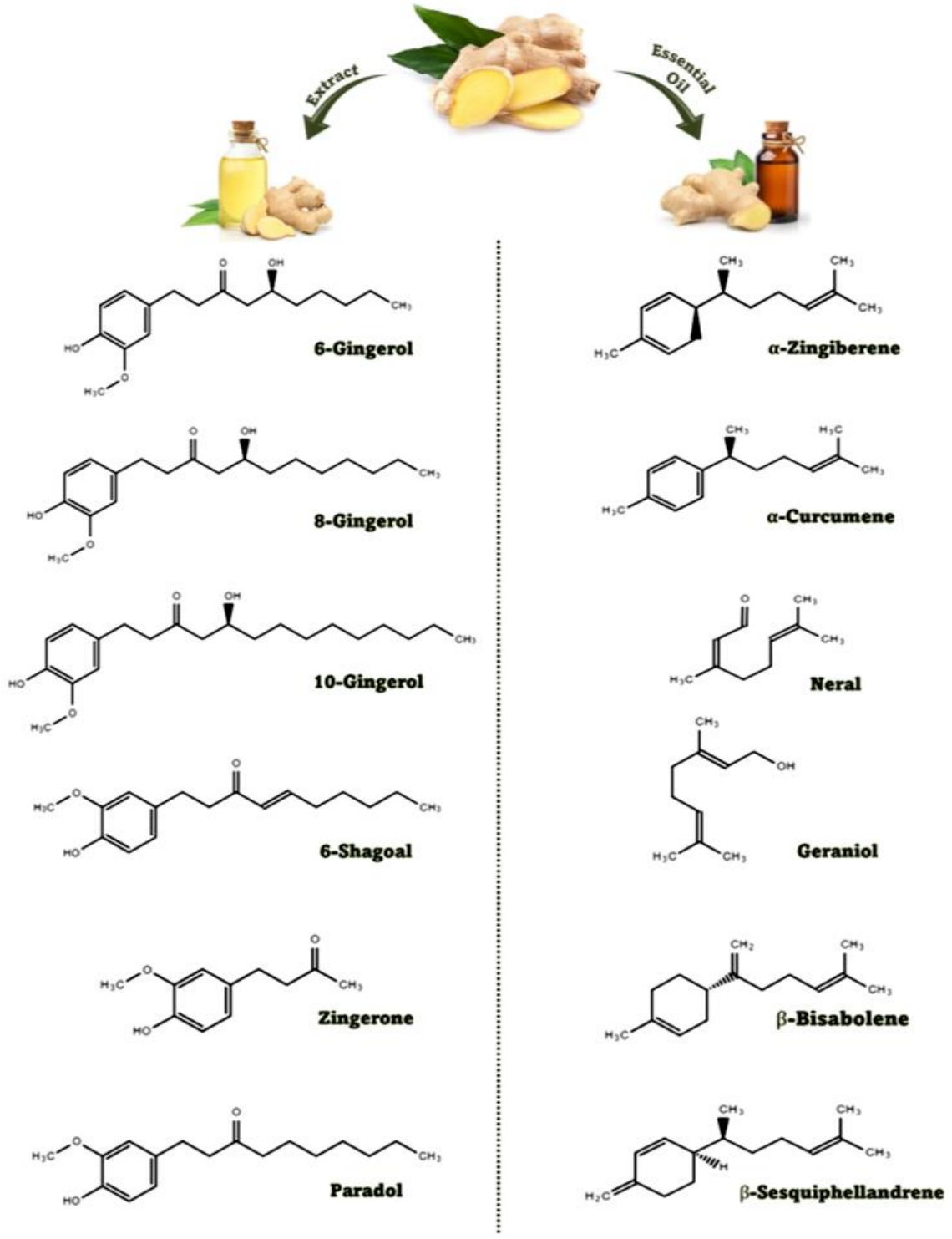

Figure 7. Molecular structure of the main bioactive compounds of ginger (*Piper nigrum*). Adapted from Shaukat et al. (2023).

Turmeric (*Curcuma longa*), also known as "haridra" or "haldi", is an herbaceous perennial evergreen flowering plant belonging to the ginger family, Zingiberaceae. Turmeric spice is known as the "Golden Spice" because of its attractive yellow pigmentation and is obtained from the plant rhizomes. This plant is widely cultivated in Asia, mostly in India and China, and it had its origin in South-East Asia, probably India, since first reports of turmeric usage

dates back nearly 4,000 years to the Vedic culture in India, where it was already used as a culinary spice and had a religious significance (Prasath et al., 2018). Since then, turmeric has been extensively utilized in traditional medicine practices in India, Pakistan, and Bangladesh, as a household remedy for biliary and hepatic disorders, diabetic wounds, anorexia, coryza, cough, rheumatism, and sinusitis, among other diseases (Rathaur et al., 2012; Verma et al., 2018). Similar to the above-mentioned spices, the main components of turmeric oleoresin are volatile oils (up to 5%) and non-volatile compounds, including diarylheptanoids, such as curcuminoids (2-9%). Volatile oils provide the spice's aroma and smell, while curcuminoids contribute to its colour (Prasath et al., 2018). There are four main groups of curcuminoids which can be found in turmeric: curcumin, demethoxycurcumin, bisdemethoxycurcumin, and tetrahydrocurcumin.

Curcumin is the main active principle of turmeric, responsible for the spice's yellow colour and of many of its therapeutic applications (Jiang, 2019; Figure 8). In this sense, since curcumin is also a diarylheptanoid, it has antioxidative and anti-inflammatory properties, as well as antimicrobial, hypocholesterolemic, hypoglycemic, antidiabetic, hypotensive, antithrombotic, hepatoprotective and antimutagenic effects (Prasath et al., 2018; Jiang, 2019). Some studies in pigs, lambs, and broiler chickens have shown that turmeric supplementation can improve their growth performance, feed utilization (Rajput et al., 2013; Alagbe et al., 2017; Odhaib et al., 2021; Recharla et al., 2021), and antioxidant capacity (Khan et al., 2012; Molosse et al., 2019; Zhang et al., 2020). In broilers, it also promotes lipid metabolism, reduces abdominal fat accumulation, enhances egg production and quality, and induces an immunomodulatory response (Khan et al., 2012; Rajput et al., 2013). Several works have shown an improved growth and feed performance in different fish species under turmeric or curcumin supplementation, including gilthead seabream (*Sparus aurata*; Ashry et al., 2021), crucian carp (*Carassius auratus*; Jiang et al., 2016), grass carp (*Ctenopharyngodon Idella*; Ming et al., 2020), rainbow trout (Yonar et al., 2019), and Nile tilapia (Diab et al., 2014) (Appendix 2). Similar to broilers, hypolipidemic effects of turmeric and curcumin have also been reported in fish (Jiang et al., 2016; Ji et al., 2021), resulting in reduced body lipid content (El-Houseiny et al., 2019; Wojno et al., 2021; Wang et al., 2023). Several studies have also demonstrated an improvement in the antioxidant status, as well as immune response and disease resistance, when supplementing aquafeeds with this spice or its active principle (Abdel-Tawwab and Abbass, 2017; Yonar et al., 2019; Ming et al., 2020).

Figure 8. Molecular structure of curcumin, the main bioactive compound of turmeric (*Curcuma longa*).

Cinnamaldehyde is the main active principle which can be obtained from the bark oil of the cinnamon tree (*Cinnamomum zeylanicum* or *Cinnamomum verum*) (Figure 9). Cinnamon is a bushy perennial evergreen flowering tree from the Lauraceae family native to Sri Lanka and South India, and it is mostly cultivated in South-East Asia, China, Australia, and South America. In this case, the most collectively valued part of the tree is the bark, which is not only used for cooking and beverages, but also in traditional medicine for treating toothache, soothe stomach irritation and urinary infections (Jakhetia et al., 2010; Thomas and Kuruvilla, 2012). Cinnamaldehyde has shown antioxidant, anti-inflammatory, antibacterial, antifungal, antiplatelet, antidiabetic, hypoglycemic and hypolipidemic properties in mammals (Jiang, 2019). In this sense, supplementation of cinnamaldehyde in mouse diets down-regulates genes involved in lipid synthesis and reduces visceral adiposity (Neto et al., 2020). Similarly, in finishing pigs, dietary cinnamaldehyde supplementation leads to decreased backfat thickness and intramuscular fat levels, as well as improving their growth performance, meat quality, antioxidant capacity, and immune status (Luo et al., 2020). In some studies in broiler chickens, positive effects of cinnamaldehyde have also been reported on their growth and feed utilization, lipid digestibility, cecal microbiota composition, and a lower incidence of enteritis, coccidiosis, and mortality (Yang et al., 2020; Yang et al., 2021). Growth and feed efficiency promoting effects have been also reported for fish, including tongue sole (*Cynoglossus semilaevis*; Wang et al., 2021a), fat greenling (*Hexagrammos otakii*; Gu et al., 2022), grass carp (Zhou et al., 2020), and Nile tilapia (Abd El-Hamid et al., 2021) (Appendix 2). Cinnamaldehyde also stimulates fish lipid digestion, as indicated by the higher lipase activity (Zhou et al., 2020; Wang et al., 2021a) reduced apparent lipid digestibility, and regulation of lipid metabolism-related gene expression (Gu et al., 2021). The antioxidant capacity and immunity are also enhanced under cinnamaldehyde dietary supplementation (Abd El-Hamid et al., 2021; Wang et al., 2021a; Amer et al., 2018; Gu et al., 2022). Additionally, the supplementation of tongue sole diets with cinnamaldehyde has been shown to increase the abundance of genera containing probiotic bacteria in the fish gut (Wang et al., 2021a).

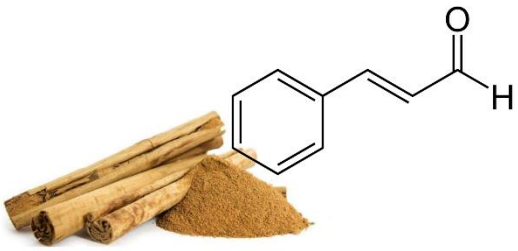

Figure 9. Molecular structure of cinnamaldehyde, the main bioactive compound of cinnamon (*Cinnamomum zeylanicum* or *Cinnamomum verum*).

Despite the multiple range of beneficial effects of spices individually, it is well-known that combining spices can led to a combined and/or synergetic impact with a much higher potential on the animal health. For instance, spices with an individual mid-low effect on bile acid secretion and bile flow rate in rats, such as capsaicin, ginger and the active principle curcumin, have been shown to result in a strong stimulation of bile acid secretion and much higher bile flow rate when combined together and with other spices (Platel and Srinivasan,

2004). Similarly, in fish the combined effect of some of the above-mentioned spices has led to better health or physiological performance results than the spices separately. As an example, greater lipid content reduction and immunostimulatory effects have been observed in African catfish (*Clarias gariepinus*) when combining black pepper and turmeric than when only supplementing the diets with turmeric (El-Houseiny et al., 2019). Furthermore, an improved growth performance, higher protein content, and an enhanced antioxidant and immune status have been reported in rainbow trout when supplementing its diets with a combination of ginger and curcumin rather than with each compound individually (Aqmasjed et al., 2023). The first of the spices' combinations evaluated in the present thesis (containing capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde) have already been tested in broiler chickens (Herrero-Encinas et al., 2023). The results of supplementing broiler diets with such combination of spices were an improved growth performance during the first 7 days, a higher gross energy and dry matter digestibility, and a potential enhancement of the antioxidant activity (Herrero-Encinas et al., 2023). This makes these spice combinations perfect candidates to be tested on other vertebrates with the aim of improving their performance and general health, as well as unraveling their effects on the regulation of lipid metabolism and fat storage.

4.2 Intestinal microbiota modulation: microbial transplantation

Gut microbiota plays a major role in animal health, having developed a multitude of close and often highly mutualistic relationships with the host throughout million years of co-evolution. Thus, both microbiota and host can be considered as a single unit known as a "holobiont" (Postler and Ghosh, 2017). Indeed, the intestinal microbiota can contribute to multiple functions in the host, such as feed digestion, nutrient metabolism, energy homeostasis, mucosal integrity, intestinal barrier function, immune system modulation, neuronal development, and endocrine system modulation, among many others (Forsythe, 2013; Mills et al., 2019).

The key role of the microbiota on the host health is clearly reflected under conditions of dysbiosis, which may be defined as a pronounced imbalance in the composition, diversity and/or function of bacteria that usually compromises the host health, leading to digestive and systemic diseases, and even promoting chronic pathologies, such as atherosclerosis and obesity (Postler and Ghosh, 2017; Mills et al., 2019). Inversely, an induced modulation of the intestinal microbiota can also improve the host health. For instance, many bacterial species from the phylum Firmicutes, including members of the Lactobacillaceae, Ruminococcaceae and Lachnospiraceae families, can metabolize complex carbohydrates which are not digestible for the host into short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate (Fusco et al., 2023). Apart from being an energy source, these SCFAs promote a multiple range of health benefits in mammals, such as promotion of an anti-inflammatory response, improved intestinal integrity and barrier functions, mineral solubilization, prevention of accumulation of toxic metabolites, and increased nutrient digestibility and absorption (Sekirov et al., 2010; Ikeda-Ohtsubo et al., 2018; Dawood, 2021). They also have a role on lipid, cholesterol, and glucose metabolism, can induce fatty acid oxidation, and reduce lipid storage in liver and muscle, as well as modulating feed intake by suppressing the appetite via the gut-brain axis

(Canfora et al., 2015; Deleu et al., 2021). On the other hand, some studies have suggested that acetate and propionate attenuate intracellular lipolysis in adipose tissue and increase adipogenesis (Canfora et al., 2015), so the effect of SCFAs may depend on the tissue where they act and on the specific fatty acid type. Considering the above, the role of Firmicutes in animal health is undoubtedly essential, so it is not strange that the Firmicutes/Bacteroidetes ratio is widely used as a marker of weight gain in mammals and as a marker of intestinal dysbiosis in fish (Naya-Català et al., 2021a). The differential significance of this ratio between mammals and fish is normal considering the great variability in the composition and associated functionality of the microbiota among different species (Ikeda-Ohtsubo et al., 2018; Figure 10). More extreme is the case of the ratio Bacteroidetes/Proteobacteria, which in humans and mice decreases upon inflammation, while in fish increases with inflammation (Brugman et al., 2018).

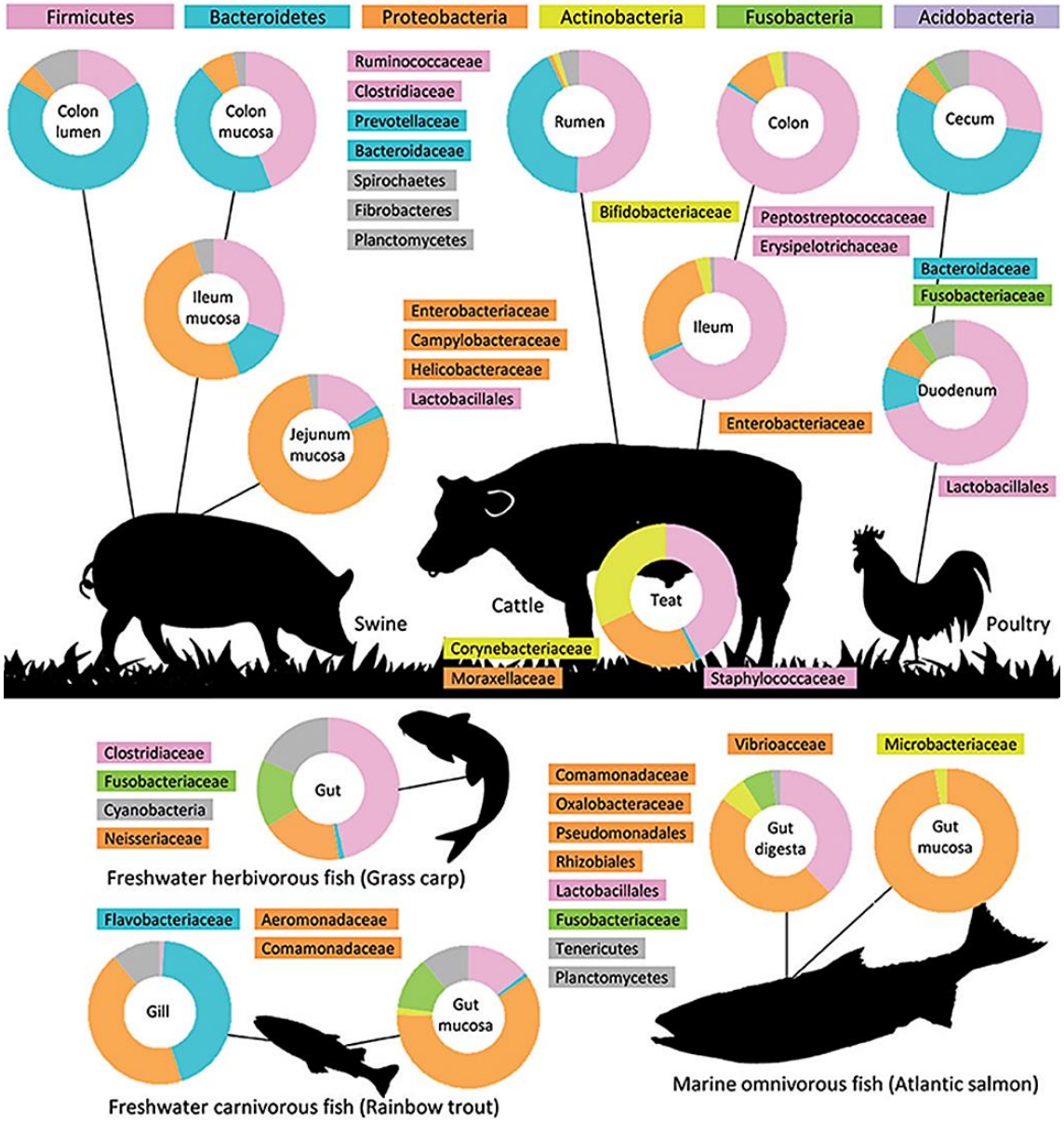

Figure 10. Overview of the variations in bacterial composition among different animal parts and species, in swine, cattle, poultry, and fish. Adapted from Ikeda-Ohtsubo et al. (2018).

The above-mentioned information were just a few examples of the important role of the microbiota in animal health and of the close relationships between host and microbiota. Consequently, many strategies to improve the animal health and condition through gut microbial modulation have to date been tested and developed, including quorum quenching, antimicrobial peptides, feed supplementation with exogenous enzymes, probiotics, prebiotics or synbiotics, and fecal (FMTs) or intestinal (IMTs) microbial transplants (Cheng et al., 2014; Figure 11).

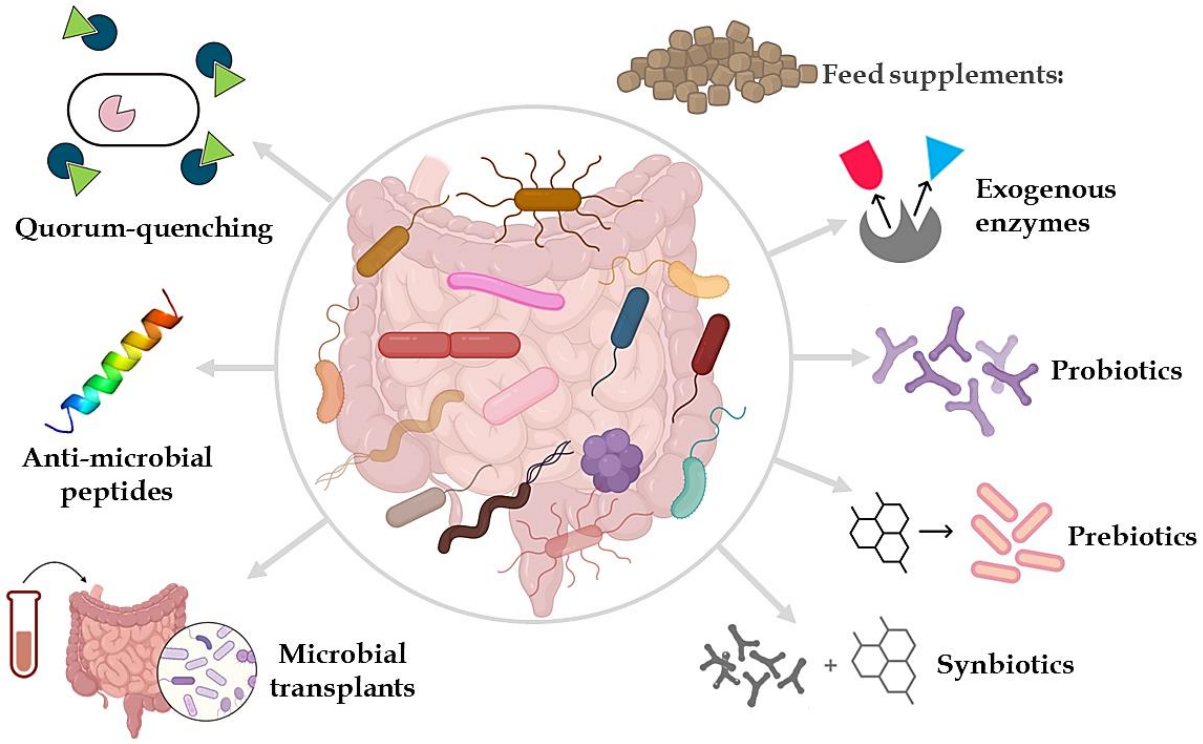

Figure 11. Representation of the main strategies which have been tested in humans and animals from the livestock industry to improve their health through induced modifications of the gut microbial communities.

Among the above-mentioned strategies to modulate intestinal microbiota, FMTs (and IMTs) have recently gained attention, since they have generally a longer-lasting effect and do not require a continuous supply, even though it is sometimes more effective to repeat the transplantation over time (Hasan and Yang, 2019). The concept of FMT generally refers to the transference of the microbiota associated to fecal matter (or intestinal content, in the case of IMTs) from a healthy donor into the gastrointestinal tract of a diseased recipient in order to colonize the gut of the recipient or modify its microbiota to improve the recipient's health (Gupta et al., 2016). Traditionally, FMT have been used in humans to treat *Clostridium difficile* infection, and experimentally tested to treat inflammatory bowel disease, obesity and metabolic syndrome, and functional gastrointestinal disorders (Gupta et al., 2016). In addition, FMTs and IMTs have also been used with production purposes in the livestock industry. In this sense, microbial transplantations have been shown to be a good strategy to improve

growth and feed performance in ruminants, through ruminal transfaunation, which is the transplant of the ruminal content (Pounden and Hibbs, 1949; Ribeiro et al., 2017), as well as in swine (Hu et al., 2018) and, in poultry (Siegerstetter et al., 2018). Like in humans, microbial transplants have also been applied to other vertebrates with therapeutic uses, in order to alleviate and prevent disorders and infections, such as horses (Mullen et al., 2018), ruminants (DePeters and George, 2014), pigs (Niederwerder et al., 2018), chicks (Rantala and Nurmi, 1973), dogs (Pereira et al., 2018), and cats (Furmanski and Mor, 2017), among other animals.

Although FMTs are still far away from being a routine clinical procedure to reduce body weight in obese and metabolic diseased humans due to their lack of efficiency in recent trials (Dalby, 2023), there are also some works which have shown promising prospects in this application (Hu et al., 2023). Otherwise, an experimental IMT performed between obese mice and germ-free mice resulted in increased body fat levels in the receiving individuals, associated to a higher Firmicutes/Bacteroidetes ratio and higher capacity for energy extraction (Turnbaugh et al., 2006). In this sense, multiple FMTs in mice have demonstrated the deleterious effect of the gut microbiota from aged donors when transplanted into younger mice, leading to obesity and increased fat body mass, as well as higher insulin levels, systemic inflammation, and neurodegeneration; while the inverse health effects are observed when transplanting the fecal microbiota from young donors into old individuals (Yan et al., 2023). In addition, inter-species FMTs from lean humans into germ-free mice have resulted in lower body weight and reduced fat deposition than germ-free mice transplanted with the microbiota of obese humans (Ridaura et al., 2013; Tremaroli et al., 2015). Similar results have been observed when performing FMT from two different breeds of pigs ("lean" and "obese") into germ-free mice (Yang et al., 2018). In that case, mice transplanted with the "obese" microbiota exhibited an elevated Firmicutes/Bacteroidetes ratio, an up-regulated expression of genes involved in the lipogenic pathway, and higher hepatic fat deposition with respect to those transplanted with the "lean" microbiota, even though no discernible differences in adipocyte morphology and fat deposition were found in the abdominal adipose tissue (Yang et al., 2018). Some studies in poultry have shown no obvious effects on growth and fat accumulation modulation (Song et al., 2023). On the other hand, other works have shown that performing FMTs from adult chickens, with high body weight, into younger chickens, resulted in an increased body weight and an up-regulation of the hepatic fat metabolism, including genes involved in lipid synthesis, catabolism, and transport (Zhang et al., 2022b). These ideas suggest that IMTs and FMTs might be good tools, yet under development, to modulate body fat levels in vertebrates, at least in the livestock industry, for potentially improving their growth, health, overall condition, and ultimately production.

5. Hypothesis and object of study

5.1 Hypothesis

Based on the hypolipidemic, antioxidant, immunostimulant, and growth-promoting properties of bile salts, and spices like capsicum, black pepper, ginger, turmeric, and cinnamaldehyde, and considering the important role of the gut microbiota on lipid metabolism and the demonstrated efficiency of intestinal microbiota transplants on reducing and preventing health disorders in higher vertebrates, the hypothesis of the present thesis is established:

It is possible to reduce fat accumulation and consequently improve the general health and condition of aquaculture fish through the supplementation of aquafeeds with additives with hypolipidemic properties (bile salts; a combination of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde; and a combination of turmeric, capsicum, black pepper, and ginger oleoresins) and by performing an intestinal microbiota transplant from fish with a lean phenotype to fish with a fatter condition.

5.2 Object of study: gilthead seabream (*Sparus aurata*)

In order to evaluate the efficiency of the above-mentioned strategies in the fish performance, reduction of fat accumulation, and general health-promoting effects, the species gilthead seabream (*Sparus aurata*; Figure 12) was selected as the object of study of the present thesis. The main reasons for selecting gilthead seabream were mainly that: 1) despite containing moderate levels of fat accumulation under typical wild and culture conditions, the body adiposity of this well-studied species can be largely modulated by the diet composition, particularly by the lipid source used (Houston et al., 2017); and 2) for its importance in the current worldwide and European aquaculture industry, explained below. In this context, assessing the proposed strategies to reduce fat accumulation in an important aquaculture species, represents a significant advantage over other existing fish models such as zebrafish, from a production and economic value perspective, as it brings the strategies evaluated herein closer to the market, facilitating and accelerating the transfer of results to the industry.

Gilthead seabream can be found in the Mediterranean Sea and along the coasts of the Eastern Atlantic, ranging from Great Britain to Senegal, and rarely in the Black Sea. Due to its euryhaline and eurythermal nature, this species from the Sparidae family inhabits both marine and brackish water environments, including coastal lagoons and estuarine areas, especially during the early stages of its life cycle. Born in the open sea between October and December, juveniles usually migrate in early spring towards sheltered coastal waters, characterized by their abundant trophic resources and milder temperatures. This species is highly sensitive to low temperatures, with a lower lethal limit of 4 °C. Consequently, in late autumn, they return to the open sea, where adult fish breed. Gilthead seabream is a benthopelagic fish and in the open sea, it is commonly found in rocky areas and seagrass meadows, and on sandy bottoms.

Young individuals tend to stay in relatively shallow areas (up to 30 m), but adults may be found to 150 m depth (FAO, 2024). Regarding feeding habits, gilthead seabream is mainly carnivorous, having a diet largely based on molluscs, crustaceans, and small fish in the wild, even though it is accessorily herbivorous. In terms of reproductive biology, this species exhibits protandrous hermaphroditism, reaching sexual maturity as males during the first two years (20-30 cm) and the turning into females at the second or third year of life (33-40 cm). Females are batch spawners capable of laying 20,000-80,000 eggs daily for a period of up to 4 months. Spawning naturally takes place from December to April, with water temperatures of 13-17 ºC (Basurco et al., 2011).

Figure 12. Gilthead seabream *(Sparus aurata).*

Concerning aquaculture, this sparid species can be farmed in extensive and semi-intensive systems, in coastal ponds and lagoons, or in intensive farming systems, in land installations and sea cages. Commercially acceptable size can range from 250 g to over 1.5 kg. The duration of the culture period varies based on location and water temperature. Generally, it takes between 18 and 24 months to reach 400 g from hatched larvae. Farmed gilthead seabreams are fed commercial diets that usually consist of extruded pellets containing around 45–50% protein and 20% lipid levels. The main ingredients used in commercial diets have traditionally been fishmeal and fish oil, but due to their limited availability and increasing costs, aquafeed manufacturers are currently incorporating alternative and sustainable sources into marine fish diets (Basurco et al., 2011). In terms of production, gilthead seabream is one of the most farmed finfish species in marine and coastal aquaculture worldwide, with more than 282 thousand tonnes produced during 2020 (FAO, 2022). In Europe and the rest of the Mediterranean area, it reached a production of 320,630 tonnes in 2022, with Spain being the fifth country (after Turkey, Greece, Egypt, and Tunisia) with the highest production of gilthead seabream (8,932 tonnes in 2022) (APROMAR, 2023; Figure 13).

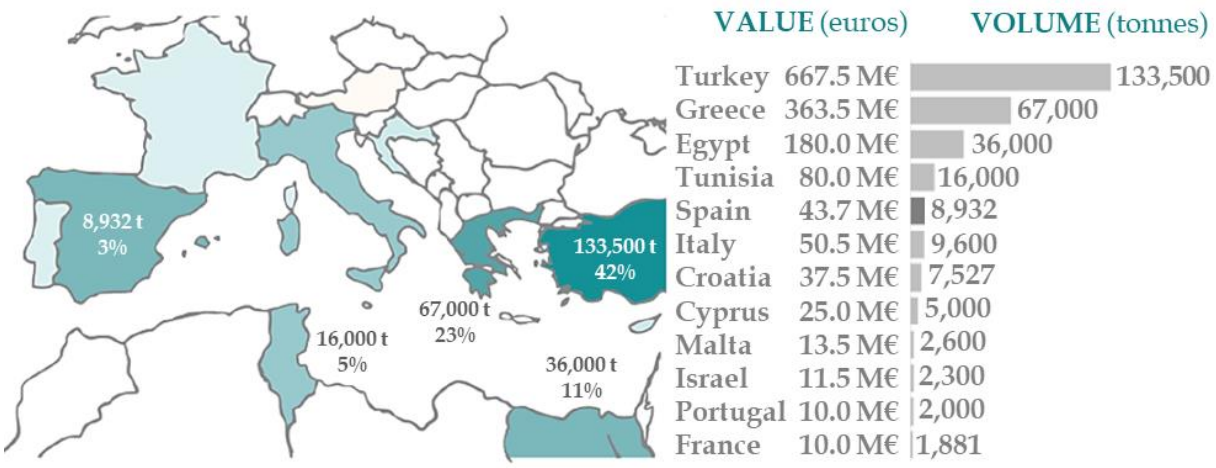

Figure 13. Distribution of aquaculture production of gilthead seabream (*Sparus aurata*) in the Mediterranean area in 2022 in volume (tonnes, "t") and value (million euros). The symbol % in the map indicates percentage of total aquaculture production of gilthead seabream. Adapted from APROMAR, 2023.

OBJECTIVES

Objectives

The general objective of the present thesis is to promote the reduction of fat accumulation in gilthead seabream (*Sparus aurata*) through the supplementation of its diets with a blend of bile salts (sodium cholate, sodium deoxycholate, and sodium taurocholate hydrate) and two different combinations of spices (capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde; and turmeric, capsicum, black pepper, and ginger oleoresins), as well as testing the feasibility of performing an intestinal microbiota transplant (IMT) from Atlantic salmon (*Salmo salar*) to gilthead seabream and its effect on gut microbial modulation.

The specific objectives are:

 To study the effect of the tested feed additives in key performance indicators (KPIs) associated to growth and feed performance in gilthead seabream..

 To evaluate the potential of the tested feed additives in reducing fat accumulation in gilthead seabream, through the study of the fillet and liver proximate composition, fatty acid profile, histomorphology of digestive tissues, and biomarkers related to lipid metabolism.

 To unravel the role of the tested feed additives in the regulation of the immune status in gilthead seabream, through the study of a selected set of biomarkers linked to epithelial integrity, barrier function, and immune response.

 To develop a methodological procedure to perform an IMT from Atlantic salmon (*Salmo salar*) to gilthead seabream, as a conceptual approach for future IMTs aimed at reducing fat accumulation through modulation of fish gut microbial communities.

 To study the modulation of gilthead seabream gut microbial communities by the feed additives and the IMT, by evaluating the diversity, structure, and composition of the gut microbiota.

DIRECTORS' REPORT

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La Ràpita, 6th May 2023

To whom it may concern,

The undersigned, Dr. Enric Gisbert and Dr. Karl B. Andree, codirectors of the thesis presented by Alberto Ruiz Hernández entitled "**Nutrition and gut microbiota modulation as tools for regulating fat accumulation in aquaculture fish**", confirm the role of the doctoral candidate as the main author of the five manuscripts published within this doctoral project as well as the veracity of the publication data for each of the manuscripts with regard to their impact factor and quartile (data retrieved from JCR® on the 2nd May 2024).

As supervisors of his doctorate, we are pleased to attest to Alberto's exemplary dedication and contribution to the research conducted within his thesis. Throughout the design, planning, and execution of all trials, Alberto has exhibited a high level of commitment and expertise. He has taken a proactive role in the analysis of biological samples obtained from each trial, demonstrating his proficiency in handling complex experimental procedures. Furthermore, Alberto has actively participated in the interpretation and discussion of results with the research team, showcasing his analytical skills and ability to critically evaluate scientific data. His insightful contributions have significantly enriched the research outcomes and facilitated a deeper understanding of the findings. It is noteworthy that Alberto's deep involvement in each study has earned him a position of prominence in the authorship of manuscripts. As the first and corresponding author in all five studies, Alberto has demonstrated leadership and ownership of the research outcomes. His leadership role reflects his exceptional grasp of the subject matter and his ability to effectively communicate research findings to the scientific community.

Overall, we commend Alberto for his outstanding performance and invaluable contributions to the research conducted within his doctoral thesis. His dedication, expertise, and leadership qualities have been instrumental in the success of the studies conducted, and we have full confidence in his continued excellence in his academic and scientific endeavors.

The details of the five publications of this thesis are presented as follows:

Chapter 1.
Ruiz, A, Andree, KB, Sanahuja, I, Holhorea, PG, Calduch-Giner, JÀ, Morais, S, Pérez-Sánchez, J, Gisbert, E. (2023). Bile salt dietary supplementation promotes growth and reduces body adiposity in gilthead seabream (*Sparus aurata*). *Aquaculture*, 566, 739203.
https://doi.org/10.1016/j.aquaculture.2022.739203
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Quartile: Q1; Decile: 1st
Category: MARINE & FRESHWATER BIOLOGY

Chapter 2.
Ruiz, A, Andree, KB, Furones, D, Holhorea, PG, Calduch-Giner, JÀ, Viñas M, Pérez-Sánchez, J, Gisbert, E. (2023). Modulation of gut microbiota and intestinal immune response in gilthead seabream (*Sparus aurata*) by dietary bile salt supplementation. *Frontiers in Microbiology*, 14, 1123716. https://doi.org/10.3389/fmicb.2023.1123716
Journal impact factor: 5.2
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Chapter 3.
Ruiz, A, Sanahuja, I, Andree, KB, Furones, D, Holhorea, PG, Calduch-Giner, JA, Pastor, JJ, Viñas, M, Pérez-Sánchez, J, Morais, S, Gisbert, E. (2023). The potential of a combination of pungent spices as a novel supplement in gilthead seabream (*Sparus aurata*) diets to aid in the strategic use of fish oil in aquafeeds: a holistic perspective. *Frontiers in Immunology*, 14, 1222173.
https://doi.org/10.3389/fimmu.2023.1222173
Journal impact factor: 7.3
Quartile: Q1; Decile: 3rd
Category: IMMUNOLOGY

Chapter 4.
Ruiz, A, Sanahuja, I, Andree, KB, Furones, D, Holhorea, PG, Calduch-Giner, JA, Pastor, JJ, Viñas, M, Pérez-Sánchez, J, Morais, S, Gisbert, E. (2024). Supplementation of gilthead seabream (*Sparus aurata*) diets with spices as a functional strategy to control excess adiposity through lipid, cholesterol and bile acid metabolism, and to induce an immunomodulatory intestinal regulation. *Aquaculture*, 581, 740378. https://doi.org/10.1016/j.aquaculture.2023.740378
Journal impact factor: 4.5
Quartile: Q1; Decile: 1st
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Chapter 5.
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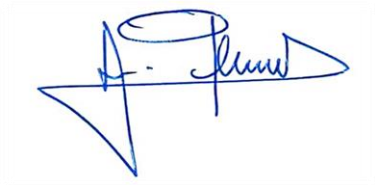
Journal impact factor: 4.6
Quartile: Q2; Decile: 4th
Category: MULTIDISCIPLINARY SCIENCES

In addition to the studies conducted within the doctoral project supported by ADIPOQUIZ project (RTI2018-095653-R-I00) funded by the the Ministerio de Ciencia, Innovación y Universidades (Spain), Alberto has done a research stage of three months (01/09/2022-01/12/2022) at the Wageningen University & Research (Aquaculture and Fisheries Subdivision) under the supervision and guidance of Dr. Fotini Kokou. His stage was focused on pursuing Alberto's training in microbiome studies and tools for microbiome analyses.



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PUBLICATIONS

CHAPTER 1

Bile salt dietary supplementation promotes growth and reduces body adiposity in gilthead seabream (*Sparus aurata*)

Aquaculture 566 (2023) 739203

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Bile salt dietary supplementation promotes growth and reduces body adiposity in gilthead seabream (*Sparus aurata*)

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ABSTRACT

The present study aimed to evaluate the effect of dietary supplementation using bile salts (BSs) on several key performance indicators like growth and feed performance, fat accumulation and tissue condition in gilthead seabream (*Sparus aurata*). A 90-day feeding trial was conducted in juveniles of gilthead seabream (initial body weight, BW = 44.0 ± 4.2 g) that were fed three isoproteic (44% crude protein), isolipidic (18% crude fat) and isoenergetic (21.4 MJ kg^{-1}) diets rich in saturated fats to favour lipid accumulation. One diet was used as a control and the others were supplemented with graded levels of a BS blend: 0.06 ($BS_{0.06\%}$) and 0.12% ($BS_{0.12\%}$). At the end of the trial, a significant increase in BW and a decrease in the levels of perivisceral fat were observed in fish fed the $BS_{0.06\%}$ diet. However, only the $BS_{0.12\%}$ diet significantly enhanced lipid digestibility, as indicated by the higher coefficient of apparent lipid digestibility and increased activity of the pancreatic bile salt-activated lipase. None of the diets modified the fatty acid profile of liver or fillet. In addition, while only the $BS_{0.06\%}$ diet decreased fat deposits in liver, both BS-supplemented diets reduced fat accumulation in the intestine. Furthermore, dietary BS inclusion modified the bile acid profile in the gallbladder and anterior intestine, increasing the content of taurodeoxycholic acid in both tissues and decreasing the content of taurochenodeoxycholic acid in the gallbladder when supplementing the diet at an inclusion level of 0.12% of the BS blend. The gene expression profile of liver was analysed by quantitative PCR, targeting biomarkers mainly related to lipid metabolism and antioxidant defense, and few differences were found among dietary treatments. It was noteworthy that the decrease of lipoprotein lipase expression in fish fed the $BS_{0.06\%}$ diet may be correlated with their reduced perivisceral fat and lipid accumulation in the liver, while the increase of the fatty acid synthase expression might help maintaining hepatic fatty acid levels stable. The higher gene expression of peroxiredoxin 5 was also noticeable and might be the cause of the lower catalase activity on the liver of fish fed the $BS_{0.06\%}$ diet. Summarizing, we recommend the 0.06% BS dose to enhance growth performance and reduce perivisceral, hepatic and intestinal fat of gilthead seabream without comprising their health, but further studies still need to be performed for deciphering the mechanisms by which BSs act in fish lipid metabolism.

1. Introduction

In aquaculture, lipids are used as the main source of energy in the diet, with fish oil being the most used source of lipids. In 2020, about 16 million tonnes of fish, which constituted 20% of capture fisheries in marine waters, were destined for fish meal and fish oil production (FAO, 2022). One of the main reasons for the widespread use of fish oil in aquaculture is the high amount of essential fatty acids that it contains and confers to farmed fish, especially of the n-3 long-chain polyunsaturated fatty acids (LC-PUFAs) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are necessary for the optimal growth, development, health, and reproduction of marine fish (Jaya-Ram et al., 2008; Peng et al., 2014). The non-existent or limited ability of many marine fish species to synthesize these types of fatty acids requires that they be supplied in the diet (Tocher, 2015). Since fish body lipid composition is directly affected by their consumption, the fatty acids

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from the diet are reflected in the fatty acid profile of the fillet, and consequently the fish flesh serves as a DHA and EPA source for the consumer (Ballester-Lozano et al., 2011; Trushenski et al., 2011). Consumption of fish with high DHA and EPA levels thus provides numerous human health benefits, such as lower risk of coronary heart disease, amelioration of symptoms of rheumatoid arthritis, growth and development of the brain, promotion of learning capacity, inhibition of tumour cell proliferation, prevention of thrombosis, arrhythmias and numerous other disorders and diseases (De Deckere et al., 2001; Horrocks and Yeo, 1999).

However, the limited availability and consequent increasing cost of fish oil, together with the sustainability problems that its use imposes (Tocher, 2015), have brought to the forefront other nutritional strategies during recent years. One of the most common is the partial or total substitution of fish oil by plant-based oils and, in some cases, rendered animal fats in the diets due to their lower price and higher availability (Kenari et al., 2011; Simó-Mirabet et al., 2018; Trushenski and Lochmann, 2009). As reviewed by Mozanzadeh et al. (2021), the replacement of fish oil by alternative lipid sources has been a successful strategy in terms of growth and feed performance indicators, although this strategy has some side-effects such as the modification of the fatty acid profile of the fillet by reducing the levels of n-3 LC-PUFA (Bell et al., 2010; Benedito-Palos et al., 2010; Kenari et al., 2011; Nasopoulou and Zabetakis, 2012), and the increased deposition of body fat (Ballester-Lozano et al., 2015; Bell et al., 2010). Furthermore, the use of other nutritional approaches consisting in decreasing the levels of dietary protein and increasing other energy sources, like lipids or carbohydrates (protein-sparing), have been reported to limit somatic growth, feed intake and feed conversion ratio (FCR), and induce some physiological disorders such as a higher susceptibility to lipid peroxidation and/or altered synthesis and secretion of lipoproteins, increased enzymatic lipogenesis activity and increased body fat deposition, among others (Du et al., 2006; Kousoulaki et al., 2015; Rueda-Jasso et al., 2004). These changes in levels of body adiposity have not only an impact on animal welfare, but also on aquaculture production, in terms of reduction of fillet yield and shelf-life, and changes to the organoleptic properties of the product, reducing the consumer appreciation and, consequently, its demand and marketability (Salmerón, 2018). Indeed, while maintenance of adipose tissue and fatty acid levels within a normal range are required for the edible part of the fish for achieving the expected flavour, taste, and texture to which the consumer is accustomed, the perivisceral fat has been noted to cause a negative visual impact on the consumer and to release a strong and unpleasant smell (Grigorakis, 2007), possibly because of its well-known rapid oxidation causing rancidity (Hsieh and Kinsella, 1989).

Dietary supplementation with bile salts (BSs) has successfully been used in poultry due to the limited capacity of young chicks to synthesize BSs (Siyal et al., 2017), as well as due to its demonstrated potential for increasing lipid digestion, decreasing fat deposition, improving growth performance and FCR, and improving meat quality (Arshad et al., 2021; Ge et al., 2019; Lai et al., 2018; Siyal et al., 2017). Regarding aquatic species, several studies have shown that the supplementation of BSs in compound diets for fish species led to similar results as those previously mentioned for poultry. In particular, the use of BSs as a feed additive for fish promoted growth performance (Ding et al., 2020; Gu et al., 2017; Iwashita et al., 2008; Jiang et al., 2018; Li et al., 2021; Yamamoto et al., 2007; Yu et al., 2019; Zhang et al., 2022; Zhou et al., 2018), improved lipid digestion (Ding et al., 2020; Gu et al., 2017; Iwashita et al., 2008; Jiang et al., 2018; Li et al., 2021; Yamamoto et al., 2007) and decreased body fat deposits (Iwashita et al., 2009; Jin et al., 2019; Yamamoto et al., 2007; Yin et al., 2021; Zhang et al., 2022).

Bile acids (BAs) are synthesized in the liver from cholesterol (hydrophobic portion) and conjugated with an amino acid, either glycine or taurine (hydrophilic portion), forming stronger acids, that when conjugated are amphipathic molecules, commonly called BSs (Frisch and Alstrup, 2018). Fish have low or no capacity to conjugate BAs with glycine, therefore most fish only produce BSs with conjugated taurine (Romano et al., 2020). BSs are stored in the gallbladder until feed ingestion and then secreted into the gut by bladder contractions stimulated by the hormone cholecystokinin (Le et al., 2019). In the intestinal lumen, they have a key role in the emulsification of fat aggregates through the formation of micelles, thereby creating a higher action surface for the lipase, as well as activation of the lipase/co-lipase complex (Romano et al., 2020). In the intestine, a fraction of primary BSs is deconjugated, and chenodeoxycholic acid (CDCA) and cholic acid (CA) are metabolized into secondary BAs, lithocholic acid (LCA) and deoxycholic acid (DCA) (Schubert et al., 2017). Only a small portion of BSs is excreted in the faeces, while the majority of conjugated and deconjugated BSs are reabsorbed through the apical surface of the enterocytes and return to the liver by enterohepatic circulation (Romano et al., 2020).

The objective of the present study was to evaluate the effect of dietary supplementation with a blend of BSs (sodium cholate, sodium deoxycholate and sodium taurocholate hydrate) on fish growth and feed performance, as well as the potential effects of the use of BSs as feed additives in order to reduce body adiposity, using the gilthead seabream (*Sparus aurata*) as a model fish species.

2. Material and methods

2.1. Animals and rearing conditions

Juveniles of gilthead seabream with an initial body weight (BW_i) of 44.0 ± 4.2 g (mean ± standard deviation) and standard length (SL) of 12.13 ± 0.48 cm were purchased from Piscicultura Marina Mediterranea SL (Andromeda Group, Valencia, Spain), and transported to the Institute of Agrifood Research and Technology (IRTA) in la Ràpita (Tarragona, Spain). Then, fish were randomly assigned to 12 tanks with a capacity of 450 L (30 fish per tank; initial density of 3 kg m^{-3}) connected to a water recirculation system (IRTAmar™), where they acclimatized for two weeks. Each tank had a sedimentation column for faeces collection. Water temperature (22.5 ± 0.5 °C), dissolved oxygen (6.3 ± 0.2 mg L^{-1}) (OXI330, Crison Instruments, Spain) and pH (7.6 ± 0.01) (pH meter 507, Crison Instruments) were monitored daily and ammonia (0.22 ± 0.08 mg NH_4^+ L^{-1}), nitrite (0.16 ± 0.1 mg NO_2^- L^{-1}) levels (HACH DR 900 Colorimeter, Hach Company, Spain) and salinity (36‰) (MASTER-20 T Hand-Held Refractometer, ATAGO Co. Ltd., Italy) were weekly controlled.

2.2. Ethics statement

All animal experimental procedures were carried out following the Spanish legislation (law 32/2007 and Royal Decree 1201/2015) and the Guiding Principles for Biomedical Research Involving Animals (EU2010/63) and approved by the Ethical Committee of IRTA, which adopts "The European Code of Conduct for Research Integrity", and by the Generalitat of Catalunya (CEEA 219/2020).

2.3. Diets

Three isoproteic (44% crude protein), isolipidic (18% crude fat) and isoenergetic (21.4 MJ kg^{-1}) experimental extruded diets (3 mm) were manufactured by Sparos Lda. (Portugal) as described in Vallejos-Vidal et al. (2022). A basal diet (control) was formulated based on fish commercial feed, but partially replacing fish oil and soybean oil with poultry fat to increase the levels of saturated fatty acids in the diet (up to 27% of total fatty acids), which is known to promote adiposity (Legrand and Rioux, 2010). The formulation of the two other diets were the same as the control but supplemented with a blend of BSs at an inclusion level of 0.06% ($BS_{0.06\%}$) and 0.12% ($BS_{0.12\%}$), respectively (Table 1). The BS blend was added in the feed prior to extrusion, and was composed of a powder BS mixture containing equal parts of sodium cholate and sodium

Table 1
Formulation, proximate composition and fatty acid profile of experimental diets: a control and two basal diets supplemented with a blend of bile salts (BSs) at a dietary inclusion level of 0.06 ($BS_{0.06\%}$) and 0.12% ($BS_{0.12\%}$).

Ingredients (%)	Control	$BS_{0.06\%}$	$BS_{0.12\%}$
Fishmeal Super Prime	7.50	7.50	7.50
Fishmeal 60	5.00	5.00	5.00
Fish protein concentrate	2.00	2.00	2.00
Feathermeal hydrolysate	5.00	5.00	5.00
Porcine blood meal	3.00	3.00	3.00
Poultry meal	15.00	15.00	15.00
Aminopro NT70 - *C. glutamicum*	4.00	4.00	4.00
Corn gluten meal	8.00	8.00	8.00
Soybean meal 48	12.00	12.00	12.00
Sunflower meal	5.00	5.00	5.00
Wheat meal	10.31	10.31	10.31
Whole peas	5.00	5.00	5.00
Pea starch (raw)	2.40	2.40	2.40
Fish oil	3.02	3.02	3.02
Soybean oil	2.35	2.35	2.35
Poultry fat	8.04	8.04	8.04
Vitamin and mineral premix	1.00	1.00	1.00
Vitamin C35	0.05	0.05	0.05
Vitamin E50	0.02	0.02	0.02
Betaine HCl	0.20	0.20	0.20
Choline chloride 60	0.10	0.10	0.10
Antioxidant	0.20	0.20	0.20
Sodium propionate	0.10	0.10	0.10
Monoammonium phosphate	0.35	0.35	0.35
L-Tryptophan	0.15	0.15	0.15
DL-Methionine	0.20	0.20	0.20
Bile salts mix	–	0.06	0.12
Yttrium oxide	0.02	0.02	0.02
Proximate composition			
Crude protein, %	44.1 ± 0.05	44.0 ± 0.08	44.1 ± 0.11
Crude fat, %	18.1 ± 0.04	18.2 ± 0.05	18.2 ± 0.01
Gross energy, MJ kg^{-1}	21.4 ± 1.11	21.5 ± 1.20	21.5 ± 1.37
Fatty acid profile (% of total fatty acids)*			
Saturated fatty acids (SFAs)	27.19 ± 0.40	26.55 ± 0.06	27.65 ± 0.31
Monounsaturated fatty acids (MUFAs)	36.61 ± 0.73	36.60 ± 0.22	36.19 ± 0.51
n-6 Polyunsaturated fatty acids (n-6 PUFAs)	26.65 ± 0.06	27.11 ± 0.45	26.32 ± 0.15
n-3 Polyunsaturated fatty acids (n-3 PUFAs)	9.55 ± 0.39	9.74 ± 0.09	9.84 ± 0.21
Total PUFAs	36.20 ± 0.45	36.86 ± 0.36	36.16 ± 0.36

* Fatty acid profile of experimental diets is detailed in Supplementary Table S1. The proximate and fatty acid composition of diets was analysed in duplicate; values are represented as mean ± standard deviation (SD).

deoxycholate (ref. 48,305, Sigma-Aldrich, USA), and sodium taurocholate hydrate (ref. 86,339, Sigma-Aldrich) in a proportion of 30/70 respectively. This composition was formulated based on the BS profile of perciform fish (Hagey et al., 2010). Yttrium oxide (Y_2O_3, Sigma Aldrich, Spain) was included in the diets at 0.2 g kg^{-1} as an inert marker for proper determination of apparent digestibility coefficients (ADCs) for macronutrients.

2.4. Feeding trial and fish sampling

Each experimental diet was randomly assigned to four replicate tanks. During the trial, which lasted 90 days, fish were fed by Arvo-Tec T Drum 2000 automatic feeders (Arvo-Tec Oy, Finland) in 12 meals spread over an hour (one each 5 min) two times per day, with a total daily rate of 3.0% of fish tank biomass. The feeding rate was regularly adjusted depending on the amount of uneaten feed pellets recovered from the bottom of the tank two hours after each meal, in order to guarantee that feed was offered in excess (Salomón et al., 2020). To evaluate diet digestibility, faeces from each tank were collected from the sedimentation columns during three consecutive days and pooled for further biochemical and yttrium analyses. Once a month, all fish in each tank were anesthetized with 100 mg L^{-1} of buffered tricaine methanesulfonate (MS-222, Sigma-Aldrich, Spain) and their somatic growth in terms of BW (g) and SL (cm) were measured.

At the end of the trial fish were fasted for 48 h following the guidelines of Naya-Català et al. (2021), then netted and anesthetized for measuring BW_f and SL_f, and the following growth performance parameters were calculated: specific growth rate (SGR, % day^{-1}) = 100 x (ln BW_f − ln BW_i) / days; Fulton's condition factor (K) = 100 x BW_f / SL_f^3. Feed intake was calculated as (FI, g $fish^{-1}$) = total feed intake per tank (g) / number of fish; and feed performance was also evaluated through the feed conversion ratio (FCR) = feed intake (g) / biomass increase (g). Six randomly selected animals from each tank were euthanized with an overdose of MS-222 (300 mg L^{-1}), while the rest of individuals were returned to their respective tanks. Euthanized animals (24 per treatment) were eviscerated, and the perivisceral fat was carefully separated from the digestive tract with a scalpel and weighed for assessing the perivisceral fat index, which is defined as PVFI (%) = 100 x weight of perivisceral fat (g) / BW (g). The liver was also extracted and weighed for calculating the hepatosomatic index (HSI, %) defined as = 100 × weight of the liver (g) / BW (g). Then, livers and dissected fillets of three fish per tank ($n = 12$ per diet) were stored in hermetically-sealed bags at −20 °C for proximate composition analysis. A section of the anterior intestine (AI) (4 cm) and liver (1.5–2 cm^2) were removed from three euthanized fish and fixed in 10% buffered formalin (pH 7.2) for histological analyses. The rest of the liver was divided into parts and stored at −80 °C ($n = 3$ per tube; 4 replicate tubes per treatment) for subsequent study of oxidative stress and hepatic metabolism biomarkers. The gallbladders of four randomly selected fish per tank from those previously slaughtered were dissected and ruptured with a blunt scalpel, and the contents coming from all animals of the same tank were collected into a tube and stored at −80 °C. Small segments (ca. 1 cm^2) of the liver of two individuals per tank (8 per treatment) were dissected and separately immersed in 5 volumes of RNAlater® (Sigma-Aldrich, USA), incubated overnight at 4 °C, and stored at −80 °C until RNA extraction.

The remaining fish in the tanks were fed for three days to restore their non-fasted physiological condition. Subsequently, 2 h after the last feeding, four fish from each tank (16 per treatment) were randomly selected and anesthetized for blood collection via caudal vein with 1 ml heparinized syringes (ref. 303,179, BD Plastik, Canada). Plasma of each fish was individually isolated after blood centrifugation (1600 xg, 10 min, 4 °C). Then, these fish were euthanized with an overdose of anaesthetic. For the analysis of intestinal BS profile, the AI was extracted and stripped gently, making a pool of the gut luminal content of the four fish per tank. For evaluating the effect of experimental diets on digestive enzyme activities, the digestive tract of an additional four euthanized animals per tank was dissected into two discrete regions (stomach with pyloric caeca, and foregut) and frozen at −80 °C until their analyses. The liver of two randomly selected slaughtered individuals per tank were dissected for RNA extraction following the previously mentioned procedure, to compare the gene expression profile of selected hepatic gene markers (Table 2) in 48 h fasted-animals and 2 h postprandial-animals.

2.5. Biochemical analyses

A pool of three livers per tank were homogenized using an Ultra Turrax® homogenizer (IKA T25 digital ULTRA-TURRAX, IKA Works, USA), whereas the three fillets per tank were processed separately. Ash content was determined according to AOAC (1990), and protein, carbohydrates, and total fat levels were estimated using the method of respectively, Lowry et al. (1951), Dubois et al. (1956) and Folch et al. (1957). Fatty acid analysis was carried out as described by Skalli et al.

Table 2
PCR-array layout for gene expression profile in the liver of gilthead seabream fed experimental diets.

Function	Gene	Symbol	GenBank
Fatty acids, cholesterol & phospholipid metabolism	Fatty acid synthase	*fasn*	JQ277708
	Elongation of very long chain fatty acids 1	*elovl1*	JX975700
	Elongation of very long chain fatty acids 4	*elovl4*	JX975701
	Elongation of very long chain fatty acids 5	*elovl5*	AY660879
	Elongation of very long chain fatty acids 6	*elovl6*	JX975702
	Fatty acid desaturase 2	*fads2*	AY055749
	Stearoyl-CoA desaturase 1a	*scd1a*	JQ277703
	Stearoyl-CoA desaturase 1b	*scd1b*	JQ277704
	Cholesterol 7-alpha-monooxygenase	*cyp7a1*	KX122017
	Phospholipid transfer protein	*pltp*	XM_030418561
Lipases	Adipose triglyceride lipase	*atgl*	JX975711
	Hepatic lipase	*hl*	EU254479
	Lipoprotein lipase	*lpl*	AY495672
	85 kDa calcium-independent phospholipase A2	*pla2g6*	JX975708
Transcription factors & nuclear receptors	Hepatocyte nuclear factor 4 alpha	*hnf4a*	FJ360721
	Sterol regulatory element-binding proteins 1	*srebp1*	JQ277709
	Sterol regulatory element-binding protein 2	*srebp2*	XM_030408996
	Farnesoid X receptor	*fxr*	XM_030426192
	Liver X receptor α	*lxrα*	FJ502320
	Peroxisome proliferator-activated receptor α	*pparα*	AY590299
	Peroxisome proliferator-activated receptor β	*pparβ*	AY590301
	Peroxisomeproliferator-activated receptor γ	*pparγ*	AY590304
Oxidative metabolism & energy sensing	Carnitine palmitoyltransferase 1A	*cpt1a*	JQ308822
	Hydroxyacyl-CoA dehydrogenase	*hadh*	JQ308829
	Fatty acid translocase/CD36	*fat/cd36*	XM_030440140
	Fatty acid binding protein, heart	*h-fabp*	JQ308834
	Citrate synthase	*cs*	JX975229
	NADH-ubiquinone oxidoreductase chain 2	*nd2*	KC217558
	NADH-ubiquinone oxidoreductase chain 5	*nd5*	KC217559
	Cytochrome c oxidase subunit I	*coxi*	KC217652
	Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1α*	JX975264
	Sirtuin1	*sirt1*	KF018666
	Sirtuin2	*sirt2*	KF018667
Antioxidant defense	Catalase	*cat*	JQ308823
	Uncoupling protein 1	*ucp1*	FJ710211
	Glutathione peroxidase 1	*gpx1*	DQ524992
	Glutathione peroxidase 4	*gpx4*	AM977818
	Peroxiredoxin 3	*prdx3*	GQ252681
	Peroxiredoxin 5	*prdx5*	GQ252683
	Superoxide dismutase [Cu—Zn]	*cu-zn-sod / sod1*	JQ308832
	Superoxide dismutase [Mn]	*mn-sod / sod2*	JQ308833
	Glucose-regulated protein, 170 kDa	*grp-170*	JQ308821
	Glucose-regulated protein, 94 kDa	*grp-94*	JQ308820
	Glucose-regulated protein, 75 kDa	*grp-75*	DQ524993

(2020). In brief, after transesterification of fatty acids (Christie, 2012), gas-liquid chromatography allowed their identification and quantification, using heneicosanoic acid (21:0) as internal standard.

Inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7700 Series, Agilent Technologies, USA) was used to determine Y_2O_3 levels in diets and faeces (Garatun-Tjeldstø et al., 2006). Lipid and protein proximate composition from faeces was also performed to calculate their ADCs according to Lupatsch et al. (1997), as ADC of nutrient (%) = 100 − [100 × (Y_2O_3 in diet / Y_2O_3 in faeces)] x [(% nutrient in faeces / % nutrient in diet)].

2.6. Histological analysis

Fixed samples of liver and AI (ca. 0.5–1 cm^2) were dehydrated in increasing-concentration of ethanol solutions, cleared with xylene (MYR STP 120, Especialidades Médicas Myr, Spain) and embedded in paraffin (MYR EC-350, Especialidades Médicas Myr). Serial sections (4 μm thickness) were obtained with a microtome (Leica RM2155, Leica Microsystems, Germany) and stained with haematoxylin and eosin using automatic staining equipment (MYR MYREVA SS-30, Especialidades Médicas Myr). Sections were examined under a light microscope (Leica DM LB, Leica Microsystems) and photographed (Olympus DP70 Digital Camera, Olympus Europa, Germany). The software Analysis (Olympus Soft Imaging Solutions, Germany) was used for measuring the thickness of AI musculature, villus height, height of enterocytes and density of goblet cells in the intestinal mucosa (Gisbert et al., 2015). Inflammation and accumulation of fat deposits were also evaluated in both tissues under blinded and order-randomized histological examination by two different persons according to Meyerholz and Beck (2018). The level of fat accumulation was semi-quantitatively classified from 1 to 5 as described in Fig. 1A and 2A (Gisbert et al., 2008).

2.7. Hepatic oxidative stress and metabolic biomarkers

A pool of three liver pieces (ca. 60 mg per liver) per tank were homogenized (IKA T25 digital ULTRA-TURRAX, IKA Works) in 5 volumes *v/w* of buffer (150 mM KCl, 1 mM EDTA, pH 7.4), centrifuged (9000 xg, 30 min, 4 °C), and the supernatant was collected for hepatic anti-oxidative status analysis. Glutathione reductase (GR) activity was measured at λ = 340 nm using NADPH (0.09 mM) as cofactor and disulphide glutathione (GSSG, 0.9 mM) as substrate (Carlberg and Mannervik, 1975). Catalase (CAT) activity was measured at λ = 240 nm, with H_2O_2 (50 mM) as substrate (Aebi, 1974). Superoxide dismutase (SOD) activity was determined by means of a commercial kit (ref. 19,160, Sigma-Aldrich, USA) based on the McCord and Fridovich (1969). Thiobarbituric acid reactive substances (TBARs) were used to estimate (λ = 586 nm) lipid peroxidation (LPO) levels (Solé et al., 2004). Total antioxidant capacity (TAC) was quantified at λ = 570 nm following the manufacturer's instructions of the Total Antioxidant Capacity Assay Kit (ref. MAK187, Sigma-Aldrich, USA). Enzyme activities and lipid peroxidation levels were normalized to soluble protein (Bradford, 1976) with the exception of SOD activity that was expressed in % of enzyme inhibition. All assays were read in triplicate at 25 °C using 96-well microplates (ref. 467,340, Thermo Scientific, Denmark) by UV/Vis spectrophotometer (Tecan Infinite M200 Plate Reader, Tecan, Switzerland) and analysed with the specific software Magellan™ (v6, Tecan).

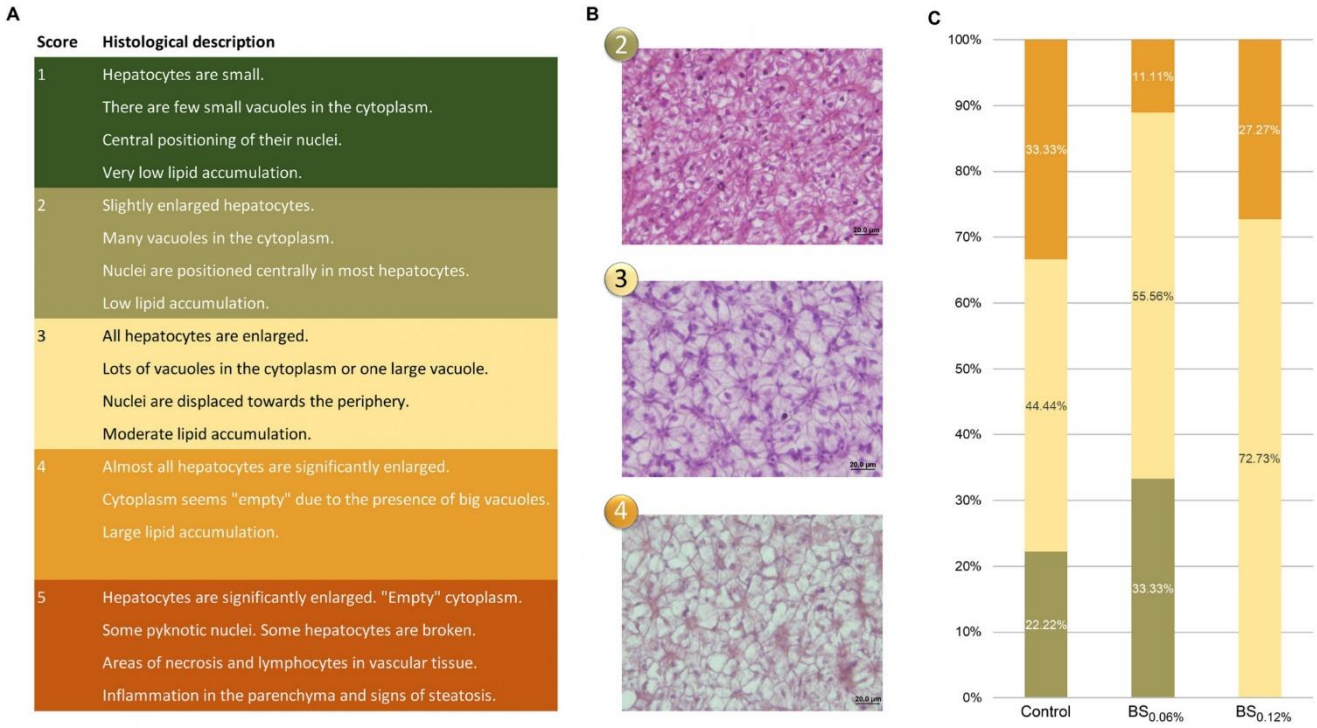

Fig. 1. (A) Semi-quantitative scoring used for evaluating the level of fat accumulation in the liver of gilthead seabream fed the experimental diets. (B) Examples of light microscope images obtained from livers of gilthead seabream classified with a score of 2, 3 and 4. (C) Results (%) of liver scoring from gilthead seabream fed a control and two diets supplemented with a blend of bile salts at a dietary inclusion level of 0.06 ($BS_{0.06\%}$) and 0.12% ($BS_{0.12\%}$).

For the analysis of metabolic biomarkers, pools of three livers (ca. 100 mg each) per tank were homogenized in 9 volumes of a buffer (pH = 7.6) composed of 1:1 *v/v* of a lysis solution (1.24 mM Triton X-100, 1 mM EDTA, 1 mM $NaHCO_3$) and a stabilizer solution (3.7 mM EDTA, 5 mM β-mercaptoethanol). Homogenates were centrifuged (5000 x*g*, 10 min, 4 °C) and enzymatic activities of lactate dehydrogenase (LDH), glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) were measured utilizing commercial kits (LDH-LQ, ref. 41,222; GOT (AST)-LQ, ref. 41,272; GPT (ALT)-LQ, ref. 41,282, respectively; Spinreact, Spain). Spinreact kits use as respective substrates pyruvate, L-aspartate and L-alanine, as described by Bergmeyer and Bernt (1974a, 1974b, 1974c). Methodological triplicates were measured (λ = 340 nm, 25 °C) with a spectrophotometer as previously described.

2.8. Plasmatic biomarkers

Cholesterol, triacylglycerides (TAGs), albumin, total proteins, GOT, GPT and total alkaline phosphatase in plasma (from four fish per tank) were quantified by reflectance spectrophotometry UV/Vis (Laboratorio Echevarne, Barcelona, Spain). Total globulin in plasma was calculated as the difference between total protein and albumin.

2.9. Bile sample analysis

Bile samples were first diluted 1/2000 in water and 100 μL of diluted bile were extracted with 400 μL of ACN (acetonitrile), including the internal standard, and vortexed. Lyophilized samples of intestinal digesta were homogenized (TissueLyzer II, QIAGEN, Germany), from which 20 mg were extracted with 800 μL of H_2O: ACN (1:1), including internal standard. After homogenization, bile and intestinal digesta samples were centrifuged (15,000 x*g*, 10 min, 4 °C) and the supernatant was diluted in H_2O: ACN (1:1). The BA profile was analysed by Ultra-Performance Liquid Chromatography connected to a Quadrupole Time-of-Flight Mass Spectrometry detector, with chenodeoxycholic acid-d_4 as internal standard, as described by Herrero-Encinas et al. (2020).

2.10. Activity of pancreatic digestive enzymes

Samples of the stomach with pyloric caeca and foregut (pool of four individuals) were homogenized in 5 volumes *v/w* of distilled water (4 °C) using an Ultra Turrax® homogenizer (IKA T25 digital ULTRA-TURRAX, IKA Works, USA) and centrifuged (3300 x*g*, 3 min at 4 °C) for removing cell debris. The supernatant was collected, aliquoted and frozen at −80 °C for the determination of the main pancreatic enzymes (Gisbert et al., 2009). Samples were processed and handled following the indications of Solovyev and Gisbert (2016) in order to prevent their degradation during storage and handling.

Total alkaline proteases were assayed using 0.5% (*w/v*) azo-casein as substrate in 50 mM Tris-HCl buffer (pH 8.0). One unit of total alkaline proteases per mL (U) was defined as 1 μmol azocasein hydrolyzed per min and mL of extract at λ = 366 nm (García-Carreño and Haard, 1993). Regarding pancreatic carbohydrases, α-amylase was measured using 0.3% soluble starch dissolved in Na_2HPO_4 buffer (pH 7.4) as substrate and its activity (U) was defined as the mg of starch hydrolysed per min and mL of extract (λ = 580 nm) (Métais and Bieth, 1968). Bile salt-activated lipase activity was determined according to Iijima et al. (1998) using *p*-nitrophenyl myristate as substrate in 0.25 mM Tris-HCl (pH 7.9), 0.25 mM 2-methoxyethanol and 5 mM sodium cholate buffer. The activity of bile salt-activated lipase (U) was defined as the μmol of substrate hydrolysed per min and mL of extract (λ = 405 nm). Enzyme activities were expressed as total specific activity (U mg $protein^{-1}$) and soluble protein in enzyme extracts was quantified by means of the Bradford's method, using bovine serum albumin as standard (Bradford, 1976). The activity of all enzymes was determined at 25 °C in triplicate per sample (methodological replicates) using a spectrophotometer (Tecan Infinite M200 Plate Reader).

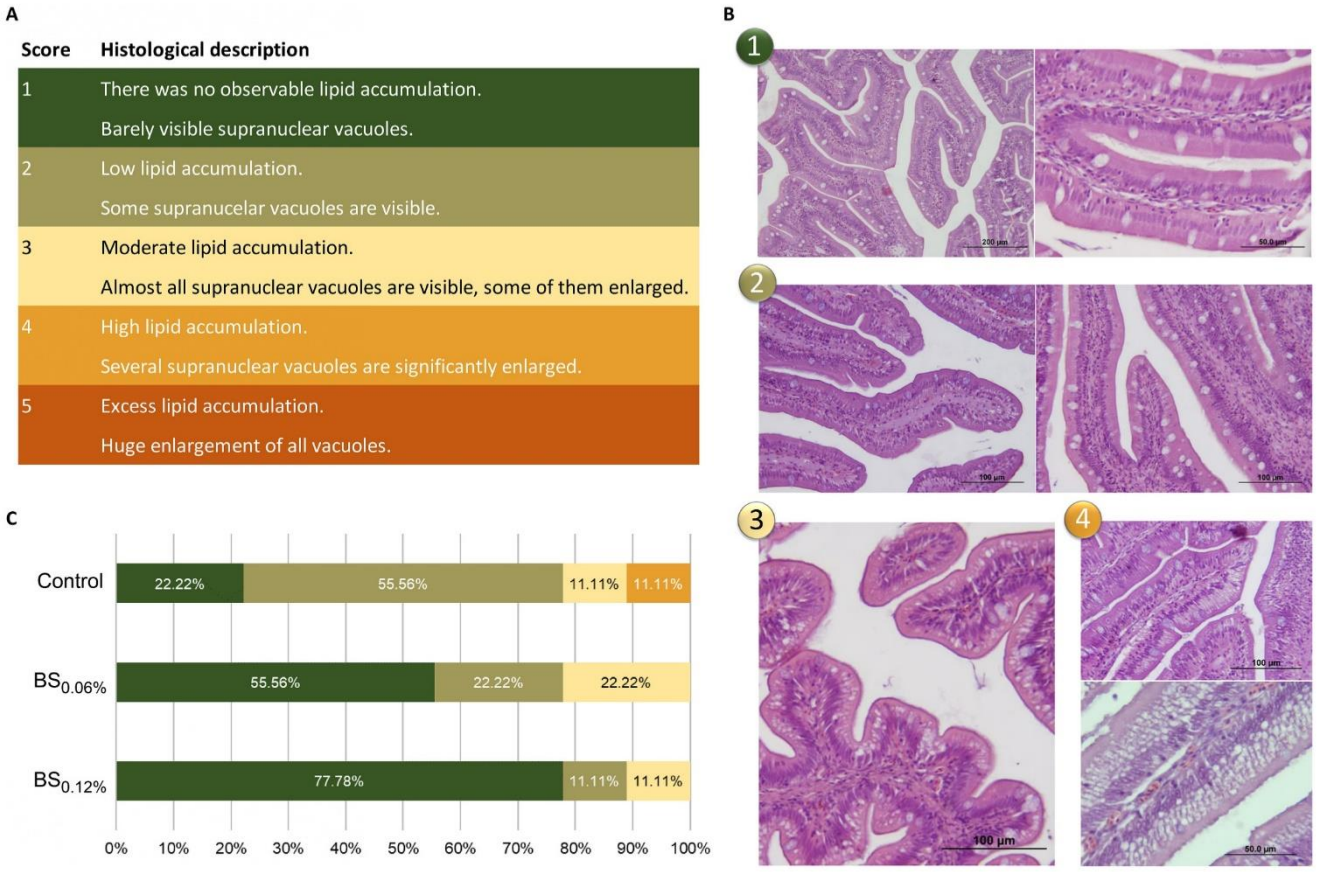

Fig. 2. (A) Semi-quantitative scoring used for evaluating the level of fat accumulation in the anterior intestine (AI) of gilthead seabream fed the experimental diets. (B) Examples of light microscope images obtained from AI of gilthead seabream classified with a score of 1, 2, 3 and 4. (C) Results (%) of AI scoring from gilthead seabream fed a control and two diets supplemented with a blend of bile salts at a dietary inclusion level of 0.06 ($BS_{0.06\%}$) and 0.12% ($BS_{0.12\%}$).

2.11. Gene expression profile of liver

RNA from liver was extracted using TRI Reagent (Sigma-Aldrich, USA) according to the manufacturer's instruction. RNA quantity and purity were measured in a Nanodrop-2000® spectrophotometer (Thermo Fisher Scientific, USA). The RNA yield was 20–100 ng/μL, with absorbance ratio (A260/A280) in the range of 1.9–2.1, and RNA integrity was verified through agarose gel electrophoresis. For cDNA synthesis, the High-Capacity cDNA Archive Kit (Applied Biosystems, USA) was used following the manufacturer's protocol, with an initial input of 500 ng of RNA. Reactions without reverse transcriptase were also run as a negative control.

Real-time quantitative PCR (qPCR) was carried out with a CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad, USA) as previously described by Naya-Català et al. (2021), using a 96-well PCR array layout designed for the simultaneous analysis of the profile of a panel of 44 genes (Table 2). Specific primer pair sequences are listed in Supplementary Table S2. Gene expression was calculated using the delta-delta Ct method (Livak and Schmittgen, 2001). The endogenous control gene *β-actin* was tested for gene expression stability using GeNorm software (M score = 0.21) and used in the normalization procedure. For multigene analysis, all values were referenced to the expression levels of *grp-170* of fish fed the control diet, with an arbitrary assigned value of 1.

2.12. Statistical analyses

Normal distribution (Shapiro-Wilk test) and homoscedasticity (Levene's test) of data were verified. According to this, to check differences among groups ($P < 0.05$), for parametric data a one-way ANOVA followed by Tukey's test for multiple comparison between groups, was performed; while for non-parametric data, a Kruskal-Wallis analysis was used, with Dunn's *post-hoc* test. These statistical analyses were performed with the software SigmaPlot version 14.5 (Systat Software Inc., USA). Data on gene expression was analysed by one-way ANOVA, with P set to 0.05 for determination of significance. Normality of the data was verified by Shapiro–Wilk test, and the Holm-Sidak post-test was used for multiple comparisons among groups. Analysis of the interaction between the diets and nutritional status were evaluated performing a two-way ANOVA, followed by a Holm-Sidak test. To study the separation among dietary groups and nutritional status, a supervised partial least squares-discriminant analysis (PLS-DA) based on statistically significant genes ($P < 0.1$) was applied using EZinfo v.3.0 (Umetrics, Sweden). Hotelling's T^2 statistic was calculated by the multivariate software package EZinfo v3.0. The confidence limit for T^2 was established at 95% and above points were discarded. The quality of the PLS-DA model was evaluated by the parameters R2Y(cum) and Q2(cum), which indicate the fit and prediction ability, respectively. A permutation test was used to validate the model by means of the *opls* function from the *ropls* R package.

3. Results

3.1. Key performance indicators, somatic indices and apparent digestibility coefficients

Results on growth and feed performance, somatic indices and ADCs

are summarized in Table 3. In brief, gilthead seabream fed the $BS_{0.06\%}$ diet were heavier and longer, and displayed higher SGR values than those fed the control diet ($P < 0.05$), whereas fish from the $BS_{0.12\%}$ diet showed intermediate values. Regarding somatic indices, whereas no differences were found for the Fulton's condition factor (K) and HSI among groups ($P > 0.05$), PVFI was affected by BS inclusion in the diet, significantly decreasing at a concentration of 0.06% ($P < 0.05$).

Regarding feed performance indicators, there were no significant differences in FI nor FCR among groups ($P > 0.05$). Values of ADC of lipids were higher in animals fed the $BS_{0.12\%}$ diet in comparison to the other experimental groups ($P < 0.05$; Table 3), while the supplementation of BSs in experimental diets had no effect on ADC of proteins ($P > 0.05$).

3.2. Fillet and liver proximate composition and fatty acid profiles

There were no significant differences in proximate composition of the fillet and liver among experimental groups (Table 4; $P > 0.05$), nor in the liver or fillet fatty acid profiles (Tables 5 and 6, respectively; $P > 0.05$).

3.3. Histological analyses of target tissues

The hepatic parenchyma of examined specimens showed a typical organization that consisted of polyhedral hepatocytes arranged along hepatic sinusoids and veins. In the evaluation of hepatic fat accumulation, only scores ranging from 2 to 4 were observed in this study. In particular, fish fed the $BS_{0.06\%}$ diet had reduced lipid deposits with respect to fish fed the other diets (Fig. 1). Regarding the AI, scoring ranged from 1 to 4. In this case, the largest observed decrease in intestinal fat deposits was in animals fed the $BS_{0.12\%}$ diet in comparison to the control diet, although in fish fed the $BS_{0.06\%}$ diet a reduction in the levels of fat deposits was also seen (Fig. 2). Otherwise, no inflammation in the liver nor in the AI was observed under any of the dietary treatments. Regarding morphometrical variables measured in the AI, there were no statistically significant differences among dietary groups (Supplementary Table S3; $P > 0.05$) with values for musculature thickness that ranged between 87.4 and 113.5 μm, for villus height ranging from 736.3 to 797.1 μm and the height of enterocytes between 31.4 and 35.7 μm. Similarly, there were no differences in goblet cell density among treatments with values ranging from 3.2 to 4.0 cells 100 μm^{-1} ($P > 0.05$).

Table 3
Growth and feed performance indicators, somatic condition indices and apparent digestibility coefficients of gilthead seabream fed a control and two diets supplemented with a blend of bile salts at a dietary inclusion level of 0.06 ($BS_{0.06\%}$) and 0.12% ($BS_{0.12\%}$).

	Control	$BS_{0.06\%}$	$BS_{0.12\%}$
Key Performance Indicators			
BW_i (g)	44.05 ± 0.04	44.03 ± 0.07	43.98 ± 0.05
SL_i (cm)	12.04 ± 0.12	12.19 ± 0.15	12.14 ± 0.06
BW_f (g)	215.80 ± 1.06^{a}	221.21 ± 3.10^{b}	220.35 ± 3.32^{ab}
SL_f (cm)	19.32 ± 0.21^{a}	19.65 ± 0.10^{b}	19.69 ± 0.22^{ab}
SGR (% day^{-1})	1.81 ± 0.01^{a}	1.83 ± 0.02^{b}	1.83 ± 0.02^{ab}
FI (g $fish^{-1}$)	195.89 ± 8.70	200.30 ± 3.94	197.21 ± 2.51
FCR	1.21 ± 0.05	1.19 ± 0.05	1.16 ± 0.03
Somatic Indices			
K	3.00 ± 0.11	2.92 ± 0.02	2.89 ± 0.08
HSI (%)	1.84 ± 0.11	1.94 ± 0.17	1.91 ± 0.05
PVFI (%)	3.01 ± 0.28^{a}	2.58 ± 0.19^{b}	2.67 ± 0.12^{ab}
Apparent Digestibility Coefficients (ADC)			
Lipid ADC (%)	81.60 ± 0.96^{a}	82.51 ± 2.03^{a}	86.50 ± 1.54^{b}
Protein ADC (%)	79.41 ± 3.60	78.33 ± 1.70	78.82 ± 5.31

Values are represented as mean ± SD and differences among groups are indicated by the different superscript letters (one-way ANOVA, $P < 0.05$).

Table 4
Proximate composition (%) of dry mass from the liver and the fillet of gilthead seabream fed a control and two experimental diets supplemented with a blend of bile salts at a dietary inclusion level of 0.06 ($BS_{0.06\%}$) and 0.12% ($BS_{0.12\%}$).

	Control	$BS_{0.06\%}$	$BS_{0.12\%}$
Liver			
Protein (%)	21.72 ± 1.35	21.63 ± 2.72	22.58 ± 1.72
Lipid (%)	42.62 ± 3.12	40.04 ± 2.78	38.79 ± 2.81
Carbohydrates (%)	24.39 ± 2.47	26.51 ± 3.07	27.51 ± 1.37
Ash (%)	2.70 ± 0.19	2.72 ± 0.35	3.64 ± 0.76
Fillet			
Protein (%)	79.81 ± 0.78	80.00 ± 0.43	79.84 ± 1.04
Lipid (%)	12.97 ± 1.75	13.24 ± 0.74	12.67 ± 2.26
Carbohydrates (%)	0.98 ± 0.15	0.87 ± 0.11	0.79 ± 0.08
Ash (%)	6.96 ± 0.42	7.20 ± 1.25	7.58 ± 0.82

Values are represented as mean ± SD.

Table 5
Fatty acid composition (mg g $lipid^{-1}$) on liver of gilthead seabream fed the control and two diets supplemented with a blend of bile salts at a dietary inclusion level of 0.06 ($BS_{0.06\%}$) and 0.12% ($BS_{0.12\%}$).

	Control	$BS_{0.06\%}$	$BS_{0.12\%}$
Myristic acid (C14:0)	8.61 ± 2.87	7.35 ± 2.40	7.60 ± 2.55
Pentadecylic acid (C15:0)	1.47 ± 0.29	1.17 ± 0.09	1.40 ± 0.45
Palmitic acid (C16:0)	125.93 ± 2.91	118.23 ± 10.89	122.45 ± 7.67
Stearic acid (C18:0)	46.63 ± 2.66	47.31 ± 4.23	51.39 ± 8.10
Saturated fatty acids (SFAs)	176.52 ± 19.53	177.02 ± 13.85	185.59 ± 12.40
Palmitoleic acid (C16:1 n-7)	26.20 ± 4.93	25.56 ± 2.56	25.36 ± 3.32
Vaccenic acid (C18:1 n-7)	34.41 ± 3.06	29.07 ± 3.13	30.72 ± 8.25
Oleic acid (C18:1 n-9)	238.38 ± 8.25	239.00 ± 30.10	244.32 ± 44.79
Eicosenoic acid (C20:1 n-9)	4.08 ± 0.39	4.20 ± 0.81	4.03 ± 0.79
Nervionic acid (C24:1 n-9)	1.76 ± 0.15	1.68 ± 0.26	1.77 ± 0.48
Monounsaturated fatty acids (MUFAs)	307.14 ± 13.72	300.02 ± 33.37	306.63 ± 50.88
Linoleic acid (C18:2 n-6)	126.14 ± 6.76	116.21 ± 18.79	118.85 ± 11.62
Gamma-linolenic acid (C18:3 n-6)	6.94 ± 1.24	7.62 ± 1.21	6.97 ± 1.04
Arachidonic acid (C20:4 n-6)	4.47 ± 0.73	4.64 ± 0.83	4.92 ± 0.82
n-6 Polyunsaturated fatty acids (n-6 PUFAs)	137.90 ± 5.82	128.46 ± 20.20	130.74 ± 11.49
Alpha-linolenic acid (C18:3 n-3)	8.48 ± 0.35	7.92 ± 1.53	7.80 ± 0.73
Stearidonic acid (C18:4 n-3)	1.52 ± 0.04	1.18 ± 0.81	1.70 ± 0.24
Eicosatetraenoic acid (C20:4 n-3)	1.67 ± 0.22	1.68 ± 0.52	1.72 ± 0.24
Eicosapentaenoic acid (C20:5 n-3)	15.17 ± 0.39	14.50 ± 2.56	15.92 ± 2.47
Docosapentaenoic acid (C22:5 n-3)	7.96 ± 1.10	7.40 ± 1.12	7.76 ± 0.74
Docosahexaenoic acid (C22:6 n-3)	15.85 ± 2.21	16.30 ± 3.29	18.31 ± 2.41
n-3 Polyunsaturated fatty acids (n-3 PUFAs)	49.15 ± 4.88	48.98 ± 8.94	53.21 ± 5.93
Total PUFAs	189.48 ± 5.85	177.44 ± 29.05	183.95 ± 17.30

Non represented fatty acids were not detected in the analysis. Values are represented as mean ± SD.

3.4. Oxidative condition, hepatic metabolism, and plasmatic biochemical parameters

In order to assess the general fish health and physiological status, parameters of oxidative stress, hepatic enzyme activities, and plasmatic parameters related with the metabolism of lipids, proteins, and amino acids were measured (Table 7). The dietary supplementation of BSs affected CAT activity, which was reduced in gilthead seabream fed the

Table 6
Fatty acid composition (mg g lipid^{-1}) on fillet of gilthead seabream fed the control and two diets supplemented with a blend of bile salts at a dietary inclusion level of 0.06 ($BS_{0.06\%}$) and 0.12% ($BS_{0.12\%}$).

	Control	$BS_{0.06\%}$	$BS_{0.12\%}$
Myristic acid (C14:0)	8.60 ± 1.35	8.50 ± 1.90	8.60 ± 2.03
Pentadecylic acid (C15:0)	1.21 ± 0.10	1.34 ± 0.30	1.18 ± 0.10
Palmitic acid (C16:0)	129.29 ± 3.49	131.40 ± 11.29	128.70 ± 2.41
Stearic acid (C18:0)	32.41 ± 1.39	31.57 ± 1.52	31.82 ± 1.69
Lignoceric acid (C24:0)	1.44 ± 0.16	1.28 ± 0.09	1.47 ± 0.18
Saturated fatty acids (SFAs)	173.63 ± 2.79	174.51 ± 14.92	172.46 ± 5.70
Palmitoleic acid (C16:1 n-7)	33.50 ± 2.27	34.57 ± 5.08	32.84 ± 1.85
Oleic acid (C18:1 n-9)	247.55 ± 10.03	257.30 ± 18.82	248.07 ± 6.98
Eicosenoic acid (C20:1 n-9)	3.39 ± 0.14	3.53 ± 0.37	3.30 ± 0.52
Nervionic acid (C24:1 n-9)	1.43 ± 0.22	1.51 ± 0.26	1.62 ± 0.25
Monounsaturated fatty acids (MUFAs)	285.59 ± 12.55	293.94 ± 28.38	285.69 ± 8.44
Linoleic acid (C18:2 n-6)	143.89 ± 7.49	145.69 ± 11.62	143.05 ± 3.45
Gamma-linolenic acid (C18:3 n-6)	3.26 ± 0.37	4.07 ± 0.64	3.26 ± 0.35
Arachidonic acid (C20:4 n-6)	5.03 ± 0.43	4.90 ± 0.34	4.99 ± 0.22
n-6 Polyunsaturated fatty acids (n-6 PUFAs)	152.56 ± 8.17	154.66 ± 12.19	151.56 ± 4.03
Alpha-linolenic acid (C18:3 n-3)	9.82 ± 0.78	10.25 ± 1.60	9.73 ± 0.34
Stearidonic acid (C18:4 n-3)	1.56 ± 0.15	1.73 ± 0.41	1.81 ± 0.13
Eicosatetraenoic acid (C20:4 n-3)	1.63 ± 0.23	1.55 ± 0.15	1.59 ± 0.15
Eicosapentaenoic acid (C20:5 n-3)	23.87 ± 2.38	23.25 ± 1.22	25.05 ± 1.68
Docosapentaenoic acid (C22:5 n-3)	9.92 ± 1.22	9.35 ± 0.35	9.28 ± 0.68
Docosahexaenoic acid (C22:6 n-3)	29.37 ± 2.75	28.08 ± 1.14	31.09 ± 2.54
n-3 Polyunsaturated fatty acids (n-3 PUFAs)	77.88 ± 9.41	74.10 ± 3.96	78.54 ± 4.81
Total PUFAs	230.43 ± 15.81	228.76 ± 15.83	230.10 ± 4.71

Non represented fatty acids were not detected in the analysis. Values are represented as mean ± SD.

$BS_{0.06\%}$ diet ($P < 0.05$), while intermediate values were displayed in fish fed the $BS_{0.12\%}$ diet. No significant differences were found in the rest of the measured variables ($P > 0.05$).

3.5. Bile salt profile

The predominant BS in the gallbladder and AI from all examined groups was the taurocholic acid (T-CA), which displayed no significant differences among groups in either of the examined tissues (Table 8; $P > 0.05$). The dietary supplementation of BSs influenced the concentration of the taurochenodeoxycholic acid (T-CDCA) in the gallbladder, which significantly decreased in fish fed the $BS_{0.12\%}$ diet with respect to fish fed the control diet ($P < 0.05$), whereas fish fed the $BS_{0.06\%}$ diet displayed intermediate values. However, no differences in the concentration of T-CDCA were found in the AI among groups ($P > 0.05$). On the other hand, the concentration of taurodeoxycholic acid (T-DCA) in both the gallbladder and AI increased in fish fed the $BS_{0.12\%}$ diet, with respect to fish fed the control diet, since T-DCA was not detected in the gallbladder nor in the AI of fish from this group ($P < 0.05$), while intermediate values were found in fish fed the $BS_{0.06\%}$ diet. No differences were found in the concentration of total BSs in gallbladder and AI among groups (Table 8; $P > 0.05$).

3.6. Activity of pancreatic enzymes

In the case of total alkaline proteases, their specific activity increased in the AI in comparison to values recorded in the stomach and pyloric caeca, although this increase was not dependent on the dietary group (Table 9; $P < 0.05$). In contrast, no differences were found in the specific activity of α-amylase regardless of the dietary group and region of the gastrointestinal tract considered (Table 9; $P > 0.05$). Regarding the activity of bile salt-activated lipase, there were no significant differences among dietary groups when measured in samples from the stomach and pyloric caeca ($P > 0.05$); however, the activity of this lipolytic enzyme increased when measured in the AI in gilthead seabream fed the $BS_{0.12\%}$ diet in comparison to the control group (Table 9; $P < 0.05$).

Table 7
Oxidative condition, activity of hepatic enzymes and blood biomarkers of gilthead seabream fed the control diet and the two diets supplemented with bile salts at an inclusion level of 0.06 ($BS_{0.06\%}$) and 0.12% ($BS_{0.12\%}$).

	Control	$BS_{0.06\%}$	$BS_{0.12\%}$
Oxidative Stress in Liver			
GR (nmol min^{-1} mg protein^{-1})	3.22 ± 0.73	2.63 ± 0.24	2.95 ± 0.22
CAT (nmol min^{-1} mg protein^{-1})	96.88 ± 5.62^{a}	63.76 ± 15.87^{b}	76.05 ± 11.55^{ab}
SOD (% enzyme inhibition)	56.58 ± 9.23	62.24 ± 3.24	59.94 ± 3.70
LPO (nmol MDA g^{-1})	15.52 ± 3.66	13.57 ± 1.34	15.25 ± 3.91
Trolox equivalents (nmol μL^{-1})	18.50 ± 1.72	20.75 ± 1.26	21.20 ± 3.25
Hepatic Metabolism			
LDH (mU mg protein^{-1})	75.49 ± 19.98	90.01 ± 2.71	81.84 ± 13.90
GOT (mU mg protein^{-1})	812.56 ± 180.70	919.20 ± 84.22	783.62 ± 108.93
GPT (mU mg protein^{-1})	251.12 ± 37.33	289.32 ± 18.54	337.84 ± 52.04
Blood Biochemistry			
Cholesterol (mg dL^{-1})	221.88 ± 32.83	220.82 ± 38.31	220.68 ± 33.64
TAGs (mg dL^{-1})	1055.06 ± 266.44	1006.65 ± 281.40	1022.63 ± 305.89
Albumin (g L^{-1})	13.44 ± 1.67	14.18 ± 1.63	14.63 ± 1.61
Total globulins (g L^{-1})	21.88 ± 5.02	23.71 ± 4.24	26.74 ± 9.40
Total proteins (g L^{-1})	35.31 ± 6.60	37.88 ± 5.75	41.37 ± 10.00
GOT (U L^{-1})	58.06 ± 44.73	56.00 ± 54.28	53.21 ± 33.17
GPT (U L^{-1})	3.31 ± 2.80	3.35 ± 2.12	3.58 ± 1.89
Total alkaline phosphatase (U L^{-1})	178.25 ± 64.16	217.59 ± 106.98	198.05 ± 48.95

Values are represented as mean ± SD and differences among groups are indicated by the different superscript letters.

Table 8
Bile salt profile in the gallbladder and the anterior intestine (AI) of gilthead seabream fed the control diet and the two diets supplemented with bile salts (BSs) at an inclusion level of 0.06 ($BS_{0.06\%}$) and 0.12% ($BS_{0.12\%}$).

	Control	$BS_{0.06\%}$	$BS_{0.12\%}$
Gallbladder (mg mL^{-1})			
T-CA	108.23 ± 9.59	101.74 ± 7.34	102.72 ± 2.22
T-CDCA	47.67 ± 3.69^{a}	42.17 ± 4.44^{ab}	35.71 ± 1.60^{b}
T-DCA	nd^{a}	5.78 ± 0.48^{ab}	8.38 ± 0.32^{b}
Total BSs	155.90 ± 9.58	149.69 ± 10.72	146.80 ± 3.87
Anterior intestine (μg mg^{-1})			
T-CA	30.00 ± 15.83	31.56 ± 6.91	45.16 ± 8.90
T-CDCA	19.94 ± 13.72	17.45 ± 4.25	18.47 ± 2.22
T-DCA	nd^{a}	1.51 ± 0.54^{ab}	4.35 ± 1.40^{b}
Total BSs	49.94 ± 29.41	50.51 ± 11.43	67.98 ± 12.20

Values are represented as mean ± SD and differences among groups are indicated by the different superscript letters. Abbreviations: T-CA: Taurocholic acid; T-CDCA: Taurochenodeoxycholic acid; T-DCA: Taurodeoxycholic acid; nd, not detected.

Table 9
Specific activity (mU · mg protein^{-1}) of total alkaline proteases, α-amylase and bile salt-activated lipase in gilthead seabream fed the control diet and the two supplemented with bile salts at an inclusion level of 0.06 ($BS_{0.06\%}$) and 0.12% ($BS_{0.12\%}$).

	Control	$BS_{0.06\%}$	$BS_{0.12\%}$
Stomach and pyloric caeca			
Total alkaline proteases	78.52 ± 8.01	78.06 ± 18.44	78.34 ± 15.17
α-amylase	398.32 ± 51.08	423.64 ± 67.01	419.49 ± 70.67
Bile salt-activated lipase	21.49 ± 6.26	19.85 ± 6.43	18.49 ± 5.81
Anterior intestine			
Total alkaline proteases	133.64 ± 13.45*	127.90 ± 12.56*	134.40 ± 20.27*
α-amylase	402.98 ± 130.44	493.27 ± 73.59	534.39 ± 67.61
Bile salt-activated lipase	50.00 ± 9.57*^{a}	60.15 ± 17.50*^{ab}	93.18 ± 22.79*^{b}

Values are represented as mean ± SD. Differences among groups are indicated by the different superscript letters, whereas differences between gut regions from the same dietary treatment are denoted by an asterisk.

3.7. Analysis of gene expression profiles in liver

All the genes included in the liver PCR-array for the analysis of the gene expression patterns from fish fed control and $BS_{0.06\%}$ diets were found at detectable levels (Supplementary Table S4) with major changes over time for 32 out of 44 analysed genes (two-way ANOVA, $P < 0.05$). This was clearly exemplified by a PLS-DA model of two components with a total explained variance [R2Y(cum)] of 90%, and a total predicted variance [Q2(cum)] of 88% (Fig. 3A), validated by a permutation test (Supplementary Fig. S1). The first component of the model (84.4% explained variance) clearly separated the fish sampled 2 h after feeding (2 h postprandial-animals) from those sampled 48 h after feeding (48 h fasted-animals) regardless of diet, whereas the second component (5.9% explained variance) did not contribute clearly to the clustering of any of the experimental groups by diet or sampling time (Fig. 3B). The only genes exhibiting a differential expression pattern between diets were *fasn*, which was up-regulated, *lpl*, *hadh*, which presented a down-regulated response caused by the $BS_{0.06\%}$ diet (one-way ANOVA, $P < 0.05$), and *fxr* to a lower degree, with a tendency to down-regulation in fish fed the $BS_{0.06\%}$ diet ($P < 0.1$). In 48 h fasted-animals, *prdx5*, whose expression increased in fish fed the $BS_{0.06\%}$ diet, was the only gene that presented significant differences with respect to fish fed the control diet (Supplementary Table S4).

4. Discussion

Several studies have postulated that BSs could be an efficient feed additive for reducing lipid deposition and promoting growth in farmed animals (Arshad et al., 2021; Ge et al., 2019; Zhang et al., 2022; Yin et al., 2021, among others). However, contrary to higher vertebrates, little is known about their mode of action in fish, or how their dietary supplementation may affect their general health and condition. Thus, in

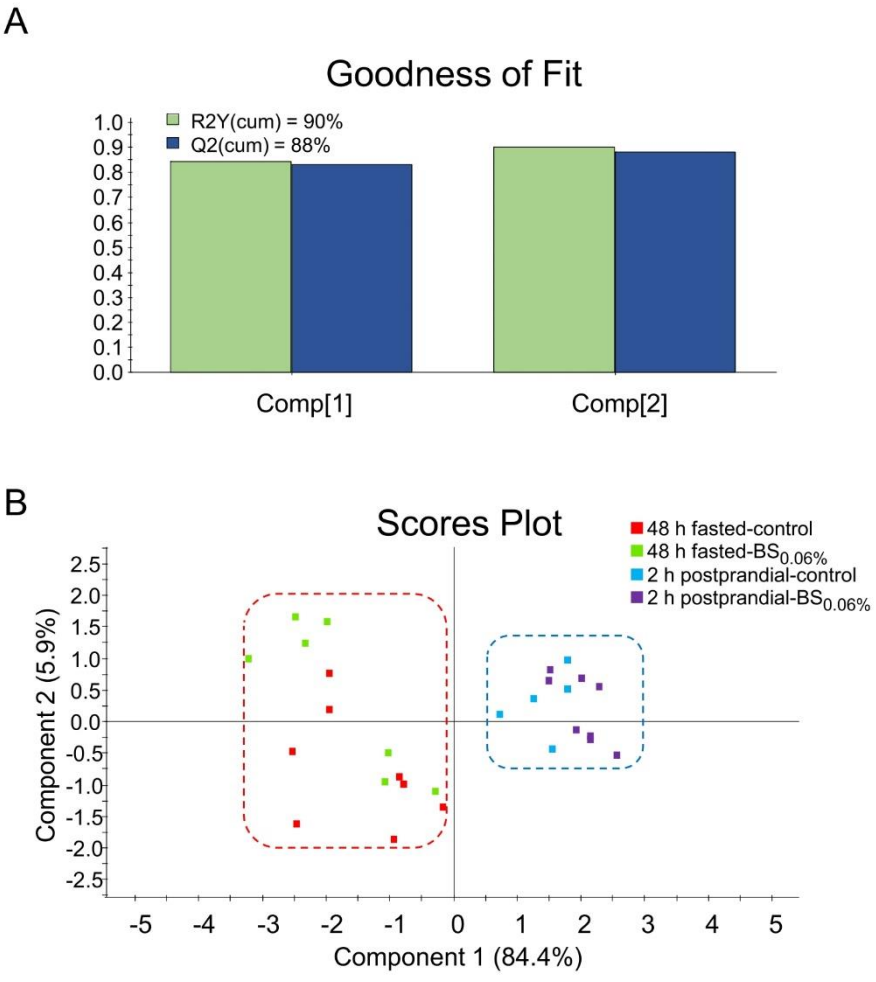

Fig. 3. (A) Graphical representation of the goodness of fit of the PLS-DA model showing the increment in explained variance [R2Y(cum)] and predicted variance [Q2(cum)] with the addition of each component to the model. (B) Scores plot for two-dimensional PLS-DA representing sample distribution between the two components of the model on the basis of their hepatic gene expression profiles based on statistically significant genes ($P < 0.1$). Samples come from liver of 48 h fasted- and 2 h postprandial-gilthead seabream that had received the control and the experimental diet supplemented with a blend of BSs at a dietary inclusion level of 0.06 ($BS_{0.06\%}$).

the current study we aimed to describe the effects of dietary BSs on several key performance indicators related to growth, somatic condition and feed performance in gilthead seabream, which is the main farmed species in the Mediterranean Sea in terms of volume and economic value.

4.1. Effects of dietary bile salt supplementation on growth and feed performance, somatic indices, and apparent digestibility coefficients

The supplementation of a blend of BSs composed of a mixture of equal parts of sodium cholate and sodium deoxycholate, and sodium taurocholate hydrate in a proportion of 30/70 in a diet with high-saturated fat content had a positive effect in several growth performance parameters (BW_f, SL_f and SGR) without affecting the FI nor FCR values in gilthead seabream. Among the two tested dietary levels of BSs, the best results in terms of growth performance were found at 0.06%. In particular, BW_f was a 2.4% higher in gilthead seabream fed the $BS_{0.06\%}$ diet with respect to the control group. As shown in the Supplementary Table S5, these results were in concordance with previous studies in which dietary BSs (0.03%) were tested in large yellow croaker (*Larimichthys crocea*) fed with a high-lipid diet (Ding et al., 2020), and largemouth bass (*Micropterus salmoides*) fed with a high-starch diet (Yu et al., 2019). Furthermore, Li et al. (2021) tested two BSs inclusion levels in the diet (0.03 and 0.09%) of a blend of BAs (70% hyodeoxycholic acid, 19% CDCA, 8% hyocholic acid) in tongue sole (*Cynoglossus semilaevis*) and found that BSs promoted somatic growth in a dose-response manner. Other studies testing only one dietary inclusion level have also proven a positive effect of BSs on somatic growth in different fish species, like turbot (*Scophthalmus maximus*) (Gu et al., 2017), rainbow trout (*Oncorhynchus mykiss*) (Iwashita et al., 2008; Yamamoto et al., 2007), Chinese perch (*Siniperca chuats*) (Zhang et al., 2022) and grass carp (*Ctenopharyngodon idella*) (Zhou et al., 2018) (Supplementary Table S5). However, BS supplementation does not always have a positive effect on fish growth. For example, Mansour et al. (2020) reported no differences in growth in marbled spinefoot rabbitfish (*Siganus rivulatus*) fed a diet supplemented with 0.15% of BSs when compared to the control group. Additionally, the dietary supplementation of 1.8% BSs (sodium T-CA) in low fishmeal diets did not improve growth performance in Atlantic salmon (*Salmo salar*) (Kortner et al., 2016). The absence of effect on growth performance in these two studies may be because the tested BS inclusion levels were not optimized, with only a single dose being tested that was chosen based on preliminary studies carried out in different species, like tilapia and rainbow trout, respectively. These results suggest that the optimal dietary inclusion of BSs is largely dependent on the fish species considered, the formulation of the basal diet and the composition of the BS blend used in each trial (Gu et al., 2017; Zhang et al., 2022).

Although in the current study the dietary inclusion of BSs increased the ADC of lipids, these results were not translated into differences in FCR among dietary groups. Furthermore, the improvement of lipid digestibility associated to the dietary supplementation of BSs was not dose-dependent. In particular, lipid digestibility was improved only when BSs were included in diets at 0.12%, whereas their inclusion at 0.06% did not result in any significant change in lipid ADC. Present results are in agreement with those of Gu et al. (2017), Iwashita et al. (2008) and Yamamoto et al. (2007) who reported that dietary BSs increased lipid ADCs (Supplementary Table S5). These results may be explained by an increase in the activity of bile salt-activated lipase found in the intestine of gilthead seabream fed the $BS_{0.12\%}$ diet, but not in the $BS_{0.06\%}$ group, as it has been previously described in tongue sole (Li et al., 2021). This can be explained by the central role of BSs in promoting digestion and absorption of lipids, as they have the capacity to form micelles and emulsify fat droplets, allowing a higher action surface for lipase (Romano et al., 2020). In addition, BSs also promote lipid digestibility by removing some surfactants that may adhere to the surface of lipids during digestion and inhibit the adsorption and action of this lipolytic enzyme (Romano et al., 2020).

4.2. Effect of dietary BSs supplementation on body adiposity and tissue condition

One of the main problems derived from the replacement of fish oil by alternative vegetal lipid sources is the modification of the fatty acid profile of fish, which has several adverse effects related to potential changes in the palatability and quality of the product (Bell et al., 2010; Kousoulaki et al., 2015). In addition, this also may result in an increased body adiposity, especially in the levels of perivisceral fat in fish like seabream, which may reduce its shelf-life as body fat is oxidized and gets rancid (Hsieh and Kinsella, 1989). Under the present experimental conditions, diets supplemented with BSs resulted in a reduction in the levels of perivisceral fat, especially in those fish fed the $BS_{0.06\%}$ diet. Interestingly, such changes in the PVFI were not correlated with changes in the lipid content of the fillet nor in the fatty acid profile. These results are of special relevance, since they indicate that the use of a blend of BSs was able to reduce the level of perivisceral fat without affecting the lipid and fatty acid profile of the edible fraction. A similar reduction of body adiposity was reported by other authors in grass carp (Zhou et al., 2018), Prussian carp (*Carassius gibelio*) (Lin et al., 2003), turbot (Huang et al., 2015) and cobia (*Rachycentron canadum*) (Zhou et al., 2010) fed BSs-supplemented diets. Fat deposition depends on the incorporation of exogenous fatty acids and de novo lipogenesis. In plasma, dietary fatty acids may be either non-esterified or incorporated as TAGs into circulating lipoproteins and very low-density lipoproteins produced by the liver. The hydrolysis of these circulating TAGs and the incorporation of fatty acids are controlled by lipoprotein lipase (LPL), a key enzyme involved in lipid deposition and metabolism (Salmerón, 2018). Under the present experimental conditions, we found a down-regulation of *lpl* in the liver of fish fed the $BS_{0.06\%}$ diet, which is in accordance with the lower fat deposits in the liver and perivisceral fat found in these animals. On the other hand, despite changes in *lpl* expression, there were no differences in plasmatic levels of cholesterol, TAGs and albumin between experimental groups. This seems to indicate that the reduction in PVFI in fish fed the $BS_{0.06\%}$ diet was not associated to changes in plasmatic dietary fatty acid levels, as suggested also by the lack of changes in the expression of fatty acid translocase/CD36 (*fat/cd36*) and fatty acid binding protein (*h-fabp*) between groups. In this sense, the up-regulation of the gene expression of the fatty acid synthase (*fasn*), could be acting as a counterregulatory mechanism to maintain stable levels of fatty acids in the liver despite the potentially lower incorporation of fatty acids caused by reduced expression of *lpl*. It is well established that *fasn* expression might be modulated by the total level of fatty acids present in the liver, as well as their profile – for instance, PUFAs are known to repress *fasn* expression (Clarke et al., 1990; Musch et al., 1974). However, different mechanisms can regulate *fasn* expression, which makes it challenging to establish a clear mode of action of BAs. It is noteworthy that *fasn* is not only regulated by several hormones and nutritional factors (Katsurada et al., 1990), but also by transcription factors, such as sterol regulatory element-binding proteins 1 (*srebp1*), liver X receptor α (*lxrα*), carbohydrate responsive element binding protein (*chrebp*) and farnesoid X receptor (*fxr*) (Joseph et al., 2002; Lehner and Quiroga, 2016; Shen et al., 2011). Shen et al. (2011) showed that *fasn* and *srebp1* are negatively regulated by *fxr* in mammalian liver, and the work of Zhang et al. (2022) suggested that this might be extrapolated to fish. In this sense, FXR can regulate *fasn* expression through different mechanisms, like the inhibition of *srebp1* or through the metabolism of glucose, among others (Cariou and Staels, 2007). In the current study, there were no differences in the expression of *srebp1* and *lxrα* between dietary treatments, but there was a decrease in *fxr* expression in fish fed the $BS_{0.06\%}$ diet, in line with the above-mentioned studies. On the other hand, Zhou et al. (2018) found that grass carp fed a diet supplemented with 0.06 g BSs kg^{-1} increased lipolysis and β-oxidation processes. In particular, dietary BAs induced the expression of adipose triglyceride lipase (*atgl*) and hormone-

sensitive lipase (*hsl*), stimulating lipid hydrolysis and mobilization, results that were coupled with the up-regulation of carnitine palmitoyl transferase (*cpt1*) that transports long chain fatty acids across the membranes of mitochondria for β-oxidation (Weil et al., 2013). These results are in contradiction to ours, since we found no changes in *atgl* and *cpt1a* expression and a down-regulation of hydroxyacyl-CoA dehydrogenase (*hadh*), which is involved in the β-oxidation of fatty acids in mitochondria releasing NADH + H^+ (Schulz, 1991). Given the lower expression of *hadh*, it could be expected less energy production in fish fed the $BS_{0.06\%}$ diet with respect to fish fed the control diet, but there was no change in the expression of NADH-ubiquinone oxidoreductase chain 2 (*nd2*), NADH-ubiquinone oxidoreductase chain 5 (*nd5*) nor cytochrome *c* oxidase subunit I (*coxi*), genes that codify proteins involved in the electron transport chain and are necessary for ATP generation. These contradictory findings illustrate the complexity of lipid metabolism and its regulation by nutritional factors, and indicate that more research is needed for further deciphering the role of BSs on the regulation of body adiposity in fish.

The dietary supplementation of BSs also had a positive effect on the condition of the liver and AI, as indicated by the histological analysis of both tissues. Regarding the liver, one of the main functions of this tissue is to metabolize, redistribute and/or store nutrients, acting as an energy reservoir for the body (Bruslé and Gonzàlez i Anadon, 1996). Therefore, any physiological disorder originating from unbalanced dietary conditions should be accurately reflected in the liver (Gisbert et al., 2008). Several studies have shown that BSs have the potential to mitigate dietary-induced hepatic disorders leading to the atrophy of hepatocytes or inflammation. In particular, dietary inclusion of BS ameliorated the liver condition in fish fed high-fat diets (Jin et al., 2019) or diets with partial or total fishmeal replacement (Iwashita et al., 2008; Yamamoto et al., 2007). However, under the current experimental conditions, the formulation of the basal diet containing lipids provided by soybean oil and poultry fat did not result in any nutritional disorder or hepatic damage, as indicated by the histopathological analysis and plasmatic biomarkers. In particular, selected plasma biochemical parameters such as levels of cholesterol, TAGs, albumin and total proteins, and levels of GOT, GPT and total alkaline phosphatase, were within the normal range of values reported for gilthead seabream (Busti et al., 2020; Peres et al., 2013). Furthermore, the histological assessment of hepatic lipid accumulation showed that fish fed the diets supplemented with BSs at 0.06% displayed a lower level of fat deposits in the hepatic parenchyma when compared with the control group, which was in agreement with previous studies on black seabream (*Acanthopagrus schlegelii*) (Jin et al., 2019), largemouth bass (Yin et al., 2021) and Chinese perch (Zhang et al., 2022). On the other hand, in the intestine both dietary levels of BSs (0.06 and 0.12%) reduced fat deposits in a dose-dependent manner, whereas no effects supported by statistics were found among experimental groups at the level of morphometric characteristics of the intestinal mucosa. Though standard deviation values were high, there was an observable trend suggesting the $BS_{0.06\%}$ diet might have resulted in enhanced intestinal epithelial morphometric parameters in this dietary group (Supplementary Table S3). A possible explanation would be that BAs may have influenced the microbiome in ways that influence the intestinal stem cell niche (Markandey et al., 2021), but further investigation is required on this aspect, which will be addressed in the future. Similar results in terms of reduction of intestinal fat deposits were found in studies by Iwashita et al. (2009) and Yamamoto et al. (2007), both in rainbow trout. The fact that fat accumulation responds to dietary BSs in a dose-dependent quadratic form in the liver and perivisceral adipose tissue, but in a linear form in the intestine, may indicate that the mechanisms that triggered these responses are tissue-specific and/or under polygenic control. Considering that LPL has been characterized in the liver and adipose tissue, but not in the intestine (Kast et al., 2001), we could hypothesize that the presence and activity of this rate limiting enzyme in plasma TAG hydrolysis, might be responsible for the differential response among tissues. On the other hand, the gene coding for apolipoprotein C-II, *apoc2*, which activates LPL, is under regulation by FXR (Kast et al., 2001), so another possibility is that an important differentiating factor in the tissue-specific mechanism by which BSs regulate fat accumulation could be the activity of FXR, whose expression decreases in the liver when supplementing the diet with a 0.06% blend of BSs. Gene expression in the intestine was not assessed in this study but it might display a different expression in this tissue, associated to intrinsic differences in the physiological roles of both organs. Future studies should look into this aspect.

4.3. Effect of dietary BS supplementation on the BA profiles in the gallbladder and AI

Information on BA profiles in fish is scarce, and available studies only offer a partial view, given that most studies are focused only on the quantification of the taurine-conjugated BAs, as the most abundant group of BAs (Gu et al., 2017; Iwashita et al., 2008; Iwashita et al., 2009; Kim et al., 2015; Yamamoto et al., 2007). However, a few studies in teleost fish offer a more general picture, encompassing conjugated and deconjugated BAs (Kortner et al., 2016; Staessen et al., 2021). The former authors showed that the most abundant BAs in salmonids are those conjugated with taurine, especially T-CA and T-DCA, while the remaining BAs appear at much lower levels and in many cases very close to the detection limit. In the current study, assays measured both conjugated and deconjugated BAs, but only the taurine-conjugated BAs were above the limit of detection. Results showed an increase in the levels of T-DCA in fish gallbladder and AI that was directly dependent on the level of dietary BS inclusion. This might be related to the composition of the dietary BS blend itself. Dietary BSs contained DCA, which may be conjugated with taurine in the liver and stored in the gallbladder, as well as CA and T-CA, the latter being deconjugated to CA and metabolized into DCA by the intestinal microbiota (Schubert et al., 2017). Then, a high proportion of conjugated and deconjugated DCA should be reabsorbed in the intestine and enter into the enterohepatic circulation, returning to the liver, where it is conjugated with taurine (Romano et al., 2020). On the other hand, T-CA levels did not vary between dietary treatments in the gallbladder nor in the AI, so it may be hypothesized that dietary T-CA and CA supplementation will also help to maintain the physiological levels of T-CA within a stable range despite CA being metabolized into DCA. As for the linear decrease of T-CDCA levels in the gallbladder of fish fed the diets supplemented with BSs, it may be speculated that there was a differential intestinal absorption rate of BAs, inversely dependent on BS supplementation. The decrease of T-CDCA levels in the gallbladder might also be attributed to the inhibition of this BS synthesis when supplementing the diets with the experimental BS blend. In this case, considering that there were no significant differences in the gene expression of the rate-limiting enzyme of the classic BA synthesis pathway, cholesterol 7-alpha-monooxygenase (*cyp7a1*), and that the levels of the primary BS T-CA remained stable, the hypothesis is that CDCA synthesis could be partly inhibited by the alternative (acidic) pathway, where CDCA has been demonstrated to be the unique final product in mammals (Chiang, 2002; Schubert et al., 2017). This hypothesis is supported by the study of Pandak et al. (2002) in which it was demonstrated that adding T-DCA and T-CA (whose deconjugated BAs and conjugated T-CA were both incorporated in the BS blend of the current study) to primary cultures of rat hepatocytes inhibited the activity of the rate-limiting enzyme oxysterol 7-alpha-hydroxylase activity (CYP7B1), involved in the acidic pathway. However, to our knowledge the synthesis of BAs by the alternative pathway has not yet been elucidated in fish. In case there was an inhibition of the BS synthesis in response to dietary BS supplementation, the absence of differences in the levels of T-CDCA in the AI between fish fed the different experimental diets might be the consequence of a normalization of the BA pool through different mechanisms involved in BS metabolism, absorption and/or excretion.

4.4. Effect of dietary BS supplementation on hepatic gene expression profiles

The current work also intended to evaluate the mechanisms through which BS supplementation reduced lipid accumulation in body tissues, as well as their effects on liver condition. For that purpose, biomarkers of lipid metabolism and oxidative stress, mainly, were included in the PCR-array layout. Based on results of growth performance, body condition, fat accumulation and oxidative stress markers, the fish fed the control and $BS_{0.06\%}$ diets were selected for further hepatic expression profiling. At first sight, a differential gene expression pattern over time was noteworthy (Fig. 3B). Indeed, while the addition of 0.06% of the BS blend to the diet had a significant impact on the expression of four analysed genes (*fasn*, *lpl*, *hadh*, *fxr*) in 2 h postprandial-fish (one-way ANOVA, $P < 0.1$), only one gene [peroxiredoxin 5 (*prdx5*)] was significantly changed after the two-day fasting period (Supplementary Table S4). These results might indicate that changes in gene expression were mainly modulated by the presence of the tested blend of BAs in the intestine and/or in the postprandial circulation, rather than being induced by long-term dietary-induced changes in fish physiology and metabolism. However, when interpreting these results, it should be kept in mind that although BA metabolism is well-known in mammals, only partial knowledge exists for fish species. In higher vertebrates it is well established that cholesterol synthesis and metabolism into primary BAs are carried out in the liver, and BA synthesis is regulated by negative feedback via the BA-receptor FXR, whose main agonist is CDCA (Rizzo et al., 2005). Within this context, the decrease in *fxr* expression in the liver of fish fed the $BS_{0.06\%}$ diet was in line with the diminishment of the gallbladder content of conjugated T-CDCA with respect to fish fed the control diet. Activation of hepatic FXR by BAs has been reported to promote expression of the nuclear receptor short heterodimer partner (*shp*), which interacts with the transcription factor liver-related homolog-1 (*lrh-1*) and *hnf4α*, inhibiting the transactivation of *cyp7a1* (Chiang and Ferrell, 2022). In teleosts, some studies have shown that dietary BS supplementation can be associated to a higher *fxr* expression, whereas this change has not always been correlated with a lower *cyp7a1* transcription (Liao et al., 2020; Zhang et al., 2022). Conversely, other studies showed that dietary BS supplementation did not modulate *fxr* expression (Zhou et al., 2018; Yin et al., 2021) nor *cyp7a1* (Yin et al., 2021), while another contradictory study showed that dietary BSs (T-CA) increased *cyp7a1* expression (Gu et al., 2017). In the present study, the down-regulation of *fxr* in the $BS_{0.06\%}$ group was not associated to a change in *hnf4α* nor *cyp7a1* expression, which indicates that dietary BS supplementation did not diminish the synthesis of BA with respect to the basal diet, at least by means of the classic pathway (Chiang and Ferrell, 2022), assuming that the lack of differences in gene expression implies non-differential BA synthesis via CYP7A1. The absence of significant differences in the expression of the mentioned genes and *ppar* and *srebp1,* which are modulated by *fxr* in mammals (Pineda Torra et al., 2003; Watanabe et al., 2004) and apparently also in fish (Zhang et al., 2022), may suggest that the differential expression of *fxr* is not enough to promote a change in FXR activity, or that other mechanisms may also be involved in its transcription. On the other hand, the activity of CYP7A1 is not only regulated by hepatic FXR, but also by intestinal FXR, that activates a fibroblast growth factor (FGF19 in humans and FGF15 in mouse), which binds to the tyrosine kinase FGF receptor 4 (FGFR4) in the membrane of hepatocytes and regulates CYP7A1 production and BAs synthesis in the liver (Frisch and Alstrup, 2018; Romano et al., 2020). Since both intestinal and hepatic FXR ultimately can regulate CYP7A1 activity, it may be speculated that intestinal FXR was activated and, consequently, an equilibrium in CYP7A1 activity regulation between hepatic and intestinal FXR was achieved, without reaching a differential expression of *cyp7a1* between dietary treatments. Another hypothesis that would explain the absence of differences in the expression of *cyp7a1* among diets is that intestinal FXR was not activated, and its regulatory role in BA synthesis was stronger than the one of hepatic FXR. This would be in line with the absence of variation in the levels of the conjugated FXR-main agonist, T-CDCA, in the AI among diets. Indeed, an in vivo study in mice proposed the FXR in the terminal ileum as the main BA target, after observing a much higher activation in this intestinal area, compared to hepatic FXR, when BSs were supplied (Houten et al., 2007). Moreover, the studies of Kim et al. (2007) and Kong et al. (2012) demonstrated that while activation of hepatic FXR has an impact on the regulation of sterol 12-alpha-hydroxylase (*cyp8b1*), necessary for cholic acid synthesis, the master regulator of *cyp7a1* would be the intestinal FXR.

After the two-day fasting period, hepatic *fxr* expression was stabilized with respect to the fish fed the control diet, meaning that the blend of BSs did not cause any lasting effects on the fish's BA, lipid, protein or carbohydrate metabolism, which are also under the regulation of FXR (Chiang and Ferrell, 2022).

In fasted fish, the only gene which displayed a differential expression was *prdx5*, being over-expressed in those fed the $BS_{0.06\%}$ diet. Peroxiredoxins are antioxidant enzyme isoforms distributed in different cell organelles. Specifically, PRDX3 and PRDX5 have been found in gilthead seabream mitochondria, and PRDX5 also in cytosol and peroxisomes (Pérez-Sánchez et al., 2011). Mouse in vivo and human hepatocellular carcinoma in vitro assays (Kim et al., 2018; Kim et al., 2020) have recently highlighted the important role of PRDX5 in maintaining the intracellular redox balance by scavenging the reactive oxygen species (ROS), and reported the implication of PRDX5 in lipid deposition, inhibiting an abnormally high fatty acid synthesis and low fatty acid oxidation, as well as preventing obesity (Kim et al., 2018) and conferring resistance to hepatic steatosis (Kim et al., 2020). Taking that into account, fish under fasting conditions previously fed the $BS_{0.06\%}$ diet may benefit from the improved antioxidative status of the liver due to the higher expression of *prdx5*, which may lead to a lower CAT activity. By contrast, *cat* expression was not differentially regulated in response to dietary treatments, which indicates that protein activity is not always related to gene expression (Farnier et al., 2003). In fact, it should be an underlying assumption when interpreting the PCR-array results, that complex pathways that regulate BA and lipid metabolism might not be completely reflected in the gene expression analysis, offering only an incomplete picture.

5. Conclusions

This nutritional study showed that the supplementation of a high-saturated fat diet with a blend of BSs composed of 30% sodium cholate and sodium deoxycholate, and 70% sodium taurocholate hydrate, at an inclusion level of 0.06% in the diet, enhanced growth performance and reduced the levels of perivisceral, hepatic and intestinal fat in gilthead seabream. The reduction of perivisceral fat and lipid deposits in liver was consistent with the observed down-regulation of hepatic *lpl*, considering that this enzyme catalyses the hydrolysis of plasma TAGs generating fatty acids that are incorporated into tissues. Furthermore, this can also link to the lower expression of hepatic *hadh*, which participates in the mitochondrial β-oxidation of fatty acids. In this sense, the up-regulation of *fasn* may be a response to the reduced expression of *lpl* in order to maintain stable levels of fatty acids in the liver. Moreover, the changes in expression of *lpl* and *fasn* might have been caused by the repression of hepatic *fxr* in the $BS_{0.06\%}$ group. However, the absence of variation in the expression of other measured genes that are also under *fxr* regulation in mammalian vertebrates, like *srebp1*, *ppar* and those regulating the BA synthesis, *hnf4α* and *cyp7a1*, may suggest that the differential expression of *fxr* is insufficient by itself to regulate their transcription, and other mechanisms may also be involved. On the other hand, the fact that adding 0.12% of BSs to the diet also reduced fat deposits in intestine, but not in liver nor perivisceral adipose tissue, may suggest a tissue-specific differential mechanism for regulating fat accumulation, which might be mediated by hepatic and intestinal FXR activity and/or by the enzyme LPL, which has been found

in liver and adipose tissue but not in the intestine. The well-known role of BSs in lipid digestion conferred by their amphipathic nature to form micelles and their innate ability to remove surfactants during digestion, was also reflected in the current results showing a higher lipid ADC and increased activity of BS-activated lipase in the anterior intestine of fish fed the $BS_{0.12\%}$ diet. This work suggests that dietary BS supplementation at a dose of 0.06% could be a functional strategy to improve the farming and production efficiency of *S. aurata*, reducing fish adiposity and potentially improving the overall fish condition when fed with a diet containing high saturated fat content, and paves the way for further studies in this field to aid the understanding of the specific mechanisms stimulated by BSs and the varying responses in different tissues.

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Authors statement

Alberto Ruiz: Sample processing, data analysis, interpretation of results and writing of the first draft. **Karl B. Andree**: Conceptualization, methodology, interpretation of results, supervision and writing of the first draft. **Ignasi Sanahuja**: Sample processing. **Paul G. Holhorea**: Data analysis on gene expression profile. **Josep À. Calduch-Giner:** Selection of biomarkers for gene expression profile, search and construction of primers. **Sofia Morais**: Decision on diet composition, selection of gene biomarkers, and interpretation of results. **Jose J. Pastor:** Sample processing, analysis and interpretation of results of bile acid profile. **Jaume Pérez-Sánchez:** Selection of biomarkers, data analysis and interpretation of results of gene expression profile. **Enric Gisbert:** Supervision, experimental design, conceptualization, interpretation of results, writing of the first draft, funding acquisition and project administration.

All authors have contributed to the final writing of the paper and have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Alberto Ruiz reports financial support was provided by Spain Ministry of Science and Innovation. Enric Gisbert reports was provided by Spain Ministry of Science and Innovation. Ignasi Sanahuja reports was provided by Spain Ministry of Science and Innovation.

Data availability

Data will be made available on request.

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References

Aebi, H., 1974. Catalase. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic Press, New York, pp. 673–684.

Arshad, M.A., Bhatti, S.A., Rehman, M.S.U., Yousaf, W., Younus, G., Sizmaz, O., Bilal, M. Q., 2021. Supplementation of bile acids and lipase in broiler diets for better nutrient utilization and performance: potential effects and future implications–a review. Ann. Anim. Sci. 21, 757–787. https://doi.org/10.2478/aoas-2020-0099.

Association of Official Analytical Chemists (AOAC), 1990. Official Methods of Analysis of the Association of Official Analytical Chemists, fifteenth ed. Association of Official Analytical Chemists, Virginia, USA.

Ballester-Lozano, G.F., Benedito-Palos, L., Navarro, J.C., Kaushik, S., Pérez-Sánchez, J., 2011. Prediction of fillet fatty acid composition of market-size gilthead sea bream (*Sparus aurata*) using a regression modelling approach. Aquaculture 319, 81–88. https://doi.org/10.1016/j.aquaculture.2011.06.015.

Ballester-Lozano, G.F., Benedito-Palos, L., Estensoro, I., Sitjà-Bobadilla, A., Kaushik, S., Pérez-Sánchez, J., 2015. Comprehensive biometric, biochemical and histopathological assessment of nutrient deficiencies in gilthead sea bream fed semi-purified diets. Br. J. Nutr. 114, 713–726. https://doi.org/10.1017/S0007114515002354.

Bell, J.G., Pratoomyot, J., Strachan, F., Henderson, R.J., Fontanillas, R., Hebard, A., Tocher, D.R., 2010. Growth, flesh adiposity and fatty acid composition of Atlantic salmon (*Salmo salar*) families with contrasting flesh adiposity: effects of replacement of dietary fish oil with vegetable oils. Aquaculture 306, 225–232. https://doi.org/10.1016/j.aquaculture.2010.05.021.

Benedito-Palos, L., Navarro, J.C., Kaushik, S., Pérez-Sánchez, J., 2010. Tissue-specific robustness of fatty acid signatures in cultured gilthead sea bream (*Sparus aurata* L.) fed practical diets with a combined high replacement of fish meal and fish oil. J. Anim. Sci. 88, 1759–1770. https://doi.org/10.2527/jas.2009-2564.

Bergmeyer, H.U., Bernt, E., 1974a. Lactate dehydrogenase: UV-assay with pyruvate and NADH. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic Press, New York, pp. 574–579.

Bergmeyer, H.U., Bernt, E., 1974b. Glutamate-oxaloacetate transaminase: UV-assay, manual method. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic Press, New York, pp. 727–733.

Bergmeyer, H.U., Bernt, E., 1974c. Glutamate-pyruvate transaminase: UV-assay, manual method. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic Press, New York, pp. 752–758.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Bruslé, J., Gonzàlez i Anadon, G., 1996. The structure and function of fish liver. In: Munshi, J.S.D., Dutta, H.M. (Eds.), Fish Morphology. Science Publishers Inc., USA, pp. 77–93.

Busti, S., Bonaldo, A., Dondi, F., Cavallini, D., Yúfera, M., Gilannejad, N., Moyano, F.J., Gatta, P.P., Parma, L., 2020. Effects of different feeding frequencies on growth, feed utilisation, digestive enzyme activities and plasma biochemistry of gilthead sea bream (*Sparus aurata*) fed with different fishmeal and fish oil dietary levels. Aquaculture 529, 735616. https://doi.org/10.1016/j.aquaculture.2021.737636.

Cariou, B., Staels, B., 2007. FXR: a promising target for the metabolic syndrome? Trends Pharmacol. Sci. 28, 236–243. https://doi.org/10.1016/j.tips.2007.03.002.

Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250, 5475–5480. https://doi.org/10.1016/S0021-9258(19)41206-4.

Chiang, J.Y., 2002. Bile acid regulation of gene expression: roles of nuclear hormone receptors. Endocr. Rev. 23, 443–463. https://doi.org/10.1210/er.2000-0035.

Chiang, J.Y., Ferrell, J.M., 2022. Discovery of farnesoid X receptor and its role in bile acid metabolism. Mol. Cell. Endocrinol. 111618 https://doi.org/10.1016/j.mce.2022.111618.

Christie, W.W., 2012. Analysis of fatty acids. In: Christie, W.W., Han, X. (Eds.), Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids. The Oily Press, Bridgwater, pp. 145–214. https://doi.org/10.1093/jn/120.2.225.

Clarke, S.D., Armstrong, M.K., Jump, D.B., 1990. Dietary polyunsaturated fats uniquely suppress rat liver fatty acid synthase and S14 mRNA content. J. Nutr. 120, 225–231.

De Deckere, E.A., Korver, O., Verschuren, P.M., Katan, M.B., 2001. Health aspects of fish and n-3 polyunsaturated fatty acids from plant and marine origin. Eur. J. Clin. Nutr. 52, 749–753. https://doi.org/10.1038/sj.ejcn.1600641.

Ding, T., Xu, N., Liu, Y., Du, J., Xiang, X., Xu, D., Liu, Q., Yin, Z., Li, J., Mai, K., Ai, Q., 2020. Effect of dietary bile acid (BA) on the growth performance, body composition, antioxidant responses and expression of lipid metabolism-related genes of juvenile large yellow croaker (*Larimichthys crocea*) fed high-lipid diets. Aquaculture 518, 734768. https://doi.org/10.1016/j.aquaculture.2019.734768.

Du, Z.Y., Clouet, P., Zheng, W.H., Degrace, P., Tian, L.X., Liu, Y.J., 2006. Biochemical hepatic alterations and body lipid composition in the herbivorous grass carp (*Ctenopharyngodon idella*) fed high-fat diets. Br. J. Nutr. 95, 905–915. https://doi.org/10.1079/BJN20061733.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.T., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356. https://doi.org/10.1021/ac60111a017.

FAO, 2022. The State of World Fisheries and Aquaculture 2022. Towards Blue Transformation. FAO, Rome. https://doi.org/10.4060/cc0461en.

Farnier, C., Krief, S., Blache, M., Diot-Dupuy, F., Mory, G., Ferre, P., Bazin, R., 2003. Adipocyte functions are modulated by cell size change: potential involvement of an integrin/ERK signalling pathway. Int. J. Obes. 27, 1178–1186. https://doi.org/10.1038/sj.ijo.0802399.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509. https://doi.org/10.1016/S0021-9258(18)64849-5.

Frisch, K., Alstrup, A.K.O., 2018. On the evolution of bile salts and the farnesoid X receptor in vertebrates. Physiol. Biochem. Zool. 91, 797–813. https://doi.org/10.1086/695810.

Garatun-Tjeldstø, O., Otterå, H., Julshamn, K., Austreng, E., 2006. Food ingestion in juvenile cod estimated by inert lanthanide markers–effects of food particle size. ICES Mar. Sci. Symp. 63, 311–319. https://doi.org/10.1016/j.icesjms.2005.11.008.

García-Carreño, F.L., Haard, N.F., 1993. Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. J. Food Biochem. 17, 97–113. https://doi.org/10.1111/j.1745-4514.1993.tb00864.x.

Ge, X.K., Wang, A.A., Ying, Z.X., Zhang, L.G., Su, W.P., Cheng, K., Feng, C.C., Zhou, Y.M., Zhang, L.L., Wang, T., 2019. Effects of diets with different energy and bile acids levels on growth performance and lipid metabolism in broilers. Poult. Sci. 98, 887–895. https://doi.org/10.3382/ps/pey434.

Gisbert, E., Ortiz-Delgado, J.B., Sarasquete, C., 2008. Nutritional cellular biomarkers in early life stages of fish. Histol. Histopathol. 23, 125–139. https://doi.org/10.14670/HH-23.1525.

Gisbert, E., Giménez, G., Fernández, I., Kotzamanis, Y., Estévez, A., 2009. Development of digestive enzymes in common dentex *Dentex dentex* during early ontogeny. Aquaculture 287, 381–387. https://doi.org/10.1016/j.aquaculture.2008.10.039.

Gisbert, E., Skalli, A., Campbell, J., Solovyev, M.M., Rodríguez, C., Dias, J., Polo, J., 2015. Spray-dried plasma promotes growth, modulates the activity of antioxidant defenses, and enhances the immune status of gilthead sea bream (*Sparus aurata*) fingerlings. J. Anim. Sci. 93, 278–286. https://doi.org/10.2527/jas.2014-7491.

Grigorakis, K., 2007. Compositional and organoleptic quality of farmed and wild gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) and factors affecting it: a review. Aquaculture 272, 55–75. https://doi.org/10.1016/j.aquaculture.2007.04.062.

Gu, M., Bai, N., Kortner, T.M., 2017. Taurocholate supplementation attenuates the changes in growth performance, feed utilization, lipid digestion, liver abnormality and sterol metabolism in turbot (*Scophthalmus maximus*) fed high level of plant protein. Aquaculture 468, 597–604. https://doi.org/10.1016/j.aquaculture.2016.11.022.

Hagey, L.R., Møller, P.R., Hofmann, A.F., Krasowski, M.D., 2010. Diversity of bile salts in fish and amphibians: evolution of a complex biochemical pathway. Physiol. Biochem. Zool. 83, 308–321. https://doi.org/10.1086/649966.

Herrero-Encinas, J., Blanch, M., Pastor, J.J., Mereu, A., Ipharraguerre, I.R., Menoyo, D., 2020. Effects of a bioactive olive pomace extract from *Olea europaea* on growth performance, gut function, and intestinal microbiota in broiler chickens. Poult. Sci. 99, 2–10. https://doi.org/10.3382/ps/pez467.

Horrocks, L.A., Yeo, Y.K., 1999. Health benefits of docosahexaenoic acid (DHA). Pharmacol. Res. 40, 211–225. https://doi.org/10.1006/phrs.1999.0495.

Houten, S.M., Volle, D.H., Cummins, C.L., Mangelsdorf, D.J., Auwerx, J., 2007. *In vivo* imaging of farnesoid X receptor activity reveals the ileum as the primary bile acid signaling tissue. Mol. Endocrinol. 21, 1312–1323. https://doi.org/10.1210/me.2007-0113.

Hsieh, R.J., Kinsella, J.E., 1989. Oxidation of polyunsaturated fatty acids: mechanisms, products, and inhibition with emphasis on fish. Adv. Food Nutr. Res. 33, 233–341. https://doi.org/10.1016/S1043-4526(08)60129-1.

Huang, B.S., Li, B.S., Zhang, L.M., Li, H.D., Sun, Y.Z., Wang, S.X., Wang, J.Y., 2015. Effects of dietary bile acid on growth performance, fatty enzymatic activities and biochemical indices of turbot *Scophthalmus maximus* L. junveniles. J. Shanghai Ocean Univ. 24, 737–744 (In Chinese).

Iijima, N., Tanaka, S., Ota, Y., 1998. Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiol. Biochem. 18, 59–69. https://doi.org/10.1023/A:1007725513389.

Iwashita, Y., Suzuki, N., Yamamoto, T., Shibata, J.I., Isokawa, K., Soon, A.H., Ikehata, Y., Furuita, H., Sugita, T., Goto, T., 2008. Supplemental effect of cholyltaurine and soybean lecithin to a soybean meal-based fish meal-free diet on hepatic and intestinal morphology of rainbow trout *Oncorhynchus mykiss*. Fish. Sci. 74, 1083–1095. https://doi.org/10.1111/j.1444-2906.2008.01628.x.

Iwashita, Y., Suzuki, N., Matsunari, H., Sugita, T., Yamamoto, T., 2009. Influence of soya saponin, soya lectin, and cholyltaurine supplemented to a casein-based semipurified diet on intestinal morphology and biliary bile status in fingerling rainbow trout *Oncorhynchus mykiss*. Fish. Sci. 75, 1307–1315. https://doi.org/10.1007/s12562-009-0158-1.

Jaya-Ram, A., Kuah, M.K., Lim, P.S., Kolkovski, S., Shu-Chien, A.C., 2008. Influence of dietary HUFA levels on reproductive performance, tissue fatty acid profile and desaturase and elongase mRNAs expression in female zebrafish *Danio rerio*. Aquaculture 277, 275–281. https://doi.org/10.1016/j.aquaculture.2008.02.027.

Jiang, M., Wen, H., Gou, G.W., Liu, T.L., Lu, X., Deng, D.F., 2018. Preliminary study to evaluate the effects of dietary bile acids on growth performance and lipid metabolism of juvenile genetically improved farmed tilapia (*Oreochromis niloticus*) fed plant ingredient-based diets. Aquac. Nutr. 24, 1175–1183. https://doi.org/10.1111/anu.12656.

Jin, M., Pan, T., Cheng, X., Zhu, T.T., Sun, P., Zhou, F., Ding, X., Zhou, Q., 2019. Effects of supplemental dietary L-carnitine and bile acids on growth performance, antioxidant and immune ability, histopathological changes and inflammatory response in juvenile black seabream (*Acanthopagrus schlegelii*) fed high-fat diet. Aquaculture 504, 199–209. https://doi.org/10.1016/j.aquaculture.2019.01.063.

Joseph, S.B., Laffitte, B.A., Patel, P.H., Watson, M.A., Matsukuma, K.E., Walczak, R., Collins, J.L., Osborne, T.F., Tontonoz, P., 2002. Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. J. Biol. Chem. 277, 11019–11025. https://doi.org/10.1074/jbc.M111041200.

Kast, H.R., Nguyen, C.M., Sinal, C.J., Jones, S.A., Laffitte, B.A., Reue, K., Gonzalez, F.J., Willson, T.M., Edwards, P.A., 2001. Farnesoid X-activated receptor induces apolipoprotein C-II transcription: a molecular mechanism linking plasma triglyceride levels to bile acids. Mol. Endocrinol. 15, 1720–1728. https://doi.org/10.1210/mend.15.10.0712.

Katsurada, A., Iritani, N., Fukuda, H., Matsumura, Y., Nishimoto, N., Noguchi, T., Tanaka, T., 1990. Effects of nutrients and hormones on transcriptional and post-transcriptional regulation of fatty acid synthase in rat liver. Eur. J. Biochem. 190, 427–433. https://doi.org/10.1111/j.1432-1033.1990.tb15592.x.

Kenari, A.A., Mozanzadeh, M.T., Pourgholam, R., 2011. Effects of total fish oil replacement to vegetable oils at two dietary lipid levels on the growth, body composition, haemato-immunological and serum biochemical parameters in Caspian brown trout (*Salmo trutta caspius* Kessler, 1877). Aquac. Res. 42, 1131–1144. https://doi.org/10.1111/j.1365-2109.2010.02701.x.

Kim, I., Ahn, S.H., Inagaki, T., Choi, M., Ito, S., Guo, G.L., Kliewer, S.A., Gonzalez, F.J., 2007. Differential regulation of bile acid homeostasis by the farnesoid X receptor in liver and intestine. J. Lipid Res. 48, 2664–2672. https://doi.org/10.1194/jlr.M700330-JLR200.

Kim, M.H., Park, S.J., Kim, J.H., Seong, J.B., Kim, K.M., Woo, H.A., Lee, D.S., 2018. Peroxiredoxin 5 regulates adipogenesis-attenuating oxidative stress in obese mouse models induced by a high-fat diet. Free Radic. Biol. Med. 123, 27–38. https://doi.org/10.1016/j.freeradbiomed.2018.05.061.

Kim, M.H., Seong, J.B., Huh, J.W., Bae, Y.C., Lee, H.S., Lee, D.S., 2020. Peroxiredoxin 5 ameliorates obesity-induced non-alcoholic fatty liver disease through the regulation of oxidative stress and AMP-activated protein kinase signaling. Redox Biol. 28, 101315 https://doi.org/10.1016/j.redox.2019.101315.

Kim, S.K., Kim, K.G., Kim, K.D., Kim, K.W., Son, M.H., Rust, M., Johnson, R., 2015. Effect of dietary taurine levels on the conjugated bile acid composition and growth of juvenile Korean rockfish *Sebastes schlegeli* (Hilgendorf). Aquac. Res. 46, 2768–2775. https://doi.org/10.1111/are.12431.

Kong, B., Wang, L., Chiang, J.Y., Zhang, Y., Klaassen, C.D., Guo, G.L., 2012. Mechanism of tissue-specific farnesoid X receptor in suppressing the expression of genes in bile-acid synthesis in mice. Hepatology 56, 1034–1043. https://doi.org/10.1002/hep.25740.

Kortner, T.M., Penn, M.H., Björkhem, I., Måsøval, K., Krogdahl, Å., 2016. Bile components and lecithin supplemented to plant based diets do not diminish diet related intestinal inflammation in Atlantic salmon. BMC Vet. Res. 12, 1–12. https://doi.org/10.1186/s12917-016-0819-0.

Kousoulaki, K., Sæther, B.S., Albrektsen, S., Noble, C., 2015. Review on European sea bass (*Dicentrarchus labrax*, Linnaeus, 1758) nutrition and feed management: a practical guide for optimizing feed formulation and farming protocols. Aquac. Nutr. 21, 129–151. https://doi.org/10.1111/anu.12233.

Lai, W., Cao, A., Li, J., Zhang, W., Zhang, L., 2018. Effect of high dose of bile acids supplementation in broiler feed on growth performance, clinical blood metabolites, and organ development. J. Appl. Poult. Res. 27, 532–539. https://doi.org/10.3382/japr/pfy040.

Le, H.T., Lie, K.K., Giroud-Argoud, J., Rønnestad, I., Sæle, Ø., 2019. Effects of cholecystokinin (CCK) on gut motility in the stomachless fish ballan wrasse (*Labrus bergylta*). Front. Neurosci. 13, 553. https://doi.org/10.3389/fnins.2019.00553.

Legrand, P., Rioux, V., 2010. The complex and important cellular and metabolic functions of saturated fatty acids. Lipids 45, 941–946. https://doi.org/10.1007/s11745-010-3444-x.

Lehner, R., Quiroga, A.D., 2016. Fatty acid handling in mammalian cells. In: Ridgway, N. D., McLeod, R.S. (Eds.), Biochemistry of Lipids, Lipoproteins and Membranes. Elsevier, Amsterdam, pp. 149–184.

Li, Y., Wang, S., Hu, Y., Cheng, J., Cheng, X., Cheng, P., Cui, Z., 2021. Dietary bile acid supplementation reveals beneficial effects on intestinal healthy status of tongue sole (*Cynoglossus semiliaevis*). Fish Shellfish Immunol. 116, 52–60. https://doi.org/10.1016/j.fsi.2021.06.020.

Liao, Z., Sun, B., Zhang, Q., Jia, L., Wei, Y., Liang, M., Xu, H., 2020. Dietary bile acids regulate the hepatic lipid homeostasis in tiger puffer fed normal or high-lipid diets. Aquaculture 519, 734935. https://doi.org/10.1016/j.aquaculture.2020.734935.

Lin, S.M., Ye, Y.T., Luo, L., 2003. The effect of bile acid preparation on the growth of *Carassius auratus gibelio*. Guang Dong Feed 12, 14–15 (In Chinese).

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275 (PMID: 14907713).

Lupatsch, I., Kissil, G.W., Sklan, D., Pfeffer, E., 1997. Apparent digestibility coefficients of feed ingredients and their predictability in compound diets for gilthead seabream, *Sparus aurata* L. Aquac. Nutr. 3, 81–89. https://doi.org/10.1046/j.1365-2095.1997.00076.x.

Mansour, A.T., El-Feky, M.M., El-Beltagi, H.S., Sallam, A.E., 2020. Synergism of dietary co-supplementation with lutein and bile salts improved the growth performance, carotenoid content, antioxidant capacity, lipid metabolism, and lipase activity of the marbled spinefoot rabbitfish, *Siganus rivulatus*. Animals 10, 1643. https://doi.org/10.3390/ani10091643.

Markandey, M., Bajaj, A., Ilott, N.E., Kedia, S., Travis, S., Powrie, F., Ahuja, V., 2021. Gut microbiota: sculptors of the intestinal stem cell niche in health and inflammatory bowel disease. Gut Microbes 13, 1990827. https://doi.org/10.1080/19490976.2021.1990827.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055. https://doi.org/10.1016/S0021-9258(18)63504-5.

Métais, P., Bieth, J., 1968. Determination of alpha-amylase by a microtechnic. Ann. Biol. Clin. 26, 133–142 (PMID: 5651930).

Meyerholz, D.K., Beck, A.P., 2018. Fundamental concepts for semiquantitative tissue scoring in translational research. ILAR J. 59, 13–17. https://doi.org/10.1093/ilar/ily025.

Mozanzadeh, M.T., Hekmatpour, F., Gisbert, E., 2021. Fish oil sparing and alternative lipid sources in aquafeeds. In: Lorenzo, J.M., Simal-Gandara, J. (Eds.), Sustainable Aquafeeds: Technological Innovation and Novel Ingredients. CRC Press, London, pp. 185–292.

Musch, K., Ojakian, M.A., Williams, M.A., 1974. Comparison of α-linolenate and oleate in lowering activity of lipogenic enzymes in rat liver: evidence for a greater effect of dietary linolenate independent of food and carbohydrate intake. Biochim. Biophys. Acta 337, 343–348. https://doi.org/10.1016/0005-2760(74)90109-X.

Nasopoulou, C., Zabetakis, I., 2012. Benefits of fish oil replacement by plant originated oils in compounded fish feeds. A review. LWT 47, 217–224. https://doi.org/10.1016/j.lwt.2012.01.018.

Naya-Català, F., do Vale Pereira, G., Piazzon, M.C., Fernandes, A.M., Calduch-Giner, J.A., Sitjà-Bobadilla, A., Conceição, L.E.C., Pérez-Sánchez, J., 2021. Cross-talk between intestinal microbiota and host gene expression in gilthead sea bream (*Sparus aurata*) juveniles: insights in fish feeds for increased circularity and resource utilization. Front. Physiol. 12, 748265 https://doi.org/10.3389/fphys.2021.748265.

Pandak, W.M., Hylemon, P.B., Ren, S., Marques, D., Gil, G., Redford, K., Mallonee, D., Vlahcevic, Z.R., 2002. Regulation of oxysterol 7α-hydroxylase (CYP7B1) in primary cultures of rat hepatocytes. Hepatology 35, 1400–1408. https://doi.org/10.1053/jhep.2002.33200.

Peng, S., Yue, Y., Gao, Q., Shi, Z., Yin, F., Wang, J., 2014. Influence of dietary n-3 LC-PUFA on growth, nutritional composition and immune function in marine fish *Sebastiscus marmoratus*. Chin. J. Oceanol. Limnol. 32, 1000–1008. https://doi.org/10.1007/s00343-014-3312-2.

Peres, H., Santos, S., Oliva-Teles, A., 2013. Selected plasma biochemistry parameters in gilthead seabream (*Sparus aurata*) juveniles. J. Appl. Ichthyol. 29, 630–636. https://doi.org/10.1111/j.1439-0426.2012.02049.x.

Pérez-Sánchez, J., Bermejo-Nogales, A., Calduch-Giner, J.A., Kaushik, S., Sitjà-Bobadilla, A., 2011. Molecular characterization and expression analysis of six peroxiredoxin paralogous genes in gilthead sea bream (*Sparus aurata*): insights from fish exposed to dietary, pathogen and confinement stressors. Fish Shellfish Immunol. 31, 294–302. https://doi.org/10.1016/j.fsi.2011.05.015.

Pineda Torra, I., Claudel, T., Duval, C., Kosykh, V., Fruchart, J.C., Staels, B., 2003. Bile acids induce the expression of the human peroxisome proliferator-activated receptor α gene via activation of the farnesoid X receptor. Mol. Endocrinol. 17, 259–272. https://doi.org/10.1210/me.2002-0120.

Rizzo, G., Renga, B., Mencarelli, A., Pellicciari, R., Fiorucci, S., 2005. Role of FXR in regulating bile acid homeostasis and relevance for human diseases. Curr. Drug Targets Immune Endocr. Metabol. Disord. 5, 289–303. https://doi.org/10.2174/1568008054863781.

Romano, N., Kumar, V., Yang, G., Kajbaf, K., Rubio, M.B., Overturf, K., Brezas, A., Hardy, R., 2020. Bile acid metabolism in fish: disturbances caused by fishmeal alternatives and some mitigating effects from dietary bile inclusions. Rev. Aquac. 12, 1792–1817. https://doi.org/10.1111/raq.12410.

Rueda-Jasso, R., Conceiçao, L.E., Dias, J., De Coen, W., Gomes, E., Rees, J.F., Soares, F., Dinis, M.T., Sorgeloos, P., 2004. Effect of dietary non-protein energy levels on condition and oxidative status of Senegalese sole (*Solea senegalensis*) juveniles. Aquaculture 231, 417–433. https://doi.org/10.1016/S0044-8486(03)00537-4.

Salmerón, C., 2018. Adipogenesis in fish. J. Exp. Biol. 221, jeb161588. https://doi.org/10.1242/jeb.161588.

Salomón, R., Firmino, J.P., Reyes-López, F.E., Andree, K.B., González-Silvera, D., Esteban, M.A., Tort, L., Quintela, J.C., Pinilla-Rosas, J.C., Vallejos-Vidal, E., Gisbert, E., 2020. The growth promoting and immunomodulatory effects of a medicinal plant leaf extract obtained from *Salvia officinalis* and *Lippia citriodora* in gilthead seabream (*Sparus aurata*). Aquaculture 524, 735291. https://doi.org/10.1016/j.aquaculture.2020.735291.

Schubert, K., Olde Damink, S.W., von Bergen, M., Schaap, F.G., 2017. Interactions between bile salts, gut microbiota, and hepatic innate immunity. Immunol. Rev. 279, 23–35. https://doi.org/10.1111/imr.12579.

Schulz, H., 1991. Beta oxidation of fatty acids. Biochim. Biophys. Acta 1081, 109–120. https://doi.org/10.1016/0005-2760(91)90015-A.

Shen, L.L., Liu, H., Gan, L., Lu, L., Zhang, Q., Li, L., He, F., Jiang, Y., 2011. Effects of farnesoid X receptor on the expression of the fatty acid synthetase and hepatic lipase. Mol. Biol. Rep. 38, 553–559. https://doi.org/10.1007/s11033-010-0140-0.

Simó-Mirabet, P., Felip, A., Estensoro, I., Martos-Sitcha, J.A., de las Heras, V., Calduch-Giner, J., Puyalto, M., Karalazos, V., Sitjà-Bobadillac, A., Pérez-Sánchez, J., 2018. Impact of low fish meal and fish oil diets on the performance, sex steroid profile and male-female sex reversal of gilthead sea bream (*Sparus aurata*) over a three-year production cycle. Aquaculture 490, 64–74. https://doi.org/10.1016/j.aquaculture.2018.02.025.

Siyal, F.A., Babazadeh, D., Wang, C., Arain, M.A., Saeed, M., Ayasan, T., Zhang, L., Wang, T., 2017. Emulsifiers in the poultry industry. Poult. Sci. J. 73, 611–620. https://doi.org/10.1017/S0043933917000502.

Skalli, A., Firmino, J.P., Andree, K.B., Salomón, R., Estévez, A., Puig, P., Sabater-Martínez, M., Hechavarria, T., Gisbert, E., 2020. The inclusion of the microalga *Scenedesmus sp.* in diets for rainbow trout, *Onchorhynchus mykiss*, juveniles. Animals 10, 1656. https://doi.org/10.3390/ani10091656.

Solé, M., Potrykus, J., Fernández-Díaz, C., Blasco, J., 2004. Variations on stress defences and metallothionein levels in the Senegal sole, *Solea senegalensis*, during early larval stages. Fish Physiol. Biochem. 30, 57–66. https://doi.org/10.1007/s10695-004-6786-6.

Solovyev, M., Gisbert, E., 2016. Influence of time, storage temperature and freeze/thaw cycles on the activity of digestive enzymes from gilthead sea bream (*Sparus aurata*). Fish Physiol. Biochem. 42, 1383–1394. https://doi.org/10.1007/s10695-016-0226-2.

Staessen, T.W., Verdegem, M.C., Nederlof, M.A., Eding, E.H., Schrama, J.W., 2021. Effect of type of dietary non-protein energy source (starch vs. fat) on the body bile acid pool size and composition, faecal bile acid loss and bile acid synthesis in rainbow trout (*Oncorhynchus mykiss*). Aquac. Nutr. 27, 865–879. https://doi.org/10.1111/anu.13231.

Tocher, D.R., 2015. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. Aquaculture 449, 94–107. https://doi.org/10.1016/j.aquaculture.2015.01.010.

Trushenski, J., Rosenquist, J., Gause, B., 2011. Growth performance, tissue fatty acid composition, and consumer appeal of rainbow trout reared on feeds containing terrestrially derived rendered fats. North Am. J. Aquac. 73, 468–478. https://doi.org/10.1080/15222055.2011.633691.

Trushenski, J.T., Lochmann, R.T., 2009. Potential, implications and solutions regarding the use of rendered animal fats in aquafeeds. Am. J. Anim. Vet. Sci. 4, 108–128. https://doi.org/10.3844/ajavsp.2009.108.128.

Vallejos-Vidal, E., Reyes-Cerpa, S., Tort, L., Polo, J., Reyes-López, F.E., Gisbert, E., 2022. Spray-dried porcine plasma promotes the association between metabolic and immunological processes at transcriptional level in gilthead sea bream (*Sparus aurata*) gut. Front. Mar. Sci. 9, 814233 https://doi.org/10.3389/fmars.2022.814233.

Watanabe, M., Houten, S.M., Wang, L., Moschetta, A., Mangelsdorf, D.J., Heyman, R.A., Moore, D.D., Auwerx, J., 2004. Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. J. Clin. Investig. 113, 1408–1418. https://doi.org/10.1172/JCI21025.

Weil, C., Lefèvre, F., Bugeon, J., 2013. Characteristics and metabolism of different adipose tissues in fish. Rev. Fish Biol. Fish. 23, 157–173. https://doi.org/10.1007/s11160-012-9288-0.

Yamamoto, T., Suzuki, N., Furuita, H., Sugita, T., Tanaka, N., Goto, T., 2007. Supplemental effect of bile salts to soybean meal-based diet on growth and feed utilization of rainbow trout *Oncorhynchus mykiss*. Fish. Sci. 73, 123–131. https://doi.org/10.1111/j.1444-2906.2007.01310.x.

Yin, P., Xie, S., Zhuang, Z., He, X., Tang, X., Tian, L., Liu, Y., Niu, J., 2021. Dietary supplementation of bile acid attenuate adverse effects of high-fat diet on growth performance, antioxidant ability, lipid accumulation and intestinal health in juvenile largemouth bass (*Micropterus salmoides*). Aquaculture 531, 735864. https://doi.org/10.1016/j.aquaculture.2020.735864.

Yu, H., Zhang, L., Chen, P., Liang, X., Cao, A., Han, J., Wu, X., Zheng, Y., Qin, Y., Xue, M., 2019. Dietary bile acids enhance growth, and alleviate hepatic fibrosis induced by a high starch diet via AKT/FOXO1 and cAMP/AMPK/SREBP1 pathway in *Micropterus salmoides*. Front. Physiol. 10, 1430. https://doi.org/10.3389/fphys.2019.01430.

Zhang, Y., Feng, H., Liang, X.F., He, S., Lan, J., Li, L., 2022. Dietary bile acids reduce liver lipid deposition via activating farnesoid X receptor, and improve gut health by regulating gut microbiota in Chinese perch (*Siniperca chuatsi*). Fish Shellfish Immunol. 121, 265–275. https://doi.org/10.1016/j.fsi.2022.01.010.

Zhou, J.S., Chen, H.J., Ji, H., Shi, X.C., Li, X.X., Chen, L.Q., Du, Z.Y., Yu, H.B., 2018. Effect of dietary bile acids on growth, body composition, lipid metabolism and microbiota in grass carp (*Ctenopharyngodon idella*). Aquac. Nutr. 24, 802–813. https://doi.org/10.1111/anu.12609.

Zhou, S.Y., Liu, Y.J., Liang, H.O., Zhao, L.M., Tian, L.X., Yang, H.J., Liang, G.Y., 2010. Effect of bile acids on growth and body composition of cobia (*Rachycentron canadum*). South China Fish. Sci. 6, 20–25 (In Chinese).

CHAPTER 2

Modulation of gut microbiota and intestinal immune response in gilthead seabream (*Sparus aurata*) by dietary bile salt supplementation

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Modulation of gut microbiota and intestinal immune response in gilthead seabream (*Sparus aurata*) by dietary bile salt supplementation

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Given their role in lipid digestion, feed supplementation with bile salts could be an economic and sustainable solution to alterations in adiposity and intestinal inflammation generated by some strategies currently used in aquaculture. An important part of the metabolism of bile salts takes place in the intestine, where the microbiota transforms them into more toxic forms. Consequently, we aimed to evaluate the gut immune response and microbial populations in gilthead seabream (*Sparus aurata*) fed a diet supplemented with a blend of bile salts with proven background as a regulator of lipid metabolism and fat content. After the 90-day feeding trial, a differential modulation of the microbiota between the anterior and posterior intestine was observed. While in the anterior intestine the relative abundance of Desulfobacterota doubled, in the posterior intestine, the levels of Firmicutes increased and Proteobacteria, Actinobacteriota, and Campylobacterota were reduced when supplementing the diet with bile salts. Even so, only in the anterior intestine, there was a decrease in estimated richness (Chao1 and ACE indices) in presence of dietary bile salts. No significant differences were displayed in alpha (Shannon and Simpson indices) nor beta-diversity, showing that bile sales did not have a great impact on the intestinal microbiota. Regarding the gene expression profile in 2h postprandial-fish, several changes were observed in the analyzed biomarkers of epithelial integrity, nutrient transport, mucus production, interleukins, cell markers, immunoglobulin production and pathogen recognition receptors. These results may indicate the development of an intestinal immune-protective status to tackle future threats. This work also suggests that this immune response is not only regulated by the presence of the dietary bile salts in the intestine, but also by the microbial populations that are in turn modulated by bile salts. After a fasting period of 2 days, the overall gene expression profile was stabilized with respect to fish fed the unsupplemented diet, indicating that the effect of bile salts was transient after short periods of fasting. On the balance, bile salts can be used as a dietary supplement to enhance *S. aurata* farming and production without compromising their intestinal health.

KEYWORDS

fish gut microbiome, immune response, aquaculture, feed additive, bile salts, *Sparus aurata*, teleost, intestinal health

1. Introduction

According to estimates made by the FAO, aquaculture production will increase with respect to 2020 by 14% (24 million tons) in 2030, which would imply an increase in fish oil manufacturing of 13% [Food and Agriculture Organization of the United Nations (FAO), 2022]. The limited availability (depletion of resources and insufficient production) and high cost of fish oil have called into question the sustainability of the major source of omega-3 (n-3) fatty acids in aquafeeds. Under this scenario, the increasing production of plant-derived oils and their lower market prices in comparison to fish oil have made these alternative oil sources an attractive ingredient for fish feed manufacturers. Indeed, several studies have shown that the partial or total replacement of fish oil by plant-based oils do not significantly affect fish growth and feeding performance (Turchini et al., 2003; Ng et al., 2004; Fonseca-Madrigal et al., 2005; Drew et al., 2007). Nonetheless, most vegetable oils have deficiencies in n-3 long-chain highly unsaturated fatty acids (LC-HUFA) with respect to fish oil, which leads to alterations in the fatty acid profile, decreasing the concentration of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA; Caballero et al., 2002; Turchini et al., 2003; Ng et al., 2004; Fonseca-Madrigal et al., 2005; Tocher et al., 2006; Drew et al., 2007). Furthermore, some physiological disorders such as a higher accumulation of lipid droplets on the hepatocytes and enterocytes have been observed under substitution of fish oil by plant-based oils (Olsen et al., 2000; Caballero et al., 2002). In the same way, although the replacement of fish oil by other animal fats in fish diets does not usually generate significant differences in fish performance, it may cause alterations in the fatty acid profile of the filet (Turchini et al., 2003; Subhadra et al., 2006; O'Neal and Kohler, 2008; Trushenski et al., 2011), thereby altering the expected nutritional content for the consumer. In addition, other nutritional alternatives, such as increasing the levels of dietary lipids or carbohydrates to spare the content of protein in the diet, may have a negative impact on growth and feeding performance, as well as modify the filet's fatty acid profile and increase the accumulation of mesenteric fat (Rueda-Jasso et al., 2004; Du et al., 2006).

The above-mentioned studies are just examples of how the aquaculture industry is moving ever closer toward finding a cheaper and more sustainable strategy than the use of fish oil in aquafeeds, and the only wall that now separates the fish farmer from the use of the proposed alternatives is the potential deregulation of the fish lipid profile and problems in adiposity that they can entail. Following the path of the livestock industry (Lothong et al., 2016; Siyal et al., 2017), the increasing use of feed additives could be a functional solution to the above-mentioned problems as long as the selected feed additives promote lipid metabolism in a holistic manner. In this context, dietary bile salt (BS) supplementation may be a successful nutritional strategy due to their well-known role in the enhancement of lipid digestion and absorption, thanks to the activation of lipase and to the formation of micelles that allows emulsification of lipid aggregates (Romano et al., 2020). Furthermore, bile acids (BAs) can act as nutrient signaling hormones, regulating several biological processes by activating specific nuclear receptors, such as the farnesoid X receptor (FXR). Some of the mechanisms under regulation of FXR are metabolism of BSs, lipids, proteins, and carbohydrates, nutrient uptake, energy homeostasis, immunity, and, indirectly, the composition of the gut microbial communities, since they are shaped depending on the BS profile (Vavassori et al., 2009; Renga et al., 2013; Lickwar et al., 2017; Schubert et al., 2017; Chiang and Ferrell, 2022). In recent years, a considerable number of studies have been conducted to test the effects of dietary BS supplementation on fish performance, pointing to the possibility of their becoming a widely extended alternative within the aquafeed production industry. Addition of BSs to aquafeeds has not only successful results in terms of growth and feeding performance, but also enhances lipid catabolism and apparent lipid digestibility (Yamamoto et al., 2007; Iwashita et al., 2008; Gu et al., 2017; Jiang et al., 2018; Ding et al., 2020; Zhang et al., 2022; Ruiz et al., 2023). These actions provide benefits in terms of performance and generally translates into a reduction in lipid content of the body, reduction in fat storage deposits in the liver and in the intestine, as well as the strengthening of antioxidant defenses (Yamamoto et al., 2007; Iwashita et al., 2009; Jiang et al., 2018; Ding et al., 2020; Yin et al., 2021; Zhang et al., 2022; Ruiz et al., 2023).

Despite all the mentioned benefits for the physiology of fish and final quality of the product of dietary BS supplementation, there are not many studies focused on the effect of these molecules at an intestinal level. The importance of this organ lies in the fact that BS metabolism is not only regulated by hepatic FXR, but also by intestinal FXR, which has been suggested to be more sensitive to BAs (Houten et al., 2007) and to be the main regulator of BS metabolism via the classical pathway (Kong et al., 2012). Moreover, it has been proven that these molecules modulate the structure of the gut microbial communities, which metabolize primary BSs into secondary BSs. Although secondary BSs are more hydrophobic and thus presumably more toxic (Hofmann, 1999; Romano et al., 2020), the gut microbiota prevents their toxicity through generation of BS species that are different from those of the host (Schubert et al., 2017; Markandey et al., 2021). In addition, some studies have suggested that the intestinal microbiota plays a fundamental role in the proper development and functionality of the gut immune system (Broom and Kogut, 2018; Markandey et al., 2021). As mentioned above, BSs may also participate in the host immune response as mediated by FXR, which has a key role in the modulation of the intestinal immunity and maintenance of homeostasis (Vavassori et al., 2009; Renga et al., 2013). Due to the conservation of FXR-mediated pathways in fish, it would not be surprising that the effect of BSs reported in higher vertebrates dealing with the modulation of the intestinal microbiome and immunity could be extrapolated to fish. That has already been anticipated by the works of Yamamoto et al. (2007) and Iwashita et al. (2008, 2009), which demonstrated the anti-inflammatory effect of dietary BS supplementation on the intestine of rainbow trout (*Oncorhynchus mykiss*). Nevertheless, it should be noticed that at certain concentrations BSs can have an antimicrobial effect on some gut bacterial strains regarding several factors such the BS combination and dose, and environmental conditions like pH (Begley et al., 2005) and may cause a cytotoxic effect on the fish intestinal mucosa (Romano et al., 2020). The noxious effect of BSs in the intestine and its microbial communities is not only caused by their accumulation, but also by their contribution to the release of other substances that can be toxic if they accumulate, like bilirubin (Romano et al., 2020) and hydrogen sulfide (Schubert et al., 2017). This may lead to alterations in the metabolic functionality of the intestine, and in the production of pathogen-associated molecular patterns (PAMPs;

Schubert et al., 2017). Under these premises, the nutritional assays testing BSs as feed additives performed by the aquaculture and, in general, livestock industry should not only focus on the animal performance but should also offer a holistic view of the health and condition of the studied holobiont (the organism and all of its associated microorganisms), including the organ which could be considered as the main target of BSs: the intestine.

In a previous work, we evaluated the effect of a blend of BSs (sodium cholate, sodium deoxycholate, and sodium taurocholate hydrate) on fish performance and studied the pathways underlying hepatic lipid metabolism in gilthead seabream (*Sparus aurata*) fed with a diet with high-saturated fat content. According to those results, fish displayed an enhanced growth performance and reduced levels of perivisceral, hepatic, and intestinal fat at a dietary BS inclusion level of 0.06% with respect to fish fed the unsupplemented diet (Ruiz et al., 2023). In this contribution, we moved a step forward by evaluating the microbial gut communities and intestinal immune status of *S. aurata* when supplementing the mentioned basal diet with the BS blend.

2. Materials and methods

2.1. Rearing conditions, feeding trial, and experimental diets

Juveniles of *S. aurata* (body weight, $BW_i = 44.0 \pm 4.2$ g; standard length, SL = 12.13 ± 0.48 cm) were obtained from Piscicultura Marina Mediterranea SL (Valencia, Spain). Once at IRTA research facilities, fish ($N = 240$ individuals) were acclimated for 2 weeks and randomly distributed in 8 tanks of 450 L (30 fish per tank; density of 3 kg m^{-3}) connected to a water recirculation system (IRTAmar™). Monitoring of water temperature (22.5 ± 0.5°C), dissolved oxygen (6.3 ± 0.2 mg L^{-1}; OXI330, Crison Instruments, Spain), and pH (7.6 ± 0.01; pH meter 507, Crison Instruments) was carried out daily, and salinity (36‰; MASTER-20 T Hand-Held Refractometer, ATAGO Co. Ltd., Italy), nitrite (0.16 ± 0.1 mg NO_2^- L^{-1}), and ammonia (0.22 ± 0.08 mg NH_4^+ L^{-1}) levels (HACH DR 900 Colorimeter, Hach Company, Spain) were measured weekly.

The trial lasted for 90 days, in which fish were fed twice a day in 12 servings of 5 min each by automatic feeders (Arvo-Tec T Drum 2000, Arvo-Tec Oy, Finland). Initial feeding rate was 3% and was regularly adjusted, as described in Salomón et al. (2020). Somatic growth was monthly monitored by netting all fish in each tank and their BW (g) and SL (cm) was measured. Once netted, fish were immediately anesthetized with 100 mg L^{-1} of buffered tricaine methanesulfonate (MS-222, Sigma-Aldrich, Spain).

Two experimental extruded diets (pellet size: 3 mm) were formulated and manufactured by Sparos Lda. (Portugal) as described by Ruiz et al. (2023). Diets were isoproteic (44.0% crude protein), isolipidic (18.0% crude fat), and isoenergetic (21.4 MJ kg^{-1}) and only differed in their content of the BSs blend at an inclusion level of 0.06% ($BS_{0.06\%}$). The ingredients and proximate composition in dry form of the two experimental diets are shown in Table 1. The BS blend was composed of a 70% of sodium taurocholate hydrate (ref. 86,339, Sigma-Aldrich, United States) and a 30% of a powder BS mixture containing equal parts of sodium cholate and sodium deoxycholate (ref. 48,305, Sigma-Aldrich, United States).

TABLE 1 Formulation, proximate composition (in dry form) and fatty acid profile of the control diet and the basal diet supplemented with a blend of bile salts (BSs) at a dietary inclusion of 0.06% ($BS_{0.06\%}$).

Ingredients (%)	Control	$BS_{0.06\%}$
Fishmeal super prime	7.50	7.50
Fishmeal 60	5.00	5.00
Fish protein concentrate	2.00	2.00
Feather meal hydrolysate	5.00	5.00
Porcine blood meal	3.00	3.00
Poultry meal	15.00	15.00
AminoPro NT70—*C. glutamicum*	4.00	4.00
Corn gluten meal	8.00	8.00
Soybean meal 48	12.00	12.00
Sunflower meal	5.00	5.00
Wheat meal	10.31	10.31
Whole peas	5.00	5.00
Pea starch (raw)	2.40	2.40
Fish oil	3.02	3.02
Soybean oil	2.35	2.35
Poultry fat	8.04	8.04
Vitamin and mineral premix	1.00	1.00
Vitamin C35	0.05	0.05
Vitamin E50	0.02	0.02
Betaine HCl	0.20	0.20
Choline chloride 60	0.10	0.10
Antioxidant	0.20	0.20
Sodium propionate	0.10	0.10
Monoammonium phosphate	0.35	0.35
L-Tryptophan	0.15	0.15
DL-Methionine	0.20	0.20
Bile salts mix	-	0.06
Yttrium oxide	0.02	0.02
Proximate composition		
Crude protein, %	44.1 ± 0.05	44.0 ± 0.08
Crude fat, %	18.1 ± 0.04	18.2 ± 0.05
Gross energy, MJ kg^{-1}	21.4 ± 1.11	21.5 ± 1.20
Fatty acid profile (% of total fatty acids)*		
Saturated fatty acids (SFAs)	27.19 ± 0.40	26.55 ± 0.06
Monounsaturated fatty acids (MUFAs)	36.61 ± 0.73	36.60 ± 0.22
n-6 Polyunsaturated fatty acids (n-6 PUFAs)	26.65 ± 0.06	27.11 ± 0.45
n-3 Polyunsaturated fatty acids (n-3 PUFAs)	9.55 ± 0.39	9.74 ± 0.09
Total PUFAs	36.20 ± 0.45	36.86 ± 0.36

*Fatty acid profile of experimental diets is detailed in Supplementary Table 1. The proximate and fatty acid composition of diets were analyzed in duplicate, and values are represented as mean ± SD.

2.2. Ethics statement

Animal procedures were performed according to the Spanish legislation (law 32/2007 and Royal Decree 1201/2015) and to the Guiding Principles for Biomedical Research Involving Animals (EU2010/63) and approved by the Ethical Committee of the Institute for Food and Agriculture Research and Technology (IRTA), which adopts "The European Code of Conduct for Research Integrity," and by the Generalitat of Catalunya (CEEA 219/2020).

2.3. Sampling

After a fasting period of 48 h at the end of the trial, five fish per tank were randomly hand-netted, euthanized with an overdose of anesthetic MS-222 (300 mg L^{-1}) and eviscerated. Then, a small segment (*ca.* 2 cm^2) from the anterior intestine (AI) of two fish per tank (8 per treatment) was immersed in five volumes of RNAlater® (Sigma-Aldrich, United States), incubated overnight at 4°C, and stored at −80°C until RNA extraction in order to evaluate their gene expression profile, following the procedures of Ruiz et al. (2023). This region of the intestine was chosen because of its demonstrated immunological specialization as described in Vallejos-Vidal et al. (2022), and it was also made to coincide with the region sampled for histological analysis in the previous study of Ruiz et al. (2023). With the goal of studying the gut microbial communities, a 4 cm long section of AI just after the pyloric caeca and a section of *ca.* 4 cm from the posterior intestine (PI), from the anus backward, were dissected from the other three slaughtered fish (12 per treatment). The purpose of the fasting period was to avoid allochthonous microbiota, just targeting the autochthonous bacterial gut communities attached to intestinal mucus (Hao and Lee, 2004). The AI and PI segments of each fish were aseptically opened lengthwise, and their inner walls were separately scraped, insistently but gently with a round edge spatula, avoiding getting host smooth muscle and epithelia, to recover only mucosal bacteria. The scraped content of each segment (12 per dietary treatment in order to ensure statistical robustness) was immediately frozen at −80°C until DNA extraction. Anterior and posterior intestines were treated separately because of the differential digestion and absorption rate in each segment, being higher in the anterior region (Bakke et al., 2010), and because the metabolization of primary BSs into secondary BSs mainly takes place in the posterior part (Hofmann, 1999), which could cause a divergence of the observed microbiota between both regions.

To restore the non-fasting physiology of fish, the remaining animals in the tanks were fed for 3 days, and after 2 h from the last feeding (2 h postprandial-animals), two fish from each tank (8 per treatment) were randomly selected and their AI were dissected and stored at −80°C for RNA extraction as explained above, for assessing the temporal effect of the diets in the intestinal gene expression profile.

2.4. Intestinal gene expression profile

Following the manufacturer's instructions of the QIAGEN RNeasy® Mini Kit (ref. 74,106, QIAGEN, Germany), RNA from AI was extracted and its concentration and purity were measured (NanoDrop-2000® spectrophotometer, Thermo Fisher Scientific, United States). The range of RNA concentrations was between 20 and 100 ng/μL, with A_{260}/A_{280} absorbance ratios of 1.9–2.1. The integrity of RNA was also verified through an agarose gel electrophoresis. For cDNA synthesis, the High-Capacity cDNA Archive Kit (Applied Biosystems, United States) was used with an initial input of 500 ng of RNA. As a negative control, reactions without reverse transcriptase were run.

As described by Naya-Català et al. (2021), real-time quantitative PCR (qPCR) was performed with a CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad, USA). Simultaneous profile of a panel of 44 genes was carried out by means of 96-well PCR array layouts, including biomarkers of epithelial integrity, nutrient transport, mucus production and innate and adaptative immunity (Table 2). Specific primer pair sequences are shown in Supplementary Table 2. Gene expression was calculated using the delta–delta Ct method (Livak and Schmittgen, 2001). The GeNorm software (M score = 0.21) was used for testing the gene expression stability of the gene *β-actin*, which was taken as an endogenous control in the normalization procedure. For multigene analysis, values were referenced to those of *hes1-b* of fish fed the control diet.

2.5. Gut microbial analyses

Extraction of the DNA from *ca.* 250 mg of the scraped product of 12 AI and 12 PI from each dietary group (three per tank) was carried out with the DNeasy PowerSoil Pro Kit (ref. 47016, QIAGEN, Germany), following the manufacturer's recommendations. Prior to performing extractions, a previous step of bead-beating for sample homogenization and cell lysis (BioSpec Mini-BeadBeater-8, BioSpec Products, United States) was performed. DNA concentration and purity were measured in a NanoDrop-2000® spectrophotometer (Thermo Fisher Scientific, United States). The values of A_{260}/A_{280} absorbance ratios were higher than 1.85, and DNA concentrations ranged up to 500 ng μL^{-1}.

The V3–V4 region of the 16S rRNA gene was amplified using the bacterial universal primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′; Klindworth et al., 2013) under the following conditions: 30 s at 98°C, followed by 30 cycles of 10 s at 98°C, 30 s at 55°C and 30 s at 72°C, and a final elongation step of 2 min at 72°C. Library generation was performed according to 16S Metagenomic Sequencing Library Preparation guide (Illumina, 2013) and pair-end 2 × 300 bp sequencing was carried out by means of Illumina-MiSeq platform. Two samples were excluded in the process due to low amplified product concentration. Raw sequencing data were deposited in the Sequence Read Archive (SRA) of NCBI under BioProject accession number PRJNA915342.

Bioinformatic analyses were performed as follows: forward and reverse primers were removed from the raw paired-ended reads by means of the Cutadapt tool in QIIME2 Software (version 2021.11). Then, data were exported to RStudio (version 4.1.2) and processed using the R package *dada2* (Callahan et al., 2016). Forward and reverse read qualities were assessed individually by sample, and by total average (Supplementary Figure 1). An individual and average quality threshold of 28 was established, excluding the reads with a lower Phred score, and those with higher expected error than 2. Then, the paired-ended reads were assembled into contigs, removing the ones with an overlap length < 12 nucleotides or with more than 0

TABLE 2 PCR-array layout for gene expression profile of the intestine of gilthead seabream (*Sparus aurata*) fed experimental diets.

Function	Gene	Symbol	GenBank
Epithelial integrity	Proliferating cell nuclear antigen	*pcna*	KF857335
	Transcription factor HES-1-B	*hes1-b*	KF857344
	Krüppel-like factor 4	*klf4*	KF857346
	Claudin-12	*cldn12*	KF861992
	Claudin-15	*cldn15*	KF861993
	Cadherin-1	*cdh1*	KF861995
	Cadherin-17	*cdh17*	KF861996
	Tight junction protein ZO-1	*tjp1*	KF861994
	Desmoplakin	*dsp*	KF861999
	Gap junction Cx32.2 protein	*cx32.2*	KF862000
	Coxsackievirus and adenovirus receptor homolog	*cxadr*	KF861998
Nutrient transport	Intestinal-type alkaline phosphatase	*alpi*	KF857309
	Liver type fatty acid-binding protein	*fabp1*	KF857311
	Intestinal fatty acid-binding protein	*fabp2*	KF857310
	Ileal fatty acid-binding protein	*fabp6*	KF857312
Mucus production	Mucin 2	*muc2*	JQ277710
	Mucin 13	*muc13*	JQ277713
Interleukins	Tumor necrosis factor-alpha	*tnf-α*	AJ413189
	Interleukin-1 beta	*il-1β*	AJ419178
	Interleukin-6	*il-6*	EU244588
	Interleukin-7	*il-7*	JX976618
	Interleukin-8	*il-8*	JX976619
	Interleukin-10	*il-10*	JX976621
	Interleukin-12 subunit beta	*il-12β*	JX976624
	Interleukin-15	*il-15*	JX976625
	Interleukin-34	*il-34*	JX976629
Cell markers	Cluster of differentiation 4–1	*cd4-1*	AM489485
	Cluster of differentiation 8 beta	*cd8b*	KX231275
	C-C chemokine receptor type 3	*ccr3*	KF857317
	C-C chemokine receptor type 9	*ccr9*	KF857318
	C-C chemokine receptor type 11	*ccr11*	KF857319
	C-C chemokine CK8 / C-C motif chemokine 20	*ck8/ccl20*	GU181393
	Macrophage colony-stimulating factor 1 receptor 1	*csf1r1*	AM050293
Ig production	Immunoglobulin M	*igm*	JQ811851
	Immunoglobulin T membrane-bound form	*igt-m*	KX599201
Pathogen-associated microbial pattern (PAMP)	Galectin-1	*lgals1*	KF862003
	Galectin-8	*lgals8*	KF862004
	Toll-like receptor 2	*tlr2*	KF857323
	Toll-like receptor 5	*tlr5*	KF857324
	Toll-like receptor 9	*tlr9*	AY751797
	CD209 antigen-like protein D	*cd209d*	KF857327
	CD302 antigen	*cd302*	KF857328
	Macrophage mannose receptor 1	*mrc1*	KF857326
	Fucolectin	*fcl*	KF857331

mismatches in the overlap region. A total of 3.3% of the sequences were identified as chimeras and discarded from analysis. Then, SILVA (v138.1) was used as the reference database for bacterial taxonomy classification of contigs into amplicon sequence variants (ASVs), establishing a bootstrapping cut-off of 80% to be considered as a reliable assignment (Smith et al., 2020); otherwise, they were classified as unassigned. Those ASVs with a total sum < 3 sequences (singletons and doubletons) were removed. In brief, from 45 samples a total of 2,900,687 sequences clustering into 14,507 ASVs were generated. According to rarefaction curves (obtained with *vegan* library; Supplementary Figure 2), sample depths were rarefied to 50,000 reads and normalized by total sum scaling (TSS) following the recommendations of McKnight et al. (2019). After rarefaction, a total of 2,250,000 sequences clustering into 14,500 AVSs were obtained. Alpha-diversity was approached by Chao1 and ACE indices for estimating richness and by Shannon and Simpson indices for assessing diversity. The ACE index takes into account rare ASVs ("rare" defined as those with fewer than 10 reads per sample; Kim et al., 2017).

2.6. Statistical analyses

Data on gene expression were analyzed by Student's *t*-test ($p < 0.05$). A Shapiro–Wilk test was used for verifying normality of the data and the Holm-Sidak *post hoc* test for multiple comparisons among groups. Analysis of the interaction between the diets and nutritional status was evaluated with a two-way ANOVA and a Holm-Sidak post-test. To study the separation among dietary groups and nutritional status, supervised partial least squares-discriminant

analysis (PLS-DA) and hierarchical clustering of statistically significant genes ($p < 0.1$) were sequentially applied using EZinfo (v.3.0, Umetrics, Sweden) and the R package *ggplot2*, respectively. Hotelling's T^2 statistic was calculated with the multivariate software package EZinfo and points above the 95% confidence limit for T^2 were considered as outliers and discarded. The quality of the PLS-DA model was evaluated by the parameters R2Y(*cum*) and Q2(*cum*), which indicate the fit and prediction ability, respectively. To assess whether the supervised model was being over-fitted, a validation test consisting of 500 random permutations was performed using the *opls* function from the *ropls* R package.

Significant differences in alpha-diversity among groups ($p < 0.05$) were determined by Kruskal—Wallis one-way analysis of variance, followed by Dunn's post-test. Beta-diversity was calculated as Bray–Curtis dissimilarity (Bray and Curtis, 1957) and represented in a principal coordinate analysis (PCoA). To check significant differences in beta-diversity, permutational multivariate analyses of variance (PERMANOVA) were performed ($p < 0.05$). Differential abundances between groups in phyla and genera with relative abundances >1% were calculated with the method *Metastats*, which includes adjustment of *p* value by false discovery rate (FDR; White et al., 2009). All the described microbial statistical analyses were executed with the R package *microeco* (Liu et al., 2021), which was used together with *ggplot2* for generation of figures.

3. Results

Results in terms of growth performance and feed efficiency are presented elsewhere (Ruiz et al., 2023). In brief, the supplementation of a blend of BSs promoted somatic growth and fish fed the $BS_{0.06\%}$ diet grew more (221.21 ± 3.10 g) than those fed the control diet (215.80 ± 1.06 g; $p < 0.05$). However, no differences in feed conversion ratio (FCR) values were found between both diets, with values ranging from 1.21 ± 0.05 in the control group to 1.19 ± 0.05 in the $BS_{0.06\%}$ diet ($p > 0.05$).

3.1. Alpha and beta-diversity of gut microbiota

Figure 1 shows the estimated richness and diversity (alpha-diversity metrics) of gut microbial communities in the AI and PI regions in both experimental groups (Kruskal–Wallis, followed by Dunn's test). Considering Chao1 and ACE indices, the addition of BSs to the diet generated a reduction in species richness in the AI in *S. aurata* (Figures 1A,B; $p < 0.05$), while no divergences in diversity were found (Figures 1C,D; $p > 0.05$). On the other hand, although no differences in richness were found between the AI and the PI (Figures 1A,B), Simpson's diversity index experienced a significant decrease in the PI with respect to the AI in fish fed both diets (Figure 1D; Supplementary Table 3).

Significant differences in beta-diversity were found among the four experimental groups (PERMANOVA, $F = 1.234$, $R^2 = 0.083$, $p = 0.024$). However, no variation in beta-diversity was registered when comparing dietary condition or region of intestine by pairwise PERMANOVA ($p > 0.05$; see PCoA in Supplementary Figure 3). In fact, the only significant differences observed were between Control-PI and $BS_{0.06\%}$-AI samples (PERMANOVA, $R^2 = 0.065$, $p = 0.037$). The tank variable was verified as an insignificant effect for beta-diversity results (PERMANOVA, $F = 1.1708$, $R^2 = 0.026$, $p = 0.114$).

3.2. Gut microbiota composition

After rarefaction (50,000 reads per sample), a total of 14,500 ASVs were obtained. Among them, 5,574 ASVs (65.0% of total microbiota composition) were found in the AI (Control-AI), and 4,191 (60.2%) in the PI (Control-PI) of fish fed the control diet; while in fish fed the $BS_{0.06\%}$ diet, 3,898 ASVs (61.4%) appeared in the AI ($BS_{0.06\%}$-AI) and 3,011 (61.2%) in the PI ($BS_{0.06\%}$-PI; Figure 2). Among them, 328 ASVs were common to the four groups (41.1%), whereas 4,668 ASVs (12.9%) were exclusive to the Control-AI; 3,289 ASVs (8.2%) were exclusive to the Control-PI; and 3,044 ASVs (11.8%) were exclusive to the $BS_{0.06\%}$-AI; and 2,239 (8.5%) to the $BS_{0.06\%}$-PI.

The ASVs were further classified by phylum (Figure 3A) and genus (Figure 3B). The most abundant phylum was Firmicutes (31.2%–49.7%), followed by Proteobacteria (16.6%–31%), Bacteroidota (17.3%–21.2%), Actinobacteriota (3.5%–5.0%), Desulfobacterota (1.9%–4.1%), Campylobacterota (1.3%–2.2%), Verrucomicrobiota (1.0%–1.9%), and Chloroflexi (0.4%–1.5%). From the most abundant genera (samples' average abundance > 1%; Figure 3B), seven of them belonged to the phylum Proteobacteria (*Pseudomonas*, *Acinetobacter*, *Catenococcus*, *Brevundimonas*, *Marivita*, *Ralstonia,* and *Sphingomonas*), five to Firmicutes (*Streptococcus*, *Fenollaria*, *Candidatus* Arthromitus, *Ezakiella,* and *Peptoniphilus*), three to Bacteroidota (*Bacteroides*, *Porphyromonas,* and *Prevotella*), one to Desulfobacterota (*Desulfovibrio*), one to Campylobacterota (*Campylobacter*), and one to Actinobacteriota (*Corynebacterium*).

When comparing the control to the $BS_{0.06\%}$ diet, significant differences were found among phyla (Supplementary Table 4). In particular, in fish fed with the $BS_{0.06\%}$ diet, Desulfobacterota abundance increased 2.2 times in the AI ($p < 0.05$; $BS_{0.06\%}$-AI vs. Control-AI), while in the PI, Firmicutes increased up to 1.4 times and Proteobacteria showed a decrease of 1.9 times ($p < 0.05$; $BS_{0.06\%}$-PI vs. Control-PI). To a lesser extent ($p < 0.1$), a reduction in Actinobacteriota and Campylobacterota abundances was also found in the PI of *S. aurata* fed the $BS_{0.06\%}$ diet ($BS_{0.06\%}$-PI) with respect to those fed the control diet (Control-PI).

At the genus level, the AI of fish fed the $BS_{0.06\%}$ diet presented higher abundances of *Bacteroides*, *Desulfovibrio*, *Brevundimonas*, and *Ralstonia* ($p < 0.05$), and an apparent slight decrease of *Streptococcus* ($p < 0.1$) was also registered with respect to fish fed the control diet (Supplementary Table 5; $BS_{0.06\%}$-AI vs. Control-AI). Otherwise, a significant reduction was found in *Porphyromonas*, *Campylobacter*, *Corynebacterium*, *Sphingomonas*, *Peptoniphilus*, and, to a lesser degree ($p < 0.1$), of *Fenollaria*, *Acinetobacter*, *Ezakiella,* and *Prevotella* in the PI of fish fed the BS-supplemented diet with respect to the control group ($BS_{0.06\%}$-PI vs. Control-PI).

3.3. Gene expression profile

All genes included in the PCR-array were found at detectable levels, as shown in Supplementary Table 6. In 2 h postprandial-animals, the relative expression of *cdh17*, *lgals8* ($p < 0.05$), and,

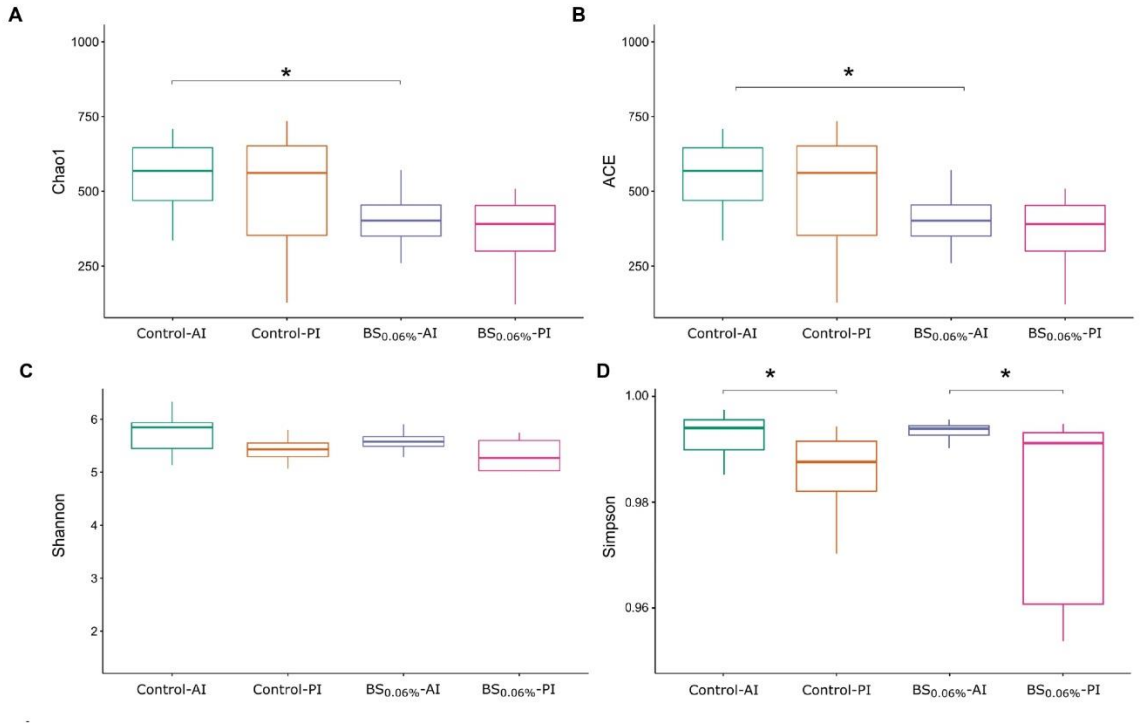

FIGURE 1
Box plots representing the minimum, maximum and the median of the sample values obtained from the richness estimators Chao1 **(A)** and ACE **(B)**, and diversity indices Shannon **(C)** and Simpson **(D)**. The asterisks represent the significant differences between dietary condition or intestinal region of gilthead seabream (*Sparus aurata*; Kruskal—Wallis, Dunn's post-test, $P<0.05$). Experimental groups ($n=12$ fish per group): anterior (Control-AI) and posterior intestine (Control-PI) of *S. aurata* fed the control diet, and anterior ($BS_{0.06\%}$-AI) and posterior intestine ($BS_{0.06\%}$-PI) of *S. aurata* fed the $BS_{0.06\%}$ diet.

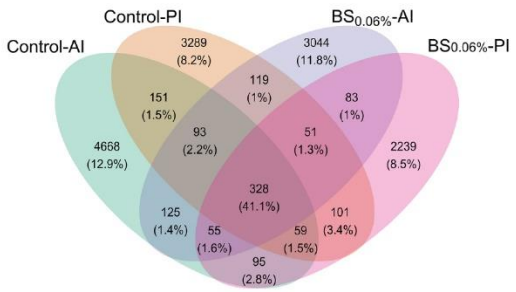

FIGURE 2
Venn diagram plotting the number and relative abundances (%) of ASVs that are unique or common among experimental groups. Experimental groups ($n=12$ fish per group): anterior (Control-AI) and posterior intestine (Control-PI) of gilthead seabream (*Sparus aurata*) fed the control diet, and anterior ($BS_{0.06\%}$-AI) and posterior intestine ($BS_{0.06\%}$-PI) of *S. aurata* fed a basal diet supplemented with a blend of bile salts at a dietary inclusion level of 0.06% ($BS_{0.06\%}$).

especially, *alpi* ($p<0.01$) was down-regulated, while *cd4-1*, *ccr9*, *ck8/ccl20* and *lgals1* were up-regulated ($p<0.05$) in *S. aurata* fed the $BS_{0.06\%}$ diet with respect to those fed the control diet. To a lesser extent ($p<0.1$), there was also a decrease in gene expression of *pcna*, *cx32.2*, *muc13*, and *tlr9*, and an increase in expression of *fabp6*, *il-8*, *ccr3*, *igt-m*, and *mrc1* in comparison with the control group. Summarizing, the addition of BSs to the diet in 2 h postprandial-fish induced an altered expression of some of the measured genes related to pathogen-associated microbial pattern (4), cell markers (4), and markers of epithelial integrity (3), among others. On the other hand, in 48 h fasted-animals the only significant differences in gene expression were the up-regulation of *cldn15* and *cxadr*, and the down-regulation of *cd8b* with respect to the control group ($p<0.05$; Supplementary Table 6).

For evaluating differences in the expression profile of the AI, a two-component PLS-DA model was constructed, with a R2Y(*cum*) of 79% and a Q2(*cum*) of 71% (Figure 4A). The fit of the model was validated by a permutation test (Supplementary Figure 4). The first component of the PLS-DA (48.0% explained variance) clustered fish separately by their feeding condition (48 h fasting vs. 2 h postprandial), whereas the second component (30.7% explained variance) separated 2 h postprandial-animals from both dietary groups (Figure 4B). On the other hand, the PLS-DA showed that in 48 h fasted-animals, there was not a divergent distribution between both dietary groups. Results from the PLS-DA were supported by the expression pattern shown in the heatmap (Figure 4C), which grouped the 2 h postprandial-fish fed the control diet separately from those fed the $BS_{0.06\%}$ diet, while the two experimental groups of 48 h fasted-fish clustered together.

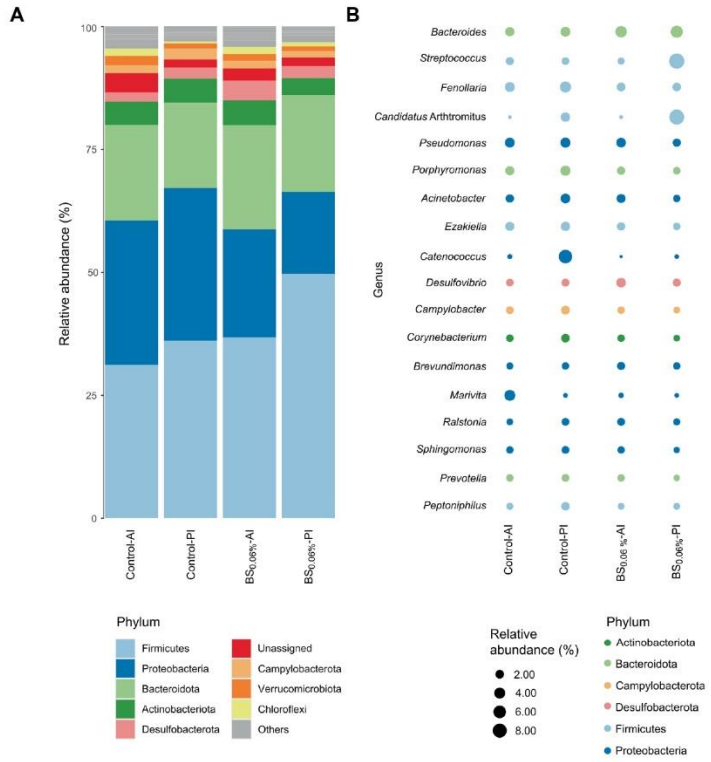

FIGURE 3
Relative abundances of gut bacterial taxa from gilthead seabream (*Sparus aurata*). Data are expressed at phylum **(A)** and genus **(B)** levels (excluding unassigned genera). Taxa appearance in the figures is in order of decreasing abundance (from bottom to top in the bar graph, and inversely in the bubble plot). Taxa with an abundance < 1% are classified as others in the bar graph and not represented in the bubble plot. Experimental groups (n=12 fish per group): anterior (Control-AI) and posterior intestine (Control-PI) of *S. aurata* fed the control diet, and anterior ($BS_{0.06\%}$-AI) and posterior intestine ($BS_{0.06\%}$-PI) of *S. aurata* fed a basal diet supplemented with a blend of bile salts at a dietary inclusion level of 0.06% ($BS_{0.06\%}$).

4. Discussion

4.1. Effect of dietary bile salt supplementation on intestinal microbiota

Under current experimental conditions, Firmicutes, Proteobacteria, Bacteroidota, and Actinobacteriota were the most abundant phyla found in the gut of *S. aurata*. These results were in agreement with previous nutritional studies in the same species (Tapia-Paniagua et al., 2020; Naya-Català et al., 2021). However, the dietary supplementation of BSs resulted in changes in the microbial richness of the intestine in *S. aurata* among dietary treatments. In this sense, a reduction in the observed (number of ASVs) and estimated richness (Chao1 and ACE indices) of bacterial communities was observed in the AI of fish fed the $BS_{0.06\%}$ diet with respect to those fed the control diet, even though such changes were not coupled to variations in bacterial diversity (Shannon and Simpson indices) among dietary groups. Previous studies conducted in fishes have reported that dietary BSs are capable of changing gut microbiota, although depending on the study considered, different changes in bacterial richness and diversity have been reported. In particular, diets supplemented with BSs resulted in differences in the bacterial community richness (Chao1 and ACE indices) in Chinese perch (*Siniperca chuatsi*; Zhang et al., 2022) and *S. aurata* (present results), whereas other studies have reported that dietary BSs only affected bacterial diversity (Shannon and Simpson indices) like in grass carp (*Ctenopharyngodon idella*) and tongue sole (*Cynoglossus semilaevis*; Xiong et al., 2018; Li et al., 2021). Such changes in bacterial richness and diversity among studies may be attributed to different blends and levels of tested BSs, as well as different basal diet formulations, fish husbandry, and physiological conditions.

Most relevant changes in gut microbiota derived from dietary BS supplementation were found in the PI, results that may be attributed to the fact that this region of the digestive tract is where primary BSs are metabolized by the microbiota into secondary BSs, which are more hydrophobic and noxious to bacteria (Romano et al., 2020). In our study, there was a significant increase in the relative abundance of Firmicutes coupled to a decrease in Proteobacteria and Actinobacteriota in the PI of fish fed the $BS_{0.06\%}$ diet. Such increase in Firmicutes relative abundance may be related to the higher tolerance and capacity of metabolizing BSs by this phylum of bacteria (Islam et al., 2011; Ridlon et al., 2014). When comparing the composition of the microbial communities at the genus level, there were also some remarkable variations between both dietary treatments. We found an increase in *Bacteroides* as it has been described in *C. idella* fed BS-supplemented diets (Xiong et al., 2018; Zhou et al., 2018), since

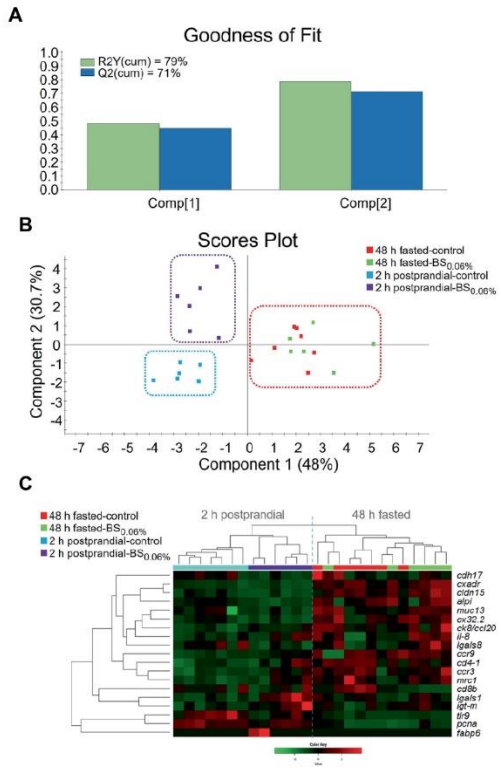

FIGURE 4
(A) Graphical representation of the goodness of fit of the PLS-DA model. **(B)** Scores plot for two-dimensional PLS-DA representing sample distribution between the two components of the model on the basis of their intestinal expression profiles based on statistically significant genes ($p<0.1$). **(C)** Heatmap plotting hierarchical clustering among statistically significant genes ($p<0.1$) on the basis of their expression pattern (Color Key scale) allowing to discriminate for sample distribution. Samples were obtained from anterior intestine (AI) of 48h fasted- and 2h postprandial-gilthead seabream (*Sparus aurata*; $n=8$ per dietary treatment) that were fed the control and the experimental diet supplemented with a blend of bile salts at a dietary inclusion level of 0.06% ($BS_{0.06\%}$).

they may use dietary BSs as substrates for their growth. In particular, *Bacteroides* is a Gram-negative group of bacteria that possess several enzymatic activities involved in the metabolization of primary BSs into secondary BSs (Kawamoto et al., 1989; Chattopadhyay et al., 2022), as well as the capacity to promote BA 7α-dehydroxylase activity in Gram-positive bacterial strains (Hirano and Masuda, 1982). Furthermore, the production of short chain fatty acids by this genus has been suggested to prevent obesity and promote lipolysis in some mammals (Al-Lahham et al., 2010; Li et al., 2013). Although this deserves further investigation in fish, the potential role of *Bacteroides* in lipolysis might explain the reduction in the perivisceral fat levels found in *S. aurata* (Ruiz et al., 2023) and in *C. idella* (Zhou et al., 2018) fed BS-supplemented diets. Furthermore, the increase in the relative abundance of the genus *Desulfovibrio* may be attributed to its capacity of using BA metabolites; in particular, the members of this Gram-negative genus are able to metabolize taurine released from deconjugated BAs, which, similarly to *Bacteroides*, may promote the growth of bacteria able to transform BAs by 7α-dehydroxylation (Hu et al., 2022). Additionally, the growth of *Desulfovibrio* may also be explained by the effect of cholic and deoxycholic acids on this genus (Chattopadhyay et al., 2022). In higher vertebrates, the increase in the relative abundance of *Desulfovibrio* has also been associated to improved health (Chen et al., 2021), findings that seemed to be in line with our previous results in *S. aurata* fed the BS-supplemented diet that showed a better condition in terms of growth and somatic indices, as well as in the levels of fat deposits in the visceral cavity and digestive organs (Ruiz et al., 2023).

While *Bacteroides* and *Desulfovibrio* are among the most reported anaerobic bacteria found in the gastrointestinal tract of marine fish (Romero et al., 2014), the aerobic genus *Brevundimonas* has not been so well studied in fish. In the present study, the increase in the levels of *Brevundimonas* in the gut of fish fed the $BS_{0.06\%}$ diet may be attributed to its role in the metabolization of BSs through 7α-hydroxysteroid dehydrogenase (7α-HSD) activity, playing an important role in secondary BA formation (Chattopadhyay et al., 2022). In addition, current results showed that using a BS-supplemented diet led to a reduction in the levels of some bacterial genera (*Acinetobacter*, *Corynebacterium*, *Peptoniphilus*, *Streptococcus*, *Sphingomonas*, *Porphyromonas*, and *Prevotella*), which are generally considered as commensal species in the gastrointestinal tract of *S. aurata* (Estruch et al., 2015; Naya-Català et al., 2021) and other fish species (Romero et al., 2014). However, as some species of the above-mentioned genera may also be potential emergent fish pathogens (Baya et al., 1992; Weinstein et al., 1997; Kozińska et al., 2014; Malick et al., 2020), the overall effect of this dietary strategy cannot be properly assessed unless further characterization of the microbial community at the species level is conducted. The above-mentioned changes in bacterial abundance may be attributed to their capacity in deconjugation and dehydroxylation of BAs (Schubert et al., 2017) as well as to the noxious effect of BAs, especially secondary BAs, on some species (Hofmann, 1999; Romano et al., 2020). Moreover, the absence of differences in beta-diversity hints that the supplementation of a high saturated fat diet with BSs does not pose any risk for dysbiosis of the fish gut microbiota.

4.2. Effect of dietary bile salt supplementation on intestinal gene expression profile

Different transcriptomic responses were observed when comparing both 48 h of fasting vs. 2 h postprandial samples. When looking at PLS-DA results, the control vs. $BS_{0.06\%}$ comparison showed different patterns of gene expression in fed fish (2 h postprandial-group), whereas this pattern disappeared when considering fish fasted for 48 h (Supplementary Table 6). That may indicate that the effect of $BS_{0.06\%}$ was transient and reversible after short fasting periods as it was previously postulated (Ruiz et al., 2023).

Only three genes (*cldn15*, *cxadr*, and *cd8b*) were found to be differentially expressed in fish fasted for 48 h. The tight junction protein claudin-15 (CLDN15) regulates Na^+ homeostasis and epithelial permeability for cations (Tamura et al., 2011; Wada et al., 2013). In addition, the uptake of BAs into enterocytes takes place by a Na^+-dependent transporter (Keating and Keely, 2009), which is under control of intestinal FXR (Sinha et al., 2008). Thus, the up-regulation of *cldn15* agrees with the up-regulation of coxsackievirus and

adenovirus receptor homolog (*cxadr*), another tight junction gene involved in the regulation of epithelial permeability and tissue homeostasis (Raschperger et al., 2006). In addition to their regulation by dietary BSs, the above-mentioned genes may also be differentially regulated as a compensatory mechanism to the fasting period to which animals were exposed before sampling (Wada et al., 2013). The third gene differentially expressed between both dietary groups after 48 h of fasting was the *cd8b*. The cluster of differentiation 8 (CD8) is a co-receptor and signal transduction molecule, expressed on the surface of $CD8^+$ T cells, playing an important regulatory role in immune responses and has antibacterial activity (Nakanishi et al., 2015). Thus, the reduction in *cd8b* expression might improve gut mucosal condition generated by the above-mentioned changes of bacterial abundance at the genus level rather than a depressed immune response.

When considering the effects of diets supplemented with BSs in 2 h postprandial-animals, we found a completely different scenario with 27% of the analyzed genes differentially expressed. The fish intestine is a complex multifunctional organ, responsible for feed digestion, nutrient absorption, water and electrolyte homeostasis, nutrient metabolism, and immunity (Buddington et al., 1997). This organ is also involved in BS metabolism through the gut-liver axis since the intestinal FXR is involved in the synthesis of BA in the liver (Romano et al., 2020). Thus, we found a differential expression pattern of some biomarkers involved in nutrient transport and absorption, such as the up-regulation of the fatty acid-binding protein (*fabp6*) and the down-regulation of intestinal-type alkaline phosphatase (*alpi*) in fish fed the BS-supplemented diet. Particularly, FABP6 is commonly regarded as a BA binding protein found in the distal portion of the intestine and is involved in the efficient apical to basolateral transport of conjugated BAs in enterocytes; thus, playing an important role in BA homeostasis (Praslickova et al., 2012). Such change in *fabp6* might be explained by changes in the BA profile in *S. aurata* fed the $BS_{0.06\%}$ (Ruiz et al., 2023), as well as by FXR (Lickwar et al., 2017), as it has recently been demonstrated in zebrafish (*Brachydanio rerio*; Wen et al., 2021). On the other hand, ALPI is located in the enterocyte brush border and participates in nutrient absorption, the maintenance of the gut barrier function, and modulation of gut microbiota (Lallès, 2020). Significantly, ALPI promotes an anti-inflammatory immune response through dephosphorylation of lipopolysaccharides (LPSs) from the outer membrane of Gram-negative bacteria, and its deficiency has been correlated with intestinal inflammation (Rader, 2017). However, under the present conditions no signs of enteric inflammation were found in fish fed the $BS_{0.06\%}$ diet (Ruiz et al., 2023); thus, the down-regulation of *alpi* in fish fed the $BS_{0.06\%}$ diet might be due to a reduced bacterial richness as indicated by microbial analyses (number of ASVs and, Chao1 and ACE indices).

The rest of the analyzed intestinal biomarkers that were also differentially expressed are mainly involved in epithelial integrity and immunity (*pcna*, *cdh17*, *cx32.2*, *muc13*, *il-8*, *cd4-1*, *ccr3*, *ccr9*, *ck8/ccl20*, *igt-m*, *lgals1*, *lgals8*, *tlr9*, and *mrc1*). Although there are not many studies in this field, some recent studies are beginning to elucidate the role of BSs in the intestine of fish (Jin et al., 2019; Peng et al., 2019; Yin et al., 2021). As Foey and Picchietti (2014) highlighted, the mucosal layer is the first line of defense of the fish immune system, which acts as a physical and chemical immune barrier, and consists of the mucus and its commensal bacteria that overlay the epithelial cells lining the gut with associated lymphoid tissue. In a previous nutritional trial of dietary BSs (Ruiz et al., 2023), the concentration of BSs used in the current work was identified as being effective at inducing an effect on body fat content while remaining within concentrations that are not toxic for the animal (Keating and Keely, 2009; Romano et al., 2020); and considering the transcriptomic results for biomarkers of gut epithelial integrity, we may state that this goal was achieved. In particular, the down-regulation of proliferating cell nuclear antigen (*pcna*) may suggest a lower epithelial turnover rate and consequently, an ameliorated health condition of enterocytes (Gisbert et al., 2017; Naya-Català et al., 2021). Lower epithelial cell turnover rate may be in line with the observed lower gene expression of the adherens- and gap-type junction proteins, cadherin-17 (*cdh17*) and gap junction Cx32.2 protein (*cx32.2*), respectively. The expression of the rest of the biomarkers related to epithelial integrity did not change among fish fed the control and the $BS_{0.06\%}$ diet. A high concentration of BSs in the intestine can cause a loss of the tight junctions between epithelial cells, leading to an increase in mucosal permeability and cell death (Keating and Keely, 2009), so the absence of differences in the expression of tight junction proteins confirmed that the gut barrier function was maintained at the concentration of BSs tested. In addition, the decrease in expression of mucin 13 (*muc13*) in intestinal cells could be correlated to a lower mucus turnover in response to a reduction of certain bacterial genera (Pérez et al., 2010).

Current data also indicated that the $BS_{0.06\%}$ diet mediated an intestinal immune response in *S. aurata*, as we observed an upregulation of the cell marker cluster of differentiation 4-1 (*cd4-1*), a cell-surface marker of T lymphocytes, and other immune cells. This finding may not be directly explained by dietary BSs, but by their effect on shaping the gut microbiota, and particularly, by the increase in *Bacteroides*. As reported by Zhou and Zhi (2016), this Gram-negative genus has an immunomodulatory role due to its ability to induce the proliferation of regulatory $CD4^+$ T cells (T_{reg}) and production of anti-inflammatory cytokines. Additionally, the up-regulation of *cd4-1*, C-C chemokine receptor type 3 (*ccr3*), C-C chemokine receptor type 9 (*ccr9*), and C-C chemokine CK8 (*ck8*) may also support that fish fed the $BS_{0.06\%}$ had an enhanced gut immune response. This hypothesis would be in line with the study of Su et al. (2021), which reported an increase in the phagocytic and antibacterial activities in plasma of Pacific white shrimp (*Litopenaeus vannamei*) fed a diet supplemented with graded levels of a mixture of BSs (67.52% hyodeoxycholic acid, 19.81% chenodeoxycholic acid, and 8.60% hyocholic acid). Indeed, the cell marker CD4 can be found on the surface of T_{reg} cells, but also T helper cells, monocytes, macrophages, and dendritic cells (Parija, 2012; Ashfaq et al., 2019). These same immune cells, together with B cells, express the cell marker *ccr9*, which can function to induce the migration of immune cells to the gut to regulate inflammation (Pathak and Lal, 2020). There was also an up-regulation of *ccr3* which drives the displacement and activation of eosinophils (Heath et al., 1997), and of *ck8*, which elicits chemoattraction of lymphocytes and granulocytes (Hieshima et al., 1997) and can activate CCR6 (Baba et al., 1997), which is involved in differentiation of T cell-lineages during gut inflammation (Kulkarni et al., 2018). On the other hand, there was an up-regulation of galectin-1 (*lgals1*) and a down-regulation of galectin-8 (*lgals8*) and toll-like receptor 9 (*tlr9*), which may suggest the induction of an anti-inflammatory response in spite of the absence of changes in

interleukin-10 (*il-10*) expression; although in the current study, the downregulation of *tlr9* and upregulation of *ck8* might be linked to the changes in bacterial abundance generated by the effect of BSs in the gut of *S. aurata* (Hemmi et al., 2000; Cuesta et al., 2010). In this sense, TLR9 recognizes bacterial unmethylated CpG motifs (Hemmi et al., 2000) and induces the production of some proinflammatory cytokines (Kumagai et al., 2008). Moreover, LGALS1 maintains the homeostasis of immune cells and the integrity of the mucosa and reduces inflammation by attenuating the synthesis of proinflammatory cytokines. It may also promote the apoptosis of T lymphocytes, the inactivation of antigen-presenting cells or the proliferation and differentiation of T cells (Seropian et al., 2018; Lu et al., 2019). Thus, the upregulation of *lgals1* may be in line with the reported up-regulation of *cd4-1* in fish fed the $BS_{0.06\%}$ diet. In contrast, we found a down-regulation in *lgals8* expression. LGALS8 favors inflammation by stimulating secretion of proinflammatory cytokines, including interleukin-6 (IL-6) and interleukin-8 (IL-8; Cattaneo et al., 2014). Thus, the hypothesis of a possible decrease in the exposure to potential pathogens due to the noxious effect of BSs might also be supported by the down-regulation of *lgals8*, considering that it can act as a sensor of membrane damage caused by infection and restricts the proliferation of infectious pathogens (Thurston et al., 2012). Although in the present study there was no variation in *il-6* expression, we found that *il*-8 was up-regulated in fish fed the $BS_{0.06\%}$ diet, and this may be associated to an increase in the expression of PRR macrophage mannose receptor 1 (*mrc1*; Gazi and Martinez-Pomares, 2009), providing evidence of an effective barrier against potential pathogens when no inflammatory disorders were found in gut (Gisbert et al., 2017). The former hypothesis may also be supported by the up-regulated expression of the membrane-bound form of immunoglobulin T (*igt-m*), widely used as a marker of mucosal immunocompetence (Zhang et al., 2010; Piazzon et al., 2016). The fact that IgM was not simultaneously up-regulated in fish fed $BS_{0.06\%}$ diet agrees with the recent studies that suggested differential regulatory mechanisms for *igt-m* and *igm* in *S. aurata* (Piazzon et al., 2016; Naya-Català et al., 2021). Considering that no inflammation was observed along the intestine of *S. aurata* fed diets supplemented with BSs (Ruiz et al., 2023), these results may indicate that dietary BS supplementation might have induced a state of immunocompetence in the intestine of *S. aurata* orchestrated by several genes related to a proinflammatory response with additional anti-inflammatory effectors, without having an overall apparent deleterious effect on gut physiology.

5. Conclusion

The present study showed that the supplementation of a high-saturated fat diet with a blend of BSs differentially modulated gut microbiota depending on the region in the intestine considered. In particular, dietary BSs decreased bacterial richness in the AI of *S. aurata*, but without remarkable changes in bacterial diversity and composition. Regarding the PI, dietary BSs had an impact on the relative abundance of some microbial taxa, resulting in an increase in the relative abundance of Firmicutes and a decrease of the phyla Proteobacteria, Actinobacteriota, and Campylobacterota, changes that were mainly linked to the fact that this region of the intestine is where the majority of primary BSs are metabolized into secondary BSs by bacterial enzymes. In addition, BSs had an antimicrobial effect on several Gram-negative bacteria, such as those from the genus *Corynebacterium*, *Sphingomonas,* and *Prevotella*. Dietary BSs promoted gut condition by mediating an intestinal immune response characterized by the regulation of several genes involved in innate and cellular immunity processes. Whether these changes are due to the immunogenic potential of BSs, by their role in shaping gut microbiota or both could not be deciphered; thus, they deserve further investigation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI—PRJNA915342.

Ethics statement

The animal study was reviewed and approved by the Ethical Committee of the Institute for Food and Agriculture Research and Technology (IRTA), which adopts "The European Code of Conduct for Research Integrity," and by the Generalitat of Catalunya (CEEA 219/2020).

Author contributions

AR: methodology, formal analysis, visualization, and writing-original draft. KA: methodology, visualization, writing—review and editing, and supervision. DF and JC-G: methodology and writing—review and editing. PH: formal analysis and writing—review and editing. MV and JP-S: methodology, visualization, and writing—review and editing. EG: conceptualization, methodology, writing—review and editing, supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1123716/full#supplementary-material

References

Al-Lahham, S. H., Peppelenbosch, M. P., Roelofsen, H., Vonk, R. J., and Venema, K. (2010). Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. *Biochim. Biophys. Acta* 1801, 1175–1183. doi: 10.1016/j.bbalip.2010.07.007

Ashfaq, H., Soliman, H., Saleh, M., and El-Matbouli, M. (2019). CD4: a vital player in the teleost fish immune system. *Vet. Res.* 50, 1–11. doi: 10.1186/s13567-018-0620-0

Baba, M., Imai, T., Nishimura, M., Kakizaki, M., Takagi, S., Hieshima, K., et al. (1997). Identification of CCR6, the specific receptor for a novel lymphocyte-directed CC chemokine LARC. *J. Biol. Chem.* 272, 14893–14898. doi: 10.1074/jbc.272.23.14893

Bakke, A. M., Glover, C., and Krogdahl, Å. (2010). "Feeding, digestion and absorption of nutrients" in *Fish physiology: The multifunctional gut of fish*. eds. M. Grosell, A. P. Farrell and C. J. Brauner (London: Academic Press), 57–110.

Baya, A. M., Lupiani, B., Bandin, I., Hetrick, F. M., Figueras, A., Carnanan, A., et al. (1992). Phenotypic and pathobiological properties *of Corynebacterium aquaticum* isolated from diseased striped bass. *Dis. Aquat. Organ.* 14, 115–126. doi: 10.3354/dao014115

Begley, M., Gahan, C. G., and Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiol. Rev.* 29, 625–651. doi: 10.1016/j.femsre.2004.09.003

Bray, J. R., and Curtis, J. T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecol. Monogr.* 27, 325–349. doi: 10.2307/1942268

Broom, L. J., and Kogut, M. H. (2018). The role of the gut microbiome in shaping the immune system of chickens. *Vet. Immunol. Immunopathol.* 204, 44–51. doi: 10.1016/j.vetimm.2018.10.002

Buddington, R. K., Krogdahl, A., and Bakke-McKellep, A. M. (1997). The intestines of carnivorous fish: structure and functions and the relations with diet. *Acta Physiol. Scand.* 638, 67–80.

Caballero, M. J., Obach, A., Rosenlund, G., Montero, D., Gisvold, M., and Izquierdo, M. S. (2002). Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 214, 253–271. doi: 10.1016/S0044-8486(01)00852-3

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Cattaneo, V., Tribulatti, M. V., Carabelli, J., Carestia, A., Schattner, M., and Campetella, O. (2014). Galectin-8 elicits pro-inflammatory activities in the endothelium. *Glycobiology* 24, 966–973. doi: 10.1093/glycob/cwu060

Chattopadhyay, I., Gundamaraju, R., Jha, N. K., Gupta, P. K., Dey, A., Mandal, C. C., et al. (2022). Interplay between dysbiosis of gut microbiome, lipid metabolism, and tumorigenesis: can gut dysbiosis stand as a prognostic marker in cancer? *Dis. Markers* 2022:2941248. doi: 10.1155/2022/2941248

Chen, Y. R., Jing, Q. L., Chen, F. L., Zheng, H., Chen, L. D., and Yang, Z. C. (2021). *Desulfovibrio* is not always associated with adverse health effects in the Guangdong gut microbiome project. *PeerJ* 9:e12033. doi: 10.7717/peerj.12033

Chiang, J. Y., and Ferrell, J. M. (2022). Discovery of farnesoid X receptor and its role in bile acid metabolism. *Mol. Cell. Endocrinol.* 548:111618. doi: 10.1016/j.mce.2022.111618

Cuesta, A., Dios, S., Figueras, A., Novoa, B., Esteban, M. A., Meseguer, J., et al. (2010). Identification of six novel CC chemokines in gilthead seabream (*Sparus aurata*) implicated in the antiviral immune response. *Mol. Immunol.* 47, 1235–1243. doi: 10.1016/j.molimm.2009.12.014

Ding, T., Xu, N., Liu, Y., Du, J., Xiang, X., Xu, D., et al. (2020). Effect of dietary bile acid (BA) on the growth performance, body composition, antioxidant responses and expression of lipid metabolism-related genes of juvenile large yellow croaker (*Larimichthys crocea*) fed high-lipid diets. *Aquaculture* 518:734768. doi: 10.1016/j.aquaculture.2019.734768

Drew, M. D., Ogunkoya, A. E., Janz, D. M., and Van Kessel, A. G. (2007). Dietary influence of replacing fish meal and oil with canola protein concentrate and vegetable oils on growth performance, fatty acid composition and organochlorine residues in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 267, 260–268. doi: 10.1016/j.aquaculture.2007.01.002

Du, Z. Y., Clouet, P., Zheng, W. H., Degrace, P., Tian, L. X., and Liu, Y. J. (2006). Biochemical hepatic alterations and body lipid composition in the herbivorous grass carp (*Ctenopharyngodon idella*) fed high-fat diets. *Br. J. Nutr.* 95, 905–915. doi: 10.1079/bjn20061733

Estruch, G., Collado, M., Peñaranda, D., Tomás Vidal, A., Jover Cerdá, M., Pérez Martínez, G., et al. (2015). Impact of fishmeal replacement in diets for gilthead sea bream (*Sparus aurata*) on the gastrointestinal microbiota determined by pyrosequencing the 16S rRNA gene. *PLoS One* 10:e0136389. doi: 10.1371/journal.pone.0136389

Foey, A., and Picchietti, S. (2014). "Immune defences of teleost fish" in *Aquaculture nutrition: Gut health, probiotics and prebiotics*. eds. D. Merrifield and E. Ringø (Oxford: Wiley-Blackwell), 14–52.

Fonseca-Madrigal, J., Karalazos, V., Campbell, P. J., Bell, J. G., and Tocher, D. R. (2005). Influence of dietary palm oil on growth, tissue fatty acid compositions, and fatty acid metabolism in liver and intestine in rainbow trout (*Oncorhynchus mykiss*). *Aquacult. Nutr.* 11, 241–250. doi: 10.1111/j.1365-2095.2005.00346.x

Food and Agriculture Organization of the United Nations (FAO). (2022). *The state of world fisheries and aquaculture 2022. Towards Blue Transformation*. Rome: FAO.

Gazi, U., and Martinez-Pomares, L. (2009). Influence of the mannose receptor in host immune responses. *Immunobiology* 214, 554–561. doi: 10.1016/j.imbio.2008.11.004

Gisbert, E., Andree, K. B., Quintela, J. C., Calduch-Giner, J. A., Ipharraguerre, I. R., and Pérez-Sánchez, J. (2017). Olive oil bioactive compounds increase body weight, and improve gut health and integrity in gilthead sea bream (*Sparus aurata*). *Br. J. Nutr.* 117, 351–363. doi: 10.1017/S0007114517000228

Gu, M., Bai, N., and Kortner, T. M. (2017). Taurocholate supplementation attenuates the changes in growth performance, feed utilization, lipid digestion, liver abnormality and sterol metabolism in turbot (*Scophthalmus maximus*) fed high level of plant protein. *Aquaculture* 468, 597–604. doi: 10.1016/j.aquaculture.2016.11.022

Hao, W. L., and Lee, Y. K. (2004). Microflora of the gastrointestinal tract: a review. *Methods Mol. Biol.* 268, 491–502. doi: 10.1385/1-59259-766-1:491

Heath, H., Qin, S., Rao, P., Wu, L., LaRosa, G., Kassam, N., et al. (1997). Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. *J. Clin. Invest.* 99, 178–184. doi: 10.1172/JCI119145

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., et al. (2000). A toll-like receptor recognizes bacterial DNA. *Nature* 408, 740–745. doi: 10.1038/35047123

Hieshima, K., Imai, T., Opdenakker, G., van Damme, J., Kusuda, J., Tei, H., et al. (1997). Molecular cloning of a novel human CC chemokine liver and activation-regulated chemokine (LARC) expressed in liver: chemotactic activity for lymphocytes and gene localization on chromosome 2. *J. Biol. Chem.* 272, 5846–5853. doi: 10.1074/jbc.272.9.5846

Hirano, S., and Masuda, N. (1982). Enhancement of the 7 alpha-dehydroxylase activity of a gram-positive intestinal anaerobe by *Bacteroides* and its significance in the 7-dehydroxylation of ursodeoxycholic acid. *J. Lipid Res.* 23, 1152–1158. doi: 10.1016/S0022-2275(20)38052-4

Hofmann, A. F. (1999). The continuing importance of bile acids in liver and intestinal disease. *Arch. Intern. Med.* 159, 2647–2658. doi: 10.1001/archinte.159.22.2647

Houten, S. M., Volle, D. H., Cummins, C. L., Mangelsdorf, D. J., and Auwerx, J. (2007). In vivo imaging of farnesoid X receptor activity reveals the ileum as the primary bile acid signaling tissue. *Mol. Endocrinol.* 21, 1312–1323. doi: 10.1210/me.2007-0113

Hu, H., Shao, W., Liu, Q., Liu, N., Wang, Q., Xu, J., et al. (2022). Gut microbiota promotes cholesterol gallstone formation by modulating bile acid composition and

biliary cholesterol secretion. *Nat. Commun.* 13, 252–213. doi: 10.1038/s41467-021-27758-8

Illumina. (2013). 16S metagenomic sequencing library preparation: preparing 16S ribosomal RNA gene amplicons for the Illumina MiSeq system. Available at: https://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf (accessed 11 November 2022).

Islam, K. S., Fukiya, S., Hagio, M., Fujii, N., Ishizuka, S., Ooka, T., et al. (2011). Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. *Gastroenterology* 141, 1773–1781. doi: 10.1053/j.gastro.2011.07.046

Iwashita, Y., Suzuki, N., Matsunari, H., Sugita, T., and Yamamoto, T. (2009). Influence of soya saponin, soya lectin, and cholyltaurine supplemented to a casein-based semipurified diet on intestinal morphology and biliary bile status in fingerling rainbow trout *Oncorhynchus mykiss*. *Fish. Sci.* 75, 1307–1315. doi: 10.1007/s12562-009-0158-1

Iwashita, Y., Suzuki, N., Yamamoto, T., Shibata, J. I., Isokawa, K., Soon, A. H., et al. (2008). Supplemental effect of cholyltaurine and soybean lecithin to a soybean meal-based fish meal-free diet on hepatic and intestinal morphology of rainbow trout *Oncorhynchus mykiss*. *Fish. Sci.* 74, 1083–1095. doi: 10.1111/j.1444-2906.2008.01628.x

Jiang, M., Wen, H., Gou, G. W., Liu, T. L., Lu, X., and Deng, D. F. (2018). Preliminary study to evaluate the effects of dietary bile acids on growth performance and lipid metabolism of juvenile genetically improved farmed tilapia (*Oreochromis niloticus*) fed plant ingredient-based diets. *Aquacult. Nutr.* 24, 1175–1183. doi: 10.1111/anu.12656

Jin, M., Pan, T., Cheng, X., Zhu, T. T., Sun, P., Zhou, F., et al. (2019). Effects of supplemental dietary L-carnitine and bile acids on growth performance, antioxidant and immune ability, histopathological changes and inflammatory response in juvenile black seabream (*Acanthopagrus schlegelii*) fed high-fat diet. *Aquaculture* 504, 199–209. doi: 10.1016/j.aquaculture.2019.01.063

Kawamoto, K., Horibe, I., and Uchida, K. (1989). Purification and characterization of a new hydrolase for conjugated bile acids, chenodeoxycholyltaurine hydrolase, from *Bacteroides vulgatus*. *J. Biochem.* 106, 1049–1053. doi: 10.1093/oxfordjournals.jbchem.a122962

Keating, N., and Keely, S. J. (2009). Bile acids in regulation of intestinal physiology. *Curr. Gastroenterol. Rep.* 11, 375–382. doi: 10.1007/s11894-009-0057-8

Kim, B. R., Shin, J., Guevarra, R. B., Lee, J. H., Kim, D. W., Seol, K. H., et al. (2017). Deciphering diversity indices for a better understanding of microbial communities. *J. Microbiol. Biotechnol.* 27, 2089–2093. doi: 10.4014/jmb.1709.09027

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi: 10.1093/nar/gks808

Kong, B., Wang, L., Chiang, J. Y., Zhang, Y., Klaassen, C. D., and Guo, G. L. (2012). Mechanism of tissue-specific farnesoid X receptor in suppressing the expression of genes in bile-acid synthesis in mice. *Hepatology* 56, 1034–1043. doi: 10.1002/hep.25740

Kozińska, A., Paździor, E., Pękala, A., and Niemczuk, W. (2014). *Acinetobacter johnsonii* and *Acinetobacter lwoffii*—the emerging fish pathogens. *J. Vet. Res.* 58, 193–199. doi: 10.2478/bvip-2014-0029

Kulkarni, N., Meitei, H. T., Sonar, S. A., Sharma, P. K., Mujeeb, V. R., Srivastava, S., et al. (2018). CCR6 signaling inhibits suppressor function of induced-T_{reg} during gut inflammation. *J. Autoimmun.* 88, 121–130. doi: 10.1016/j.jaut.2017.10.013

Kumagai, Y., Takeuchi, O., and Akira, S. (2008). TLR9 as a key receptor for the recognition of DNA. *Adv. Drug Deliv. Rev.* 60, 795–804. doi: 10.1016/j.addr.2007.12.004

Lallès, J. P. (2020). Intestinal alkaline phosphatase in the gastrointestinal tract of fish: biology, ontogeny, and environmental and nutritional modulation. *Rev. Aquac.* 12, 555–581. doi: 10.1111/raq.12340

Li, X., Chen, H., Guan, Y., Li, X., Lei, L., Liu, J., et al. (2013). Acetic acid activates the AMP-activated protein kinase signaling pathway to regulate lipid metabolism in bovine hepatocytes. *PLoS One* 8:e67880. doi: 10.1371/journal.pone.0067880

Li, Y., Wang, S., Hu, Y., Cheng, J., Cheng, X., Cheng, P., et al. (2021). Dietary bile acid supplementation reveals beneficial effects on intestinal healthy status of tongue sole (*Cynoglossus semiliaevis*). *Fish Shellfish Immunol.* 116, 52–60. doi: 10.1016/j.fsi.2021.06.020

Lickwar, C. R., Camp, J. G., Weiser, M., Cocchiaro, J. L., Kingsley, D. M., Furey, T. S., et al. (2017). Genomic dissection of conserved transcriptional regulation in intestinal epithelial cells. *PLoS Biol.* 15:e2002054. doi: 10.1371/journal.pbio.2002054

Liu, C., Cui, Y., Li, X., and Yao, M. (2021). Microeco: an R package for data mining in microbial community ecology. *FEMS Microbiol. Ecol.* 97:fiaa255. doi: 10.1093/femsec/fiaa255

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Lothong, M., Tachampa, K., Assavacheep, P., and Angkanaporn, K. (2016). Effects of dietary betaine supplementation on back fat thickness and serum IGF-1 in late finishing pigs. *Thai J. Vet. Med.* 46, 427–434.

Lu, H., Dai, X., Li, X., Sun, Y., Gao, Y., and Zhang, C. (2019). Gal-1 regulates dendritic cells-induced Treg/Th17 balance though NF-κB/RelB-IL-27 pathway. *Ann. Transl. Med.* 7:628. doi: 10.21037/atm.2019.11.02

Malick, R. C., Bera, A. K., Chowdhury, H., Bhattacharya, M., Abdulla, T., Swain, H. S., et al. (2020). Identification and pathogenicity study of emerging fish pathogens *Acinetobacter junii* and *Acinetobacter pittii* recovered from a disease outbreak in *Labeo catla* (Hamilton, 1822) and *Hypophthalmichthys molitrix* (Valenciennes, 1844) of freshwater wetland in West Bengal, India. *Aquacult. Res.* 51, 2410–2420. doi: 10.1111/are.14584

Markandey, M., Bajaj, A., Ilott, N. E., Kedia, S., Travis, S., Powrie, F., et al. (2021). Gut microbiota: sculptors of the intestinal stem cell niche in health and inflammatory bowel disease. *Gut Microbes* 13:1990827. doi: 10.1080/19490976.2021.1990827

McKnight, D. T., Huerlimann, R., Bower, D. S., Schwarzkopf, L., Alford, R. A., and Zenger, K. R. (2019). Methods for normalizing microbiome data: an ecological perspective. *Methods Ecol. Evol.* 10, 389–400. doi: 10.1111/2041-210X.13115

Nakanishi, T., Shibasaki, Y., and Matsuura, Y. (2015). T cells in fish. *Biology* 4, 640–663. doi: 10.3390/biology4040640

Naya-Català, F., do Vale Pereira, G., Piazzon, M. C., Fernandes, A. M., Calduch-Giner, J. A., Sitjà-Bobadilla, A., et al. (2021). Cross-talk between intestinal microbiota and host gene expression in gilthead sea bream (*Sparus aurata*) juveniles: insights in fish feeds for increased circularity and resource utilization. *Front. Physiol.* 12:748265. doi: 10.3389/fphys.2021.748265

Ng, W. K., Sigholt, T., and Gordon Bell, J. (2004). The influence of environmental temperature on the apparent nutrient and fatty acid digestibility in Atlantic salmon (*Salmo salar* L.) fed finishing diets containing different blends of fish oil, rapeseed oil and palm oil. *Aquacult. Res.* 35, 1228–1237. doi: 10.1111/j.1365-2109.2004.01131.x

Olsen, R. E., Myklebust, R., Ringø, E., and Mayhew, T. M. (2000). The influences of dietary linseed oil and saturated fatty acids on caecal enterocytes in Arctic char (*Salvelinus alpinus* L.): a quantitative ultrastructural study. *Fish Physiol. Biochem.* 22, 207–216. doi: 10.1023/A:1007879127182

O'Neal, C. C., and Kohler, C. C. (2008). Effect of replacing menhaden oil with catfish oil on the fatty acid composition of juvenile channel catfish, *Ictalurus punctatus*. *J. World. Aquac. Soc.* 39, 62–71. doi: 10.1111/j.1749-7345.2007.00137.x

Parija, S. C. (2012). *Textbook of microbiology and immunology*. India: Elsevier Health Sciences.

Pathak, M., and Lal, G. (2020). The regulatory function of $CCR9^+$ dendritic cells in inflammation and autoimmunity. *Front. Immunol.* 11:536326. doi: 10.3389/fimmu.2020.536326

Peng, X. R., Feng, L., Jiang, W. D., Wu, P., Liu, Y., Jiang, J., et al. (2019). Supplementation exogenous bile acid improved growth and intestinal immune function associated with NF-κB and TOR signalling pathways in on-growing grass carp (*Ctenopharyngodon idella*): enhancement the effect of protein-sparing by dietary lipid. *Fish Shellfish Immunol.* 92, 552–569. doi: 10.1016/j.fsi.2019.06.047

Pérez, T., Balcázar, J. L., Ruiz-Zarzuela, I., Halaihel, N., Vendrell, D., De Blas, I., et al. (2010). Host–microbiota interactions within the fish intestinal ecosystem. *Mucosal Immunol.* 3, 355–360. doi: 10.1038/mi.2010.12

Piazzon, M. C., Galindo-Villegas, J., Pereiro, P., Estensoro, I., Calduch-Giner, J. A., Gómez-Casado, E., et al. (2016). Differential modulation of IgT and IgM upon parasitic, bacterial, viral, and dietary challenges in a perciform fish. *Front. Immunol.* 7:637. doi: 10.3389/fimmu.2016.00637

Praslickova, D., Torchia, E. C., Sugiyama, M. G., Magrane, E. J., Zwicker, B. L., Kolodzieyski, L., et al. (2012). The ileal lipid binding protein is required for efficient absorption and transport of bile acids in the distal portion of the murine small intestine. *PLoS One* 7:e50810. doi: 10.1371/journal.pone.0050810

Rader, B. A. (2017). Alkaline phosphatase, an unconventional immune protein. *Front. Immunol.* 8:897. doi: 10.3389/fimmu.2017.00897

Raschperger, E., Thyberg, J., Pettersson, S., Philipson, L., Fuxe, J., and Pettersson, R. F. (2006). The coxsackie-and adenovirus receptor (CAR) is an in vivo marker for epithelial tight junctions, with a potential role in regulating permeability and tissue homeostasis. *Exp. Cell Res.* 312, 1566–1580. doi: 10.1016/j.yexcr.2006.01.025

Renga, B., Mencarelli, A., Cipriani, S., D'Amore, C., Carino, A., Bruno, A., et al. (2013). The bile acid sensor FXR is required for immune-regulatory activities of TLR-9 in intestinal inflammation. *PLoS One* 8:e54472. doi: 10.1371/journal.pone.0054472

Ridlon, J. M., Kang, D. J., Hylemon, P. B., and Bajaj, J. S. (2014). Bile acids and the gut microbiome. *Curr. Opin. Gastroenterol.* 30, 332–338. doi: 10.1097/MOG.0000000000000057

Romano, N., Kumar, V., Yang, G., Kajbaf, K., Rubio, M. B., Overturf, K., et al. (2020). Bile acid metabolism in fish: disturbances caused by fishmeal alternatives and some mitigating effects from dietary bile inclusions. *Rev. Aquac.* 12, 1792–1817. doi: 10.1111/raq.12410

Romero, J., Ringø, E., and Merrifield, D. (2014). "The gut microbiota of fish" in *Aquaculture nutrition: Gut health, probiotics and prebiotics*. eds. D. Merrifield and E. Ringø (Oxford: Wiley-Blackwell), 75–100.

Rueda-Jasso, R., Conceiçao, L. E., Dias, J., De Coen, W., Gomes, E., Rees, J. F., et al. (2004). Effect of dietary non-protein energy levels on condition and oxidative status of Senegalese sole (*Solea senegalensis*) juveniles. *Aquaculture* 231, 417–433. doi: 10.1016/S0044-8486(03)00537-4

Ruiz, A., Andree, K. B., Sanahuja, I., Holhorea, P. G., Calduch-Giner, J. A., Morais, S., et al. (2023). Bile salt dietary supplementation promotes growth and reduces body

adiposity in gilthead seabream (*Sparus aurata*). *Aquaculture* 566:739203. doi: 10.1016/j.aquaculture.2022.739203

Salomón, R., Firmino, J. P., Reyes-López, F. E., Andree, K. B., González-Silvera, D., Esteban, M. A., et al. (2020). The growth promoting and immunomodulatory effects of a medicinal plant leaf extract obtained from *Salvia officinalis* and *Lippia citriodora* in gilthead seabream (*Sparus aurata*). *Aquaculture* 524:735291. doi: 10.1016/j.aquaculture.2020.735291

Schubert, K., Olde Damink, S. W., von Bergen, M., and Schaap, F. G. (2017). Interactions between bile salts, gut microbiota, and hepatic innate immunity. *Immunol. Rev.* 279, 23–35. doi: 10.1111/imr.12579

Seropian, I. M., González, G. E., Maller, S. M., Berrocal, D. H., Abbate, A., and Rabinovich, G. A. (2018). Galectin-1 as an emerging mediator of cardiovascular inflammation: mechanisms and therapeutic opportunities. *Mediators Inflamm.* 2018:8696543. doi: 10.1155/2018/8696543

Sinha, J., Chen, F., Miloh, T., Burns, R. C., Yu, Z., and Shneider, B. L. (2008). β-Klotho and FGF-15/19 inhibit the apical sodium-dependent bile acid transporter in enterocytes and cholangiocytes. *Am. J. Physiol. Gastrointest. Liver Physiol.* 295, G996–G1003. doi: 10.1152/ajpgi.90343.2008

Siyal, F. A., Babazadeh, D., Wang, C., Arain, M. A., Saeed, M., Ayasan, T., et al. (2017). Emulsifiers in the poultry industry. *Worlds Poult. Sci. J.* 73, 611–620. doi: 10.1017/S0043933917000502

Smith, P. E., Waters, S. M., Gómez Expósito, R., Smidt, H., Carberry, C. A., and McCabe, M. S. (2020). Synthetic sequencing standards: a guide to database choice for rumen microbiota amplicon sequencing analysis. *Front. Microbiol.* 11:606825. doi: 10.3389/fmicb.2020.606825

Su, C., Liu, X., Li, J., Zhang, M., Pan, L., Lu, Y., et al. (2021). Effects of bile acids on the growth performance, lipid metabolism, non-specific immunity and intestinal microbiota of Pacific white shrimp (*Litopenaeus vannamei*). *Aquacult. Nutr.* 27, 2029–2041. doi: 10.1111/anu.13338

Subhadra, B., Lochmann, R., Rawles, S., and Chen, R. (2006). Effect of dietary lipid source on the growth, tissue composition and hematological parameters of largemouth bass (*Micropterus salmoides*). *Aquaculture* 255, 210–222. doi: 10.1016/j.aquaculture.2005.11.043

Tamura, A., Hayashi, H., Imasato, M., Yamazaki, Y., Hagiwara, A., Wada, M., et al. (2011). Loss of claudin-15, but not claudin-2, causes Na^+ deficiency and glucose malabsorption in mouse small intestine. *Gastroenterology* 140, 913–923. doi: 10.1053/j.gastro.2010.08.006

Tapia-Paniagua, S. T., Balebona, M. D. C., Firmino, J. P., Rodríguez, C., Polo, J., Moriñigo, M. A., et al. (2020). The effect of spray-dried porcine plasma on gilthead seabream (*Sparus aurata*) intestinal microbiota. *Aquacult. Nutr.* 26, 801–811. doi: 10.1111/anu.13039

Thurston, T. L., Wandel, M. P., von Muhlinen, N., Foeglein, Á., and Randow, F. (2012). Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. *Nature* 482, 414–418. doi: 10.1038/nature10744

Tocher, D. R., Dick, J. R., MacGlaughlin, P., and Bell, J. G. (2006). Effect of diets enriched in Δ6 desaturated fatty acids (18: 3n− 6 and 18: 4n− 3), on growth, fatty acid composition and highly unsaturated fatty acid synthesis in two populations of Arctic charr (*Salvelinus alpinus* L.). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 144, 245–253. doi: 10.1016/j.cbpb.2006.03.001

Trushenski, J., Rosenquist, J., and Gause, B. (2011). Growth performance, tissue fatty acid composition, and consumer appeal of rainbow trout reared on feeds containing terrestrially derived rendered fats. *N. Am. J. Aquac.* 73, 468–478. doi: 10.1080/15222055.2011.633691

Turchini, G. M., Mentasti, T., Frøyland, L., Orban, E., Caprino, F., Moretti, V. M., et al. (2003). Effects of alternative dietary lipid sources on performance, tissue chemical composition, mitochondrial fatty acid oxidation capabilities and sensory characteristics in brown trout (*Salmo trutta* L.). *Aquaculture* 225, 251–267. doi: 10.1016/S0044-8486(03)00294-1

Vallejos-Vidal, E., Reyes-Cerpa, S., Tort, L., Polo, J., Reyes-López, F. E., and Gisbert, E. (2022). Spray-dried porcine plasma promotes the association between metabolic and immunological processes at transcriptional level in gilthead sea bream (*Sparus aurata*) gut. *Front. Mar. Sci.* 9:814233. doi: 10.3389/fmars.2022.814233

Vavassori, P., Mencarelli, A., Renga, B., Distrutti, E., and Fiorucci, S. (2009). The bile acid receptor FXR is a modulator of intestinal innate immunity. *J. Immunol.* 183, 6251–6261. doi: 10.4049/jimmunol.0803978

Wada, M., Tamura, A., Takahashi, N., and Tsukita, S. (2013). Loss of claudins 2 and 15 from mice causes defects in paracellular Na^+ flow and nutrient transport in gut and leads to death from malnutrition. *Gastroenterology* 144, 369–380. doi: 10.1053/j.gastro.2012.10.035

Weinstein, M. R., Litt, M., Kertesz, D. A., Wyper, P., Rose, D., Coulter, M., et al. (1997). Invasive infections due to a fish pathogen, *streptococcus iniae*. *N. Engl. J. Med.* 337, 589–594. doi: 10.1056/NEJM199708283370902

Wen, J., Mercado, G. P., Volland, A., Doden, H. L., Lickwar, C. R., Crooks, T., et al. (2021). Fxr signaling and microbial metabolism of bile salts in the zebrafish intestine. *Sci. Adv.* 7:eabg1371. doi: 10.1126/sciadv.abg1371

White, J. R., Nagarajan, N., and Pop, M. (2009). Statistical methods for detecting differentially abundant features in clinical metagenomic samples. *PLoS Comput. Biol.* 5:e1000352. doi: 10.1371/journal.pcbi.1000352

Xiong, F., Wu, S.-G., Zhang, J., Jakovlic, I., Li, W.-X., Zou, H., et al. (2018). Dietary bile salt types influence the composition of biliary bile acids and gut microbiota in grass carp. *Front. Microbiol.* 9:2209. doi: 10.3389/fmicb.2018.02209

Yamamoto, T., Suzuki, N., Furuita, H., Sugita, T., Tanaka, N., and Goto, T. (2007). Supplemental effect of bile salts to soybean meal-based diet on growth and feed utilization of rainbow trout *Oncorhynchus mykiss*. *Fish. Sci.* 73, 123–131. doi: 10.1111/j.1444-2906.2007.01310.x

Yin, P., Xie, S., Zhuang, Z., He, X., Tang, X., Tian, L., et al. (2021). Dietary supplementation of bile acid attenuate adverse effects of high-fat diet on growth performance, antioxidant ability, lipid accumulation and intestinal health in juvenile largemouth bass (*Micropterus salmoides*). *Aquaculture* 531:735864. doi: 10.1016/j.aquaculture.2020.735864

Zhang, Y., Feng, H., Liang, X. F., He, S., Lan, J., and Li, L. (2022). Dietary bile acids reduce liver lipid deposition via activating farnesoid X receptor, and improve gut health by regulating gut microbiota in Chinese perch (*Siniperca chuatsi*). *Fish Shellfish Immunol.* 121, 265–275. doi: 10.1016/j.fsi.2022.01.010

Zhang, Y. A., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., et al. (2010). IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nat. Immunol.* 11, 827–835. doi: 10.1038/ni.1913

Zhou, J. S., Chen, H. J., Ji, H., Shi, X. C., Li, X. X., Chen, L. Q., et al. (2018). Effect of dietary bile acids on growth, body composition, lipid metabolism and microbiota in grass carp (*Ctenopharyngodon idella*). *Aquacult. Nutr.* 24, 802–813. doi: 10.1111/anu.12609

Zhou, Y., and Zhi, F. (2016). Lower level of *Bacteroides* in the gut microbiota is associated with inflammatory bowel disease: a meta-analysis. *Biomed. Res. Int.* 2016:5828959. doi: 10.1155/2016/5828959

CHAPTER 3

The potential of a combination of pungent spices as a novel supplement in gilthead seabream (*Sparus aurata*) diets to aid in the strategic use of fish oil in aquafeeds: a holistic perspective

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The potential of a combination of pungent spices as a novel supplement in gilthead seabream (*Sparus aurata*) diets to aid in the strategic use of fish oil in aquafeeds: a holistic perspective

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This work studied the potential of a combination of pungent spices (capsicum, black pepper, ginger, and cinnamaldehyde) to be used as a supplement in diets of gilthead seabream (*Sparus aurata*; 44.1 ± 4.2 g). During 90 days, fish were fed three experimental diets with low inclusion of fish oil and containing poultry fat as the main source of lipids, supplemented with graded levels of the tested supplement: 0 (control), 0.1 ($SPICY_{0.1\%}$), and 0.15% ($SPICY_{0.15\%}$). As a result, the pungent spices enhanced the growth performance, the activity of the bile-salt-activated lipase in the intestine, and decreased fat deposit levels within enterocytes. The $SPICY_{0.1\%}$ diet reduced the feed conversion ratio and the perivisceral fat index and lipid deposits in the liver. Moreover, the ratio of docosahexaenoic acid/eicosapentaenoic acid in fillet increased in fish fed the $SPICY_{0.1\%}$ diet, while the hepatic levels of docosahexaenoic acid and total n-3 polyunsaturated fatty acids increased in fish fed the $SPICY_{0.15\%}$ diet. Furthermore, there was an effect on the expression of some biomarkers related to lipid metabolism in 2-h postprandial fish (*fasn*, *elovl6*, *scd1b*, *cyp7a1*, *lpl*, and *pparβ*), and in 48 h fasted-fish fed with the $SPICY_{0.1\%}$ diet, a regulation of the intestinal immune response was indicated. However, no significant differences were found in lipid apparent digestibility and proximate macronutrient composition. The spices did not affect biomarkers of hepatic or oxidative stress. No differences in microbial diversity were found, except for an increase in Simpson's Index in the posterior intestine of fish fed the $SPICY_{0.1\%}$ diet, reflected in the increased relative abundance of the phylum Chloroflexi and lower relative abundances of the genera *Campylobacter*, *Corynebacterium*, and *Peptoniphilus*. In conclusion, the supplementation of gilthead seabream diets with pungent spices at an inclusion of 0.1% was beneficial to enhance growth performance and feed utilization;

reduce fat accumulation in the visceral cavity, liver, and intestine; and improve the fish health status and condition. Results suggest that the tested supplement can be used as part of a nutritional strategy to promote a more judicious use of fish oil in fish diets due to its decreasing availability and rising costs.

KEYWORDS

phytogenics, spices, anti-inflammatory, DHA/EPA ratio, digestion, bile salts, docosahexaenoic acid, microbiota

1 Introduction

It is well-established that n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs), particularly eicosapentaenoic (C20:5n-3; EPA) and docosahexaenoic (C22:6 n-3; DHA) acids, have multiple beneficial effects in vertebrate health, such as promoting a correct neurophysiological development and in the prevention of cancers, cardiovascular, and inflammatory diseases (1–3). The main source for humans of these n-3 LC-PUFAs has traditionally been the dietary intake of fish and seafood. However, fish are also dependent on their dietary intake, since many marine fishes have a restricted ability to biosynthesize DHA and EPA due to their low capacity to desaturate and elongate linoleic (C18:2 n-6) and alpha-linolenic (C18:3 n-3) acids (4). Thus, n-3 LC-PUFAs have been usually incorporated to aquafeeds as fish oil. Nevertheless, the decreasing availability of fish oil to sustain the growth of aquaculture worldwide, coupled with its rising cost, have been a strong driver to search for functional ingredients to reduce fish oil in aquafeeds while maintaining fish health and welfare, and n-3 LC-PUFA levels within the recommended and expected range for the consumer.

During recent decades, the use of plant-based oils as an alternative source to fish oils for dietary energy has been widespread among aquafeed manufactures, given their lower cost and higher availability. The partial replacement of fish oil by vegetal oils does not usually affect fish performance in terms of growth and feed utilization (5, 6). Nonetheless, some studies have reported important changes in the fatty acid profile of the fillet, with a reduction in the levels of EPA and DHA and an increase in alpha-linolenic, linoleic, and oleic (C18:1 n-9) acids (5–7). Rendered fats from animals are another affordable and widely available resource, with a growing interest in its improved use in the context of a circular bioeconomy. Moreover, besides containing more n-3 LC-PUFAs and less n-6 PUFAs than plant-based oils (8), they have a higher level of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), which are preferential substrates for metabolic energy production (β-oxidation), sparing LC-PUFAs from catabolism (9). For these reasons, rendered animal fats have been advocated as a better alternative, compared to vegetable oils, to partially replace fish oil in fish feeds. Nonetheless, the substitution of fish oil by rendered animal fats still leads to a reduction in the levels of DHA and EPA in the fillet, even if less marked than with vegetable oils, although it does not normally compromise fish performance (10). Furthermore, n-3 LC-PUFA are important regulators of metabolic pathways, with well-known hypolipidemic and hypocholesteremic effects in vertebrates (11), including fish (12). Therefore, fish oil replacement by either vegetal or land-based animal oils can cause important changes in lipid and energy metabolism (13, 14), which may lead to physiological disorders, such as a high accumulation of fat deposits within enterocytes, hepatic vacuolization and steatosis, and high levels of perivisceral fat (10, 15).

However, the reduction in fish oil levels in aquafeeds is inevitable, and although alternative sources of n-3 LC-PUFA are emerging (e.g., algal oils, heterotrophic single cell organisms, genetically modified oilseeds), available volumes and production costs are still not at a level that they could bridge the gap in the near future. Therefore, in the meantime, new strategies are required to mitigate some of the negative effects associated with the reduction in fish oils in fish diets. The present study explores the use of supplements with a lipotropic function. Several studies have demonstrated that the incorporation of ingredients with a lipotropic function in fish diets can regulate the fishes' lipid profile, reducing physiological disorders such as inflammation and improving their performance (16, 17). Considering studies in higher vertebrates, spices are promising candidates as feed additives, which should be further explored in this context. Pungent vegetal substances are valued in cooking for their intense flavor and aroma and for their properties as food preservatives, but they also have many well-demonstrated physiological benefits (18, 19). For instance, mammalian studies have established that pungent spices act as hypocholesterolemic and hypotriglyceridemic agents (similarly to n-3 LC-PUFA in fish oils) and stimulate digestion, and having antioxidant, antimicrobial, and anti-inflammatory effects. Thanks to these properties, spices are also commonly used as nutraceuticals in humans, with many therapeutic and prophylactic applications, such as prevention or reduction in obesity, diabetes, and carcinogenesis, among others (18, 19).

The aim of this work is to offer a holistic insight of the effects of supplementing a reduced fish oil diet containing poultry fat as the major lipid source (high in SFAs) with a product based on a combination of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde, in terms of fish performance and overall physiological status, including nutrient metabolism, immune response, and gut microbial profile. The study was performed in gilthead seabream (*Sparus aurata*), since this is one of the most

important farmed marine fish species in the Mediterranean area.

2 Materials and methods

2.1 Ethics statement

All procedures involving fish manipulation and tissue sampling complied with the Spanish (law 32/2007 and Royal Decree 1201/2015) and the current European legislation (EU2010/63) and were authorized by the Ethical Committee of the Institute of Agrifood Research and Technology and the Generalitat of Catalunya (CEEA 219/2020).

2.2 Animals, diets, and experimental design

The 90-day feeding trial was carried out at the Institute of Agrifood Research and Technology (IRTA) in La Ràpita (Tarragona, Spain). After an acclimation period of 2 weeks, juveniles of gilthead seabream (initial body weight, BW_i = 44.1 ± 4.2 g; mean ± standard deviation, SD) obtained from a commercial fish farm (Piscicultura Marina Mediterranea SL, Andromeda Group, Valencia, Spain) were randomly distributed in 12 tanks of 450 L (30 fish per tank; initial density = 3 kg m^{-3}; N = 360). To guarantee water quality maintenance, tanks were connected to an IRTAmar™ water recirculation system, following the natural photoperiod (June to August at 40.63N–0.66E). Water temperature, dissolved oxygen, and pH were kept at 22.5 ± 0.5°C, 6.3 ± 0.2 mg/L (OXI330, Crison Instruments, Barcelona, Spain) and 7.6 ± 0.01 (pH meter 507, Crison Instruments), respectively. Salinity, nitrite, and ammonia levels were 36‰ (MASTER-20 T Hand-Held Refractometer, ATAGO Co. Ltd., Italy), 0.16 ± 0.1 mg NO_2^-/L, and 0.22 ± 0.08 mg NH_4^+/L (HACH DR 900 Colorimeter, Hach Company, Spain), respectively.

Three experimental diets (Table 1; 3 mm pellet size) were designed to evaluate the potential use of an encapsulated combination of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde (Lucta S.A., Spain; Patent Number WO/2022/117810) as a feed additive. The encapsulation serves to enable the manipulation of the pungent spices during feed preparation but has no functional effect (in the animal), as the

TABLE 1 Ingredient formulation, proximate, and fatty acid composition of experimental diets: a control and two basal diets supplemented with a mixture of pungent spices (capsicum, black pepper, ginger, and cinnamaldehyde) at a dietary inclusion level of 0.1 ($SPICY_{0.1\%}$) and 0.15% ($SPICY_{0.15\%}$).

	Experimental diets		
Ingredients (%)	Control	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$
Fishmeal Super Prime	7.50	7.50	7.50
Fishmeal 60	5.00	5.00	5.00
Fish protein concentrate	2.00	2.00	2.00
Feathermeal hydrolysate	5.00	5.00	5.00
Porcine blood meal	3.00	3.00	3.00
Poultry meal	15.00	15.00	15.00
Aminopro NT70—*C. glutamicum*	4.00	4.00	4.00
Corn gluten meal	8.00	8.00	8.00
Soybean meal 48	12.00	12.00	12.00
Sunflower meal	5.00	5.00	5.00
Wheat meal	10.31	10.31	10.31
Whole peas	5.00	5.00	5.00
Pea starch (raw)	2.40	2.40	2.40
Fish oil	3.02	3.02	3.02
Soybean oil	2.35	2.35	2.35
Poultry fat	8.04	8.04	8.04
Vitamin and mineral premix	1.00	1.00	1.00
Vitamin C35	0.05	0.05	0.05
Vitamin E50	0.02	0.02	0.02
Betaine HCl	0.20	0.20	0.20

(Continued)

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TABLE 1 Continued

	Experimental diets		
Ingredients (%)	Control	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$
Choline chloride 60	0.10	0.10	0.10
Antioxidant	0.20	0.20	0.20
Sodium propionate	0.10	0.10	0.10
Monoammonium phosphate	0.35	0.35	0.35
L-Tryptophan	0.15	0.15	0.15
DL-Methionine	0.20	0.20	0.20
Mixture of pungent spices (Lucta)	-	0.10	0.15
Yttrium oxide	0.02	0.02	0.02
Proximate composition			
Crude protein, %	44.14 ± 0.05	44.11 ± 0.06	44.26 ± 0.21
Crude fat, %	18.10 ± 0.04	18.09 ± 0.06	18.19 ± 0.09
Gross energy, MJ kg^{-1}	21.38 ± 1.11	21.46 ± 0.92	21.45 ± 0.83
Fatty acid profile (% of total fatty acids) *			
Saturated fatty acids (SFAs)	27.19 ± 0.40	27.79 ± 0.01	27.76 ± 1.36
Monounsaturated fatty acids (MUFAs)	36.61 ± 0.73	35.97 ± 0.59	36.18 ± 0.51
n-6 polyunsaturated fatty acids (n-6 PUFAs)	26.65 ± 0.06	26.69 ± 0.45	26.45 ± 0.52
n-3 polyunsaturated fatty acids (n-3 PUFAs)	9.55 ± 0.39	9.55 ± 0.03	9.61 ± 0.00
Total PUFAs	36.20 ± 0.45	36.24 ± 0.48	36.06 ± 0.52

*Complete fatty acid profile of experimental diets is detailed in Supplementary Table S1. The proximate and fatty acid composition of diets were analyzed in duplicate; values are represented as mean ± standard deviation (SD).

active ingredients should be released during the extrusion process. A basal diet was formulated, which included a high content of poultry fat and lower levels of fish oil and soybean oil. This formula was chosen to increase the levels of SFAs (27% of total fatty acids; Table 1) compared to traditional fish oil replacement strategies based on vegetable oils, and was hypothesized to result in increased body fat deposition. The two other experimental diets had the same ingredient formulation and proximate composition, but were supplemented with the encapsulated product at two different inclusion levels before extrusion: 0.1 ($SPICY_{0.1\%}$) and 0.15% ($SPICY_{0.15\%}$). The choice of these inclusion levels was based on a previous dose–response trial performed in the same species in which the same combination of pungent spices was tested at doses between 0.05% and 0.15% (20), obtaining the best results in performance at 0.1% and 0.15%. In particular, at these inclusion levels, an improvement on feeding efficiency and lipid apparent digestibility, a reduction in hepatic lipid stores, decreased fat accumulation in fillet, and increased levels of n-3 PUFA (including EPA and DHA) in fillet. The three diets were isonitrogenous (44% crude protein), isolipidic (18% crude fat), and isoenergetic (21.4 MJ/kg), and were manufactured by Sparos Lda. (Portugal) following the procedures described by Salomón et al. (21). Yttrium oxide (Y_2O_3, Sigma Aldrich, Spain) was included in the diets at 0.2 g/kg as an inert marker to assess apparent digestibility coefficients (ADCs) of macronutrients. Each experimental diet was randomly assigned to four tanks before the beginning of the nutritional assay. During the 90-day trial, feed was distributed twice a day in 12 takes spread over an hour (one each 5 min) with automatic feeders (Arvo-Tec T Drum 2000, Finland).

Once a month, fish were anesthetized with buffered tricaine methanesulfonate (MS-222, Sigma-Aldrich, Spain; 100 mg/L) for measuring growth in body weight (BW) and standard length (SL) in order to monitor somatic growth. In addition, the uneaten dried pellets from each tank were collected and weighed to ensure that sufficient amount of feed was being offered and to calculate the daily feed intake (22). For assessing ADCs of lipids and proteins, feces were collected by means of a sedimentation column 10–12 h after removal of uneaten feed during three consecutive days. Then, pooled samples from the same tank were frozen at −20°C until biochemical analyses.

2.3 Sampling and fish performance indicators

At the end of the trial, fish were fasted for 48 h and anesthetized with 100 mg/L of MS-222, and their final body weight (BW_f) and standard length (SL_f) were individually measured. The following key performance indicators were also calculated:

$$\text{Specific growth rate (SGR; \%/day)} = 100 \times \ln[BW_f(g) - \ln BW_i(g)]/\text{time (days)}.$$

$$\text{Fulton's condition factor (K)} = 100 \times BW_f(g)/SL_f(g)^3$$

$$\text{Feed intake (FI, g/fish)} = \text{total feed intake per tank (g)}/\text{number of fish per tank (g)}$$

$$\text{Feed conversion ratio (FCR)} = \text{total feed intake per tank (g)}/\text{fish biomass increase per tank (g)}$$

In addition, eight fish per tank (32 per dietary treatment) were randomly selected and euthanized with an overdose of MS-222 (300 mg/L) for sampling different tissues for the analyses described below. In particular, the perivisceral fat was gently separated from the gastrointestinal tract in six fish per tank (four replicate tanks per diet) with the aid of a round-ended scalpel and individually weighed to calculate the perivisceral fat index (PVFI; %) = perivisceral fat weight (g)/BW_f (g). The livers of six fish per tank were also weighed to calculate the hepatosomatic index (HSI; %) = liver weight (g)/BW_f (g). Then, a piece of liver (1.5–2 cm^2) and a piece of anterior intestine (AI; approximately 4 cm) from three individuals per tank (12 fish per diet) were fixed in 10% neutral buffered formalin (pH = 7.2) and stored at 4°C until histological analysis. The AI was selected because this region of the intestine has high rates of fat digestion and absorption (23). The rest of the liver was divided in pieces and frozen at −80°C to further assess hepatic antioxidant status and metabolic biomarkers (four replicate tanks per diet). The fillet and the liver of three fish per tank were stored at −20°C until analyses of proximate and fatty acid composition. To evaluate the profile of bile acids (BAs), the walls of the gallbladders of four fish per tank were broken with the edge of a scalpel, and the content of all fish from the same tank were emptied together into one tube (four replicate tanks per diet) and frozen at −80°C. With the purpose of studying the effect of the diet on hepatic and intestinal gene expression profile of 48 h fasted fish, a piece of the liver and a piece of AI (approximately 1 and 2 cm^2, respectively) of two individuals per tank (eight per dietary treatment) were separately immersed in 5 volumes of RNAlater® (Sigma-Aldrich, USA), incubated at 4°C for 24 h and stored at −80°C. A section of AI (approximately 4 cm long cut from the pyloric caeca) and posterior intestine (PI; approximately 4 cm long cut from the anus anteriorly) from three fish per tank (12 per treatment) were taken and opened lengthwise under sterile conditions. The mucosal content was gently scraped with a round-edge spatula and immediately frozen at −80°C for further microbial analysis. Sampling was performed at 48 h after the last feeding to ensure sample stability and avoid contamination by allochthonous bacteria from the feces in the case of intestinal microbiota (24).

The rest of the fish were returned to their respective tanks and fed for 3 days. Then, 10 2-h postprandial fish per tank (40 per treatment) were netted and euthanized with 300 mg/L of MS-222 for tissue sampling. The luminal content of the AI of four fish per tank was stripped with tweezers into one tube (four replicates per diet) and frozen at −80°C for future analysis of the BA profile. The digestive tract of four fish per tank was divided in two regions: i) the stomach and pyloric caeca and ii) the AI. Both regions were separately stored at −80°C until analysis of pancreatic digestive enzymes. A piece of the liver and AI from two fish per tank (eight per treatment) were dissected and conserved as previously described for gene expression analysis.

2.4 Proximate and fatty acid composition

For biochemical analysis, a pool of three liver pieces per tank were homogenized together, while three fillets per tank were individually homogenized (IKA T25 digital ULTRA-TURRAX, IKA Works, USA). The protocols described by Lowry et al. (25), Folch et al. (26), Dubois et al. (27), and AOAC (28) were followed to determine the levels of total protein, lipids, carbohydrates, and ash content in livers and fillets. Fatty acid profile was obtained as described by Ramos-Júdez et al. (29). In brief, transmethylated fatty acids from total lipids were extracted, purified, and finally quantified by gas-liquid chromatography on a Thermo Trace GC (Thermo Fisher, Spain) coupled to a TRACE™ TR-FAME GC Column (Thermo Scientific, Spain), using heneicosylic acid (21:0) as internal standard (ref. H5,149, Sigma-Aldrich, Spain).

Protein and lipid ADCs were calculated according to Cheng and Hardy (30) using Y_2O_3 as inert marker:

$$\text{ADC of nutrient (\%)} = 100 \times [1 - (\%\ Y_2O_3 \text{ in diet}/\%\ Y_2O_3 \text{ in feces}) \times (\%\ \text{nutrient in feces}/\%\ \text{nutrient in diet})]$$

The Y_2O_3 concentration was determined using an Agilent 7700 ICP-MS (Agilent Technologies, USA).

2.5 Hepatic metabolism and antioxidant stress

Biomarkers of hepatic metabolic and antioxidant stress were evaluated following the methodology described by Ruiz et al. (31). In brief, homogenized pools of three liver pieces (approximately 100 mg each) per tank resuspended in a lysis solution (1.24 mM Triton X-100, 1 mM EDTA, and 1 mM $NaHCO_3$) with a stabilizer solution (3.7 mM EDTA, 5 mM β-mercaptoethanol), 1:1 *v/v*, were centrifuged (5,000×*g*, 10 min, 4°C), and supernatants were collected. Lactate dehydrogenase, aspartate transaminase, and alanine transaminase activities were quantified following the methodology of Bergmeyer and Bernt (32–34) with commercial kits (ref. 41,222, ref. 41,272, ref. 41,282; Spinreact, Spain). To evaluate the antioxidant condition of the liver, homogenized pools of three pieces (approximately 60 mg each) per tank resuspended in 5 volumes *v/w* of buffer (150 mM KCl, 1 mM EDTA, pH 7.4) were centrifuged (9,000×*g*, 30 min, 4°C), and the supernatants were collected. Then, superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) activities were quantified following the protocols of McCord and Fridovich (35), Aebi (36), and Carlberg and Mannervik (37). Lipid peroxidation

(LPO) levels were estimated through thiobarbituric acid reactive substances (TBARs) as described by Solé et al. (38), and total antioxidant capacity (TAC) was measured following the manufacturer's instructions of the Total Antioxidant Capacity Assay Kit (ref. MAK187, Sigma-Aldrich, USA). The above-mentioned oxidative stress biomarkers were normalized to soluble protein content (39), except for SOD activity, which was expressed as percent of enzyme inhibition. All described measures were run in triplicate at 25°C by UV/Vis spectrophotometry (Infinite M200 Plate Reader, Tecan Switzerland) and analyzed with the Magellan™ software (v6, Tecan).

2.6 Pancreatic digestive enzymes

Pools of four samples of i) stomach and pyloric ceca and ii) AI were separately homogenized (IKA T25 digital ULTRA-TURRAX, IKA Works) in 5 volumes *v/w* of ice-cold distilled water and centrifuged (3,300×*g*, 3 min, 4°C), and the supernatant was collected for quantification of pancreatic digestive enzymes (total alkaline proteases, α-amylase, and bile-salt-activated lipase) following the guidelines of Ruiz et al. (31). Samples (enzymatic crude extracts) were handled according to Solovyev and Gisbert (40) in order to prevent their degradation during storage and handling.

2.7 Bile acid quantification

Bile acid (BA) quantification was performed as previously described by Herrero-Encinas et al. (41) with modifications. BA extraction from gallbladder bile samples was performed by extraction of 100 µL of water-diluted bile (1/2,000) with 400 µL of acetonitrile (ACN) containing internal standard (chenodeoxycholic acid-d4, CDCA-d4). After vortex and centrifugation, supernatants were diluted 1/10 in H_2O:ACN (1:1 *v/v*) and directly injected in the liquid chromatograph–mass spectrometer (LC-MS). Intestinal digesta samples were first lyophilized and homogenized on a TissueLyzer II (QIAGEN, Germany). Then, 20 mg of homogenate was extracted with 800 µL of H_2O:ACN (1:1 *v/v*) including the internal standard for 15 min and centrifuged (15,000×*g*, 10 min, 4°C), and the supernatants diluted again in H_2O:ACN (1/1,000). Quantification of BAs was performed by LC-MS using response comparison against calibration curves generated using pure BA standards. Chromatographic separation was performed on an ACQUITY UPLC I-Class connected to a Xevo-G2 QTof mass spectrometer, using QuanLynx v4.2 software for operations and quantification (Waters Corp., USA).

2.8 Histological analyses

To evaluate the histological condition, small segments of fixed liver and AI (approximately 0.5–1 cm^2) were dehydrated in ethanol solutions of graded concentrations, cleared with xylene, and embedded in paraffin. Serial sections of 4 µm stained with hematoxylin and eosin were examined under light microscopy (Leica DM LB, Leica Microsystems) by means of a digital camera at 600 dpi (Olympus DP70, Olympus Europa, Germany). Inflammation and accumulation of fat deposits were semi-quantitatively evaluated from 1 to 5 following the classification described by Ruiz et al. (31). Semi-quantitative analyses were performed following a random order of samples, under blinded conditions by two different observers (42). In the images of AI, the following parameters were also measured using the software ANALYSIS (Olympus Soft Imaging Solutions, Germany): thickness of musculature, height of villi, height of enterocytes, and density of goblet cells in the intestinal mucosa (43).

2.9 Extraction of DNA and analysis of gut microbiota

The DNeasy PowerSoil Pro Kit (ref. 47,016, QIAGEN, Germany) was used for extracting the DNA of up to 250 mg of the scraped product of the AI and the PI of three fish from each tank (12 individuals per diet). The concentration of DNA ranged up to 500 ng/µL, and A_{260}/A_{280} absorbance ratios were higher than 1.85.

The region V3–V4 of the 16S rRNA gene was amplified (primers 341F/805R) and sequenced (llumina-MiSeq platform; 2 × 300 bp paired-end) according to Ruiz et al. (44), and data analysis was carried out with a workflow based on the R package dada2 (v1.16; 45). In brief, all reads with a Phred quality score <28 or with an expected error >2 were excluded from the analysis. After merging of paired-ended reads, the sequences with an overlap length <12 nucleotides, more than 0 mismatches, or identified as chimeras, were also removed. Finally, for bacterial taxonomy classification of amplicon sequence variants (ASVs), the SILVA database (v138.1) was used as a reference library. Those ASVs with a bootstrapping confidence <80% were classified as unassigned (46). According to rarefaction curves (Supplementary Figure S1), the number of reads per sample were rarefied to the minimum sample depth (49,337 reads) using the R package vegan (v2.6-4) and normalized by total sum scaling (47). Raw sequencing data are available in the Sequence Read Archive (SRA) of NCBI under the Bioproject accession numbers PRJNA915342 and PRJNA971862.

2.10 Gene expression profile of the liver and anterior intestine

The TRI Reagent (Sigma-Aldrich, USA) and QIAGEN RNeasy® Mini Kit (ref. 74,106, QIAGEN, Germany) were, respectively, used for extracting RNA from the liver and AI. Concentrations of RNA ranged between 20 and 100 ng/µL, with A_{260}/A_{280} absorbance ratios of 1.9–2.1 (Nanodrop-2000®, Thermo Fisher Scientific, USA). Integrity was verified through agarose gel electrophoresis (48). For cDNA synthesis, the High-Capacity cDNA Archive Kit (Applied Biosystems, USA) was used following the manufacturer's instructions with an initial input of 500 ng of RNA.

Real-time quantitative PCR was carried out with a CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad, USA),

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using 96-well PCR array layouts designed for simultaneously profiling a panel of 44 genes for liver (Table 2) and intestine (Table 3) as described by Ruiz et al. (44). To improve data reproducibility, all the pipetting operations were performed using an EpMotion 5070 Liquid Handling Robot (Eppendorf, Germany). Expression values were calculated with the delta–delta Ct method (49), taking *beta-actin* as a housekeeping gene, after testing its expression stability (GeNorm software; M score = 0.21). To compare the expression of multiple genes, all values in the liver and AI were referenced to the expression levels of *grp-170* and *hes1-b* of fish fed the control diet, respectively.

2.11 Statistical analyses

After confirmation of normal distribution and homoscedasticity of data by Shapiro–Wilk test and Levene's test, a one-way ANOVA followed by Tukey's range test for multiple comparison among

TABLE 2 PCR-array layout for gene expression profile in the liver of gilthead seabream fed experimental diets.

Function	Gene	Symbol	GenBank
Fatty acids, cholesterol and phospholipid metabolism	Fatty acid synthase	*fasn*	JQ277708
	Elongation of very long chain fatty acids 1	*elovl1*	JX975700
	Elongation of very long chain fatty acids 4	*elovl4*	JX975701
	Elongation of very long chain fatty acids 5	*elovl5*	AY660879
	Elongation of very long chain fatty acids 6	*elovl6*	JX975702
	Fatty acid desaturase 2	*fads2*	AY055749
	Stearoyl-CoA desaturase 1a	*scd1a*	JQ277703
	Stearoyl-CoA desaturase 1b	*scd1b*	JQ277704
	Cholesterol 7-alpha-monooxygenase	*cyp7a1*	KX122017
	Phospholipid transfer protein	*pltp*	XM_030418561
Lipases	Adipose triglyceride lipase	*atgl*	JX975711
	Hepatic lipase	*hl*	EU254479
	Lipoprotein lipase	*lpl*	AY495672
	85kDa calcium-independent phospholipase A2	*pla2g6*	JX975708
Transcription factors & nuclear receptors	Hepatocyte nuclear factor 4 alpha	*hnf4a*	FJ360721
	Sterol regulatory element-binding proteins 1	*srebp1*	JQ277709
	Sterol regulatory element-binding protein 2	*srebp2*	XM_030408996
	Farnesoid X receptor	*fxr*	XM_030426192
	Liver X receptor α	*lxrα*	FJ502320
	Peroxisome proliferator-activated receptor α	*pparα*	AY590299
	Peroxisome proliferator-activated receptor β	*pparβ*	AY590301
	Peroxisome proliferator-activated receptor γ	*pparγ*	AY590304
Oxidative metabolism & energy sensing	Carnitine palmitoyltransferase 1A	*cpt1a*	JQ308822
	Hydroxyacyl-CoA dehydrogenase	*hadh*	JQ308829
	Fatty acid translocase/CD36	*fat/cd36*	XM_030440140
	Fatty acid binding protein, heart	*h-fabp*	JQ308834
	Citrate synthase	*cs*	JX975229
	NADH-ubiquinone oxidoreductase chain 2	*nd2*	KC217558
	NADH-ubiquinone oxidoreductase chain 5	*nd5*	KC217559
	Cytochrome c oxidase subunit I	*coxi*	KC217652

(Continued)

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TABLE 2 Continued

Function	Gene	Symbol	GenBank
	Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1α*	JX975264
	Sirtuin1	*sirt1*	KF018666
	Sirtuin2	*sirt2*	KF018667
Antioxidant defense	Catalase	*cat*	JQ308823
	Uncoupling protein 1	*ucp1*	FJ710211
	Glutathione peroxidase 1	*gpx1*	DQ524992
	Glutathione peroxidase 4	*gpx4*	AM977818
	Peroxiredoxin 3	*prdx3*	GQ252681
	Peroxiredoxin 5	*prdx5*	GQ252683
	Superoxide dismutase [Cu-Zn]	*cu-zn-sod/sod1*	JQ308832
	Superoxide dismutase [Mn]	*mn-sod/sod2*	JQ308833
	Glucose-regulated protein, 170 kDa	*grp-170*	JQ308821
	Glucose-regulated protein, 94 kDa	*grp-94*	JQ308820
	Glucose-regulated protein, 75 kDa	*grp-75*	DQ524993

Specific primer sequences for marker genes of liver are listed in Supplementary Table S2.

TABLE 3 PCR-array layout for gene expression profile in the intestine of gilthead seabream fed experimental diets.

Function	Gene	Symbol	GenBank
Epithelial integrity	Proliferating cell nuclear antigen	*pcna*	KF857335
	Transcription factor HES-1-B	*hes1-b*	KF857344
	Krüppel-like factor 4	*klf4*	KF857346
	Claudin-12	*cldn12*	KF861992
	Claudin-15	*cldn15*	KF861993
	Cadherin-1	*cdh1*	KF861995
	Cadherin-17	*cdh17*	KF861996
	Tight junction protein ZO-1	*tjp1*	KF861994
	Desmoplakin	*dsp*	KF861999
	Gap junction Cx32.2 protein	*cx32.2*	KF862000
	Coxsackievirus and adenovirus receptor homolog	*cxadr*	KF861998
Nutrient transport	Intestinal-type alkaline phosphatase	*alpi*	KF857309
	Liver type fatty acid-binding protein	*fabp1*	KF857311
	Intestinal fatty acid-binding protein	*fabp2*	KF857310
	Ileal fatty acid-binding protein	*fabp6*	KF857312
Mucus production	Mucin 2	*muc2*	JQ277710
	Mucin 13	*muc13*	JQ277713
Interleukins	Tumor necrosis factor-alpha	*tnf-α*	AJ413189
	Interleukin-1 beta	*il-1β*	AJ419178
	Interleukin-6	*il-6*	EU244588

(Continued)

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TABLE 3 Continued

Function	Gene	Symbol	GenBank
	Interleukin-7	*il-7*	JX976618
	Interleukin-8	*il-8*	JX976619
	Interleukin-10	*il-10*	JX976621
	Interleukin-12 subunit beta	*il-12β*	JX976624
	Interleukin-15	*il-15*	JX976625
	Interleukin-34	*il-34*	JX976629
Cell markers	Cluster of differentiation 4-1	*cd4-1*	AM489485
	Cluster of differentiation 8 beta	*cd8b*	KX231275
	C–C chemokine receptor type 3	*ccr3*	KF857317
	C–C chemokine receptor type 9	*ccr9*	KF857318
	C–C chemokine receptor type 11	*ccr11*	KF857319
	C–C chemokine CK8/C–C motif chemokine 20	*ck8/ccl20*	GU181393
	Macrophage colony-stimulating factor 1 receptor 1	*csf1r1*	AM050293
Ig production	Immunoglobulin M	*igm*	JQ811851
	Immunoglobulin T membrane-bound form	*igt-m*	KX599201
Pattern recognition	Galectin-1	*lgals1*	KF862003
receptors (PRRs)	Galectin-8	*lgals8*	KF862004
	Toll-like receptor 2	*tlr2*	KF857323
	Toll-like receptor 5	*tlr5*	KF857324
	Toll-like receptor 9	*tlr9*	AY751797
	CD209 antigen-like protein D	*cd209d*	KF857327
	CD302 antigen	*cd302*	KF857328
	Macrophage mannose receptor 1	*mrc1*	KF857326
	Fucolectin	*fcl*	KF857331

Specific primer sequences for marker genes of intestine are listed in Supplementary Table S3.

groups ($p \leq 0.05$) was performed. When data was non-parametric, a Kruskal–Wallis one-way analysis of variance by ranks and Dunn's *post-hoc* test were performed. Correlations between variables were tested by the Pearson product–moment correlation test ($p \leq 0.05$).

For gene expression analyses, a Student's t-test was performed. A two-way ANOVA followed by a Holm–Sidak test was used for evaluating interaction between the diet and feeding time (2 h postprandial or 48 h fasted). The *p*-value was set to 0.05 to determine significant differences among dietary groups, whereas $p \leq 0.1$ was considered as a tendency for gene expression data. In the case of gene expression values with $p \leq 0.1$, a partial least squares-discriminant analysis (PLS-DA) was constructed with the software EZinfo (v3.0, Umetrics, Sweden) to achieve the maximum separation among experimental groups. Cluster separation was assessed by calculating Hotelling's T^2 statistic. Points with a T^2 above 95% confidence limit were considered as outliers and discarded. By means of the R package *ropls* (v1.22.0), each PLS-DA model was validated by a permutations test, making sure that there was no over-fitting (Supplementary Figures S2, S3).

Regarding gut microbial communities, significant differences among dietary groups in alpha diversity metrics (indices of Chao1, ACE, Shannon, Simpson, and Faith's phylogenetic diversity; 50, 51) were determined by Wilcoxon test ($p \leq 0.05$). As a beta diversity index, the weighted UniFrac distance was used to estimate similarities among samples based on the phylogenetic relationships of their ASVs (52). A permutational multivariate analyses of variance (PERMANOVA) was performed to check significant differences in beta diversity ($p \leq 0.05$; 53). Differential abundances among groups in phyla and genera with a relative abundance ≥1% were calculated with the method Metastats, adjusting the *p*-value by False Discovery Rate (FDR) (54). All the described statistics for gut microbiota data were executed with the R package microeco (55), which was used together with ggplot2 for generation of figures.

3 Results

3.1 Fish performance

The supplementation of pungent spices into the basal diet had a positive effect in fish somatic growth in terms of BW_f and SGR at both dietary inclusion levels when compared to the control diet (Table 4; $p < 0.05$). Furthermore, the administration of the $SPICY_{0.1\%}$ diet reduced perivisceral fat levels ($p < 0.05$), while fish fed the $SPICY_{0.15\%}$ diet showed PVFI values that were intermediate between the abovementioned and the control diet ($p > 0.05$). No significant differences were found in the values of SL_f, Fulton's condition factor, and HSI among dietary groups ($p > 0.05$). Regarding feed performance, whereas there were no differences in feed intake ($p > 0.05$), FCR values followed a similar pattern to that described for the PVFI; in particular, the lowest FCR values were found in fish fed the $SPICY_{0.1\%}$ diet ($p < 0.05$). On the other hand, diet supplementation with pungent spices did not significantly affect lipid and protein ADCs ($p > 0.05$).

3.2 Proximate and fatty acid composition

There were no differences in the proximate composition of the liver nor fillets among dietary groups (Supplementary Table S4; $p > 0.05$). Considering the fatty acid profile of the liver, only DHA was found at different levels among dietary groups. In particular, the highest levels of DHA were found in fish fed the $SPICY_{0.15\%}$ diet ($p < 0.05$), while fish fed the $SPICY_{0.1\%}$ diet displayed intermediate values between both dietary treatments (Table 5; $p > 0.05$). Indeed, the content of DHA in the liver was positively correlated to the dietary inclusion levels of the combination of pungent spices (Pearson correlation coefficient $r = 0.72$, $p = 0.008$). Likewise, the total levels of n-3 polyunsaturated fatty acids (n-3 PUFAs) in the liver followed a similar trend, increasing with higher concentrations of pungent spices in the diets ($r = 0.73$, $p = 0.007$). The fatty acid profile of fillet was also very conserved, although the DHA/EPA ratio significantly increased in fish fed the $SPICY_{0.1\%}$ diet (Table 6; $p < 0.05$).

3.3 Hepatic metabolism, oxidative stress biomarkers, and activity of pancreatic digestive enzymes

Dietary supplementation with the combination of pungent spices did not alter the specific activity of the measured metabolic or oxidative stress enzymes, nor were there changes in LPO and TAC in the liver (Supplementary Table S5; $p > 0.05$). Regarding pancreatic digestive enzymes, there were no significant differences in the specific activities of total alkaline proteases, bile-salt-activated

TABLE 4 Growth and feed performance indicators, somatic condition indices, and macronutrient apparent digestibility coefficients of gilthead seabream fed the control and two basal diets supplemented with a mixture of pungent spices (capsicum, black pepper, ginger, and cinnamaldehyde) at a dietary inclusion level of 0.1 ($SPICY_{0.1\%}$) and 0.15% ($SPICY_{0.15\%}$).

	Control	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$
Growth performance			
BW_i (g)	44.05 ± 0.04	44.08 ± 0.10	44.06 ± 0.10
SL_i (cm)	12.04 ± 0.12	12.22 ± 0.16	12.08 ± 0.06
BW_f (g)	215.80 ± 1.06^a	221.96 ± 3.46^b	223.02 ± 3.50^b
SL_f (cm)	19.32 ± 0.21	19.66 ± 0.30	19.51 ± 0.09
SGR (% day^{-1})	1.81 ± 0.01^a	1.84 ± 0.02^b	1.84 ± 0.02^b
Somatic indices			
K	3.00 ± 0.11	2.93 ± 0.12	3.01 ± 0.07
HSI (%)	1.84 ± 0.11	1.92 ± 0.09	1.95 ± 0.14
PVFI (%)	3.01 ± 0.28^b	2.32 ± 0.37^a	2.68 ± 0.28^{ab}
Feed performance			
FI (g $fish^{-1}$)	195.89 ± 8.70	192.29 ± 9.94	198.50 ± 6.23
FCR	1.21 ± 0.05^b	1.13 ± 0.02^a	1.20 ± 0.04^{ab}
Apparent digestibility coefficients (ADCs)			
Lipid ADC (%)	81.60 ± 0.96	81.14 ± 1.33	79.73 ± 0.89
Protein ADC (%)	79.41 ± 3.60	81.09 ± 2.82	76.08 ± 2.18

Values are represented as mean ± SD (n = 4 tanks per dietary group) and differences among groups ($p \leq 0.05$) are indicated by the different superscript letters. BW_i, initial body weight; SL_i, initial standard length; BW_f, final body weight; SL_f, final standard length; SGR, specific growth rate; K, Fulton's condition factor; HSI, hepatosomatic index; PVFI, perivisceral fat index; FI, feed intake; FCR, feed conversion ratio.

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TABLE 5 Fatty acid profile (mg/g lipid) of the liver in gilthead seabream fed the control and two basal diets supplemented with a mixture of pungent spices (capsicum, black pepper, ginger, and cinnamaldehyde) at a dietary inclusion level of 0.1 ($SPICY_{0.1\%}$) and 0.15% ($SPICY_{0.15\%}$).

	Control	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$
Myristic acid (C14:0)	8.61 ± 2.87	6.96 ± 2.62	8.66 ± 1.59
Pentadecylic acid (C15:0)	1.47 ± 0.29	1.35 ± 0.31	1.27 ± 0.25
Palmitic acid (C16:0)	125.93 ± 2.91	117.43 ± 9.82	132.13 ± 9.26
Stearic acid (C18:0)	46.63 ± 2.66	50.24 ± 6.98	52.10 ± 5.64
Saturated fatty acids (SFAs)	176.52 ± 19.53	179.57 ± 13.97	197.27 ± 12.73
Palmitoleic acid (C16:1 n-7)	26.20 ± 4.93	24.56 ± 2.82	28.42 ± 1.74
Vaccenic acid (C18:1 n-7)	34.41 ± 3.06	33.67 ± 8.12	35.80 ± 5.39
Oleic acid (C18:1 n-9)	238.38 ± 8.25	246.14 ± 35.72	261.72 ± 30.28
Eicosenoic acid (C20:1 n-9)	4.08 ± 0.39	5.06 ± 1.96	4.43 ± 0.77
Nervionic acid (C24:1 n-9)	1.76 ± 0.15	1.99 ± 0.37	1.93 ± 0.33
Monounsaturated fatty acids (MUFAs)	307.14 ± 13.72	311.83 ± 44.12	332.29 ± 36.43
Linoleic acid (C18:2 n-6)	126.14 ± 6.76	124.04 ± 10.12	138.17 ± 9.49
Gamma-linolenic acid (C18:3 n-6)	6.94 ± 1.24	7.84 ± 2.32	7.10 ± 0.77
Arachidonic acid (C20:4 n-6; ARA)	4.47 ± 0.73	5.02 ± 0.73	5.47 ± 0.37
n-6 polyunsaturated fatty acids (n-6 PUFAs)	137.90 ± 5.82	136.90 ± 12.21	150.74 ± 9.92
Alpha-linolenic acid (C18:3 n-3)	8.48 ± 0.35	8.06 ± 0.53	8.99 ± 0.38
Stearidonic acid (C18:4 n-3)	1.52 ± 0.04	1.43 ± 0.26	1.73 ± 0.22
Eicosatetraenoic acid (C20:4 n-3)	1.67 ± 0.22	1.75 ± 0.13	1.81 ± 0.16
Eicosapentaenoic acid (C20:5 n-3; EPA)	15.17 ± 0.39	15.48 ± 1.39	16.96 ± 1.80
Docosapentaenoic acid (C22:5 n-3)	7.96 ± 1.10	9.02 ± 1.93	8.92 ± 0.49
Docosahexaenoic acid (C22:6 n-3; DHA)	15.85 ± 2.21^{a}	17.77 ± 0.63^{ab}	20.80 ± 2.58^{b}
n-3 polyunsaturated fatty acids (n-3 PUFAs)	49.15 ± 4.88^{a}	53.65 ± 2.72^{ab}	59.21 ± 4.61^{b}
Total PUFAs	189.48 ± 5.85	190.55 ± 14.73	209.95 ± 14.45
DHA/EPA	1.11 ± 0.09	1.15 ± 0.07	1.23 ± 0.09
EPA + DHA	32.05 ± 0.81	33.24 ± 1.95	37.76 ± 4.15
n6/n3	2.67 ± 0.12	2.55 ± 0.12	2.55 ± 0.06

Non-represented fatty acids were not detected in the analysis. Values are represented as mean ± SD (n = 4 tanks per dietary group) and differences among groups ($p \leq 0.05$) are indicated by the different superscript letters.

TABLE 6 Fatty acid profile (mg/g lipid) of the fillet in gilthead seabream fed the control and two basal diets supplemented with a mixture of pungent spices (capsicum, black pepper, ginger, and cinnamaldehyde) at a dietary inclusion level of 0.1 ($SPICY_{0.1\%}$) and 0.15% ($SPICY_{0.15\%}$).

	Control	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$
Myristic acid (C14:0)	8.60 ± 1.35	8.34 ± 1.59	8.72 ± 1.88
Pentadecylic acid (C15:0)	1.21 ± 0.10	1.14 ± 0.12	1.14 ± 0.11
Palmitic acid (C16:0)	129.29 ± 3.49	125.17 ± 5.42	128.34 ± 9.20
Stearic acid (C18:0)	32.41 ± 1.39	32.20 ± 1.07	32.70 ± 0.64
Lignoceric acid (C24:0)	1.44 ± 0.16	1.49 ± 0.05	1.48 ± 0.28
Saturated fatty acids (SFAs)	173.63 ± 2.79	169.19 ± 7.07	172.77 ± 11.93
Palmitoleic acid (C16:1 n-7)	33.50 ± 2.27	31.87 ± 2.63	34.05 ± 5.60

(Continued)

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TABLE 6 Continued

	Control	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$
Oleic acid (C18:1 n-9)	247.55 ± 10.03	239.58 ± 12.32	249.47 ± 23.17
Eicosenoic acid (C20:1 n-9)	3.39 ± 0.14	3.51 ± 0.35	3.26 ± 0.39
Nervionic acid (C24:1 n-9)	1.43 ± 0.22	1.54 ± 0.11	1.58 ± 0.14
Monounsaturated fatty acids (MUFAs)	285.59 ± 12.55	276.50 ± 13.65	287.93 ± 28.38
Linoleic acid (C18:2 n-6)	143.89 ± 7.49	141.64 ± 5.88	143.51 ± 11.97
Gamma-linolenic acid (C18:3 n-6)	3.26 ± 0.37	3.44 ± 0.63	3.34 ± 0.12
Arachidonic acid (C20:4 n-6; ARA)	5.03 ± 0.43	5.17 ± 0.34	5.06 ± 0.96
n-6 polyunsaturated fatty acids (n-6 PUFAs)	152.56 ± 8.17	150.26 ± 6.44	151.91 ± 11.40
Alpha-linolenic acid (C18:3 n-3)	9.82 ± 0.78	9.41 ± 0.75	9.92 ± 0.89
Stearidonic acid (C18:4 n-3)	1.56 ± 0.15	1.60 ± 0.25	1.64 ± 0.24
Eicosatetraenoic acid (C20:4 n-3)	1.63 ± 0.23	1.65 ± 0.15	1.58 ± 0.17
Eicosapentaenoic acid (C20:5 n-3; EPA)	23.87 ± 2.38	22.58 ± 0.48	23.71 ± 3.64
Docosapentaenoic acid (C22:5 n-3)	9.92 ± 1.22	9.60 ± 0.13	9.32 ± 0.55
Docosahexaenoic acid (C22:6 n-3; DHA)	29.37 ± 2.75	30.83 ± 1.58	30.37 ± 4.95
n-3 polyunsaturated fatty acids (n-3 PUFAs)	77.88 ± 9.41	75.68 ± 2.29	76.39 ± 8.28
Total PUFAs	230.43 ± 15.81	225.94 ± 8.28	228.30 ± 12.78
DHA/EPA	1.23 ± 0.03^{a}	1.37 ± 0.08^{b}	1.28 ± 0.06^{ab}
EPA + DHA	53.24 ± 5.08	53.41 ± 1.62	54.07 ± 8.50
n6/n3	1.97 ± 0.18	1.99 ± 0.06	2.01 ± 0.29

Non-represented fatty acids were not detected in the analysis. Values are represented as mean ± SD (n = 4 tanks per dietary group), and differences among groups ($p \leq 0.05$) are indicated by the different superscript letters.

lipase, and α-amylase among diets in the stomach and pyloric ceca samples (Table 7; $p > 0.05$). On the other hand, the activity of bile-salt-activated lipase increased in the AI in gilthead seabream fed both supplemented diet with respect to the control group ($p < 0.05$), whereas the activities of total alkaline proteases and α-amylase followed the same numerical trend, but differences were not significant due to large interindividual variability ($p > 0.05$).

3.4 Composition of bile

Two primary BAs in their tauro-conjugated form were detected in the bile of gilthead seabream: the taurocholic acid (T-CA) and the taurochenodeoxycholic acid (T-CDCA), both at similar levels among dietary groups (Table 8; $p > 0.05$). While the concentration of BAs did not change among dietary treatments in

TABLE 7 Specific activity (mU/mg protein) of total alkaline proteases, α-amylase, and bile-salt-activated lipase in gilthead seabream fed the control and two basal diets supplemented with a mixture of pungent spices (capsicum, black pepper, ginger, and cinnamaldehyde) at a dietary inclusion level of 0.1 ($SPICY_{0.1\%}$) and 0.15% ($SPICY_{0.15\%}$).

	Control	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$
Stomach and pyloric caeca			
Total alkaline proteases	78.52 ± 8.01	90.20 ± 15.61	89.73 ± 12.03
α-amylase	398.32 ± 51.08	410.19 ± 25.24	418.92 ± 30.81
Bile salt-activated lipase	21.49 ± 6.26	23.48 ± 1.54	22.90 ± 4.83
Anterior intestine			
Total alkaline proteases	133.64 ± 13.45	145.24 ± 11.82	160.35 ± 25.63
α-amylase	402.98 ± 130.44	492.51 ± 80.53	475.20 ± 60.72
Bile salt-activated lipase	50.00 ± 9.57^{a}	65.19 ± 4.84^{b}	63.94 ± 2.81^{b}

Values are represented as mean ± SD (n = 4 tanks per dietary group) and differences among groups ($p \leq 0.05$) are indicated by the different superscript letters.

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TABLE 8 Bile acid profile in the gallbladder and the anterior intestine in gilthead seabream fed the control and two basal diets supplemented with a mixture of pungent spices (capsicum, black pepper, ginger, and cinnamaldehyde) at a dietary inclusion level of 0.1 ($SPICY_{0.1\%}$) and 0.15% ($SPICY_{0.15\%}$).

	Control	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$
Gallbladder (mg/mL)			
T-CA	108.23 ± 9.59	108.89 ± 1.11	100.23 ± 8.14
T-CDCA	47.67 ± 3.69	46.76 ± 5.14	46.76 ± 9.04
Total BAs	155.90 ± 9.58	155.65 ± 5.62	146.98 ± 15.27
Anterior intestine (μg/mg)			
T-CA	30.00 ± 15.83	44.44 ± 13.05	34.32 ± 12.06
T-CDCA	19.94 ± 13.72	23.90 ± 6.18	17.26 ± 4.26
Total BAs	49.94 ± 29.41	68.34 ± 19.07	51.58 ± 16.28

Non-represented bile acids were not detected in the analysis. Values are represented as mean ± SD (n = 4 tanks per dietary group). T-CA, taurocholic acid; T-CDCA, taurochenodeoxycholic acid; total BAs, total bile acids.

the gallbladder, the numerical values in the AI of each group notably varied even though this trend was not statistically significant due to large interindividual variability ($p > 0.05$). In particular, the mean levels of T-CA and T-CDCA increased 50% and 20%, respectively, in fish fed the $SPICY_{0.15\%}$ diet, with respect to the control diet. That resulted in an increase of more than 35% of the mean levels of total BAs, although statistically there were no significant differences due to the high deviation among specimens within each dietary group.

3.5 Histomorphology of the liver and anterior intestine

Under the present experimental conditions, the hepatic parenchyma showed a typical histological organization in all dietary groups. In particular, hepatocytes were polyhedral in shape, with varying degrees of vacuolization in their cytoplasm, and arranged in anastomosed plates separated by sinusoidal capillaries leading to central veins. No signs of inflammation nor infiltration of lymphocytes were observed in any of the diets. The $SPICY_{0.1\%}$ diet had a clear effect on the accumulation of fat deposits within hepatocytes, halving the number of individuals with high lipid accumulation in liver (score classification of 4) and increasing the number of those with low and moderate lipid accumulation (classifications 2 and 3; Figures 1A, B).

The histological organization of AI was also typical, without signs of enteritis. In brief, the mucosa was lined with the columnar epithelial layer, supported by connective tissue of the lamina propria-submucosa and surrounded by the tunica muscularis. The epithelium was mainly composed of enterocytes with acidic microvilli, a basal basophilic nucleus, eosinophilic cytoplasm, and different amount and size of clear supranuclear vacuoles depending on the dietary treatment. In particular, both diets supplemented with the mixture of pungent spices were able to reduce vacuolization with respect to the control group (Figures 1C, D). No differences in villus or enterocyte height, density of goblet cells, and thickness of the tunica muscularis were found among treatments (Supplementary Table S6; $p > 0.05$).

3.6 Microbial diversity, structure, and composition

Based on the rest of the results, only the control and $SPICY_{0.1\%}$ diets were selected for the microbiota and gene expression analyses.

After rarefaction, a total of 2,269,502 reads clustering into 19,379 ASVs were obtained. The alpha diversity indices of ACE, Shannon, and Faith's phylogenetic diversity (PD) were not different among dietary treatments (control vs. $SPICY_{0.1\%}$) regardless of the region of the intestine considered (Figures 2A, B, D; $p > 0.05$), while in the PI, the values from Simpson's Diversity Index increased in fish fed the $SPICY_{0.1\%}$ diet (0.99 ± 0.00; mean ± SEM) in comparison to the control group (0.96 ± 0.02) (Figure 2C; $p < 0.05$; Supplementary Table S7). Results of beta diversity based on the weighted UniFrac analysis did not show separation among specimens regarding diets in the AI (Figure 2E; PERMANOVA, $F = 0.666$, $R^2 = 0.029$, $p = 0.827$) nor in the PI (Figure 2F; PERMANOVA, $F = 0.950$, $R^2 = 0.045$, $p = 0.408$).

In terms of microbial composition, the most abundant phyla were Firmicutes, Proteobacteria, and Bacteroidota, accounting for 81% of the total microbial population (Figure 3A). There were no significant differences in the relative abundance of any of these three phyla among dietary treatments (Supplementary Table S8; $p > 0.05$). On the other hand, there was a significant increase in the phylum Chloroflexi in the PI in fish fed the $SPICY_{0.1\%}$ diet with respect to the control group ($p < 0.05$). The Firmicutes/Bacteroidetes (F/B) ratio was maintained at 1.67–1.70 in the AI and at 1.92–2.13 in the PI ($p > 0.05$). Similarly, the relative abundances of the most dominant genera (≥1.0%) did not change among diets in the AI (Figure 3B; Supplementary Table S9; $p > 0.05$). Nevertheless, the relative abundances of the genera *Campylobacter*, *Corynebacterium*, and *Peptoniphilus* in the PI decreased when adding the combination of pungent spices to the diet ($p < 0.05$).

3.7 Gene expression profile of the liver and anterior intestine

The expression patterns of 33 out of 44 genes analyzed in the liver were affected by the feeding time (2 h postprandial vs. 48 h fasted) (Supplementary Table S10; $p < 0.1$). Analysis of differences among diets for each feeding time highlighted an upregulation of *fasn*, *elovl6*, and *cyp7a1* ($p < 0.05$) and to a lower degree of *scd1b* ($p < 0.1$), while *lpl* ($p < 0.05$) and *pparβ* ($p < 0.1$) were downregulated in 2-h postprandial fish fed the $SPICY_{0.1\%}$ diet with respect to those fed the control diet. In 48-h fasted animals, experimental diets only affected *srebp1* and *prdx5* expression patterns, which were upregulated in fish fed the $SPICY_{0.1\%}$ diet ($p < 0.05$). The PLS-DA model of liver expression was based on three components, with an explained variance [R2Y(cum)] of 66% and a predicted variance [Q2(cum)] of 55% (Figure 4A). Separation among individuals regarding the different assayed feeding times (2 h postprandial vs. 48 h fasted) was not clear, nor was there a differential distribution of individuals based on diet (Figure 4B).

In the AI, feeding time caused major changes on the expression of 29 out of the 44 analyzed genes (Supplementary Table S11; $p < 0.1$). Regarding diet differences at each feeding time, the $SPICY_{0.1\%}$ diet

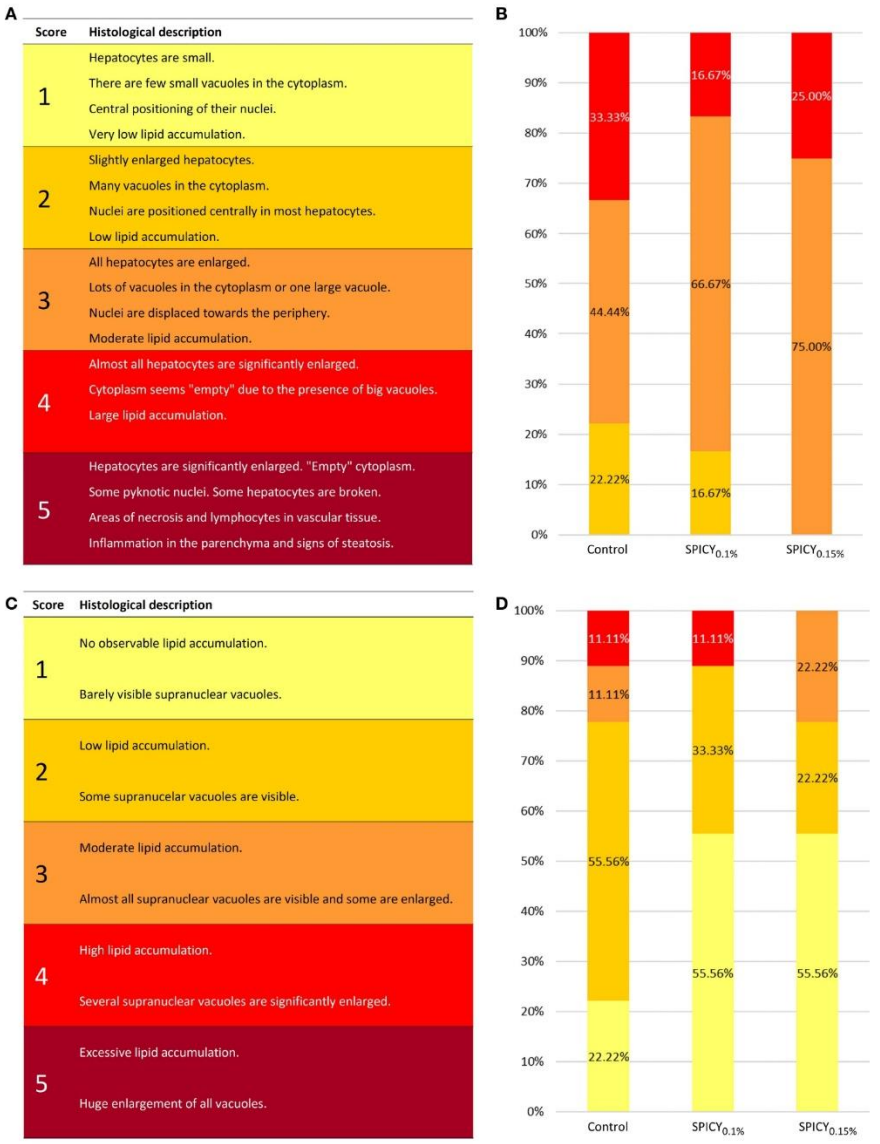

FIGURE 1
(A) Semi-quantitative scoring used for evaluating the levels of fat accumulation in the liver of gilthead seabream [adapted from Ruiz et al. (31)]. **(B)** Results (%) of hepatic scoring from gilthead seabream (n = 12 fish per dietary group) fed the control and two basal diets supplemented with a mixture of pungent spices (capsicum, black pepper, ginger, and cinnamaldehyde) at a dietary inclusion level of 0.1 ($SPICY_{0.1\%}$) and 0.15% ($SPICY_{0.15\%}$). **(C)** Semi-quantitative scoring used for evaluating the levels of fat accumulation in the anterior intestine of gilthead seabream [adapted from Ruiz et al. (31)]. **(D)** Results (%) of intestinal scoring from gilthead seabream (n = 12 fish per dietary group) fed the experimental diets.

only upregulated *il-1β* and *ccr9* with respect to the control 2-h postprandial fish (Student's t-test, $p < 0.1$), whereas after the 48-h fasting period, the $SPICY_{0.1\%}$ diet caused a significant downregulation of *fabp1*, *fabp2*, *il-34*, *cd4-1*, *cd8b*, and *cd302* ($p < 0.05$) and, to a lesser extent, *cxadr* and *il-15*, and an upregulation of *pcna* ($p < 0.1$). The PLS-DA model of the AI expression was based on two components, with an R2Y(cum) of 63% and a Q2(cum) of 54% (Figure 5A). There was a clear separation among specimens regarding feeding time along component 1, which explained 47.9% of the total variance (Figure 5B), although there were no differences among individuals for 2-h postprandial fish, and a certain overlap among individuals fed the control and $SPICY_{0.1\%}$ diets in 48-h fasted fish. Consequently, clustering was able to discriminate feeding time but not diet differences at each feeding time (Figure 5C).

4 Discussion

Present results revealed that the combination of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde promoted somatic growth in gilthead seabream (BW_f and SGR) at both dietary levels tested (0.1% and 0.15%). Such results are partially in accordance with those obtained when testing the same combination of pungent spices in broiler chickens, with production phase differences that can be attributed to inherent differences in species-specific physiology, production system, diet formulation, among other factors (41). In fish species, there is still scarce information on the pungent spices herein tested. However, a recent study was conducted in gilthead seabream under winter farming conditions (16 ± 2°C) and fed diets with a high content of SFAs (40%; 16% crude fat), in which the same blend of pungent spices was tested at different inclusion levels of 0.05%, 0.1%, and 0.15% (20). In this study, somatic growth presented a positive dose–response trend, but effects were not significant.

To our knowledge, there are no other studies in fish species testing the combined effect of all four spices evaluated herein. Nonetheless, the effects of capsicum, black pepper, and ginger (or their active principles) have been separately assessed on fish performance, with controversial results (Supplementary Table S12). In this sense, some studies have reported that the dietary inclusion of capsicum did not lead to a significant improvement in BW nor SGR in gilthead seabream, blue streak hap (*Labidochromis caeruleus*), Mozambique tilapia (*Oreochromis mossambicus*), rainbow trout (*Oncorhynchus mykiss*), and jewel cichlid (*Hemichromis guttatus*) (56–60). On the other hand, Talebi et al. (61) showed that the dietary supplementation of red bell pepper (*Capsicum annum*) at an inclusion level of 44 or 55 mg/kg for 20–60 days significantly increased the BW and total length, but not the SGR in rainbow trout (*Oncorhynchus mykiss*). When supplementing fish diets with black pepper or its extracts, growth was not enhanced in African catfish (*Clarias gariepinus*), rainbow trout, and common carp (*Cyprinus carpio*) (62–64), but weight gain was significantly increased in rohu fish (*Labeo rohita*) when adding the extract to the diet at an inclusion level of 1% or 2% (65). Among the four spices tested in the current study, ginger has been the most studied regarding its growth enhancing effect in teleosts (66–70, among others). Reviewing the literature, different results have been reported, even in the same species, depending on the study. Such changes in growth performance may be attributed to the specific conditions and design of each nutritional assay, namely, feeding period duration, basal diet formulation, supplement dosage, and form (e.g., dried powder, essential oil, or oleoresin), and to the

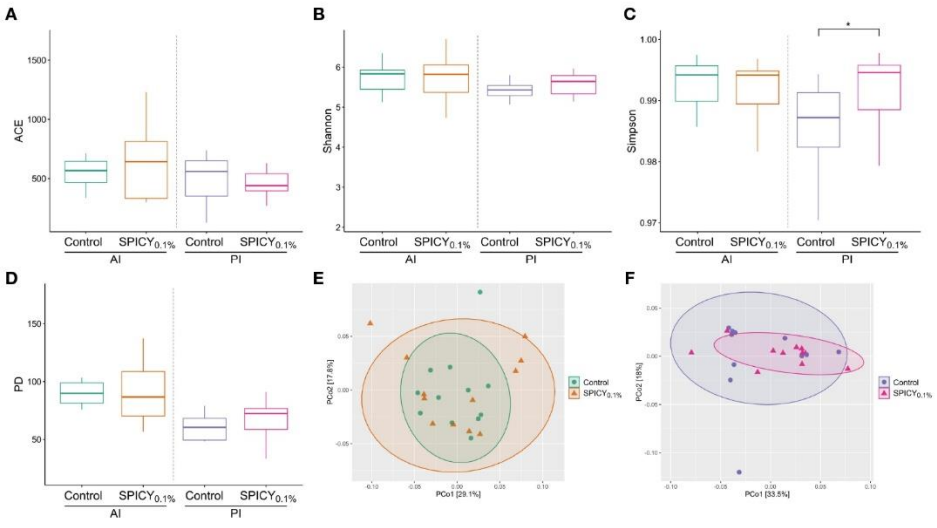

FIGURE 2
Microbial alpha diversity in the anterior (AI) and posterior intestine (PI) in gilthead seabream (n = 12 fish per dietary group) fed the control and the basal diet supplemented with a mixture of pungent spices (capsicum, black pepper, ginger, and cinnamaldehyde) at a dietary inclusion level of 0.1% ($SPICY_{0.1\%}$): **(A)** ACE index, **(B)** Shannon index, **(C)** Simpson's index, **(D)** Faith's phylogenetic diversity (PD); and PCoA analyses showing the spatial distribution of microbiota samples from **(E)** the AI and **(F)** the PI based on weighted UniFrac distances. Asterisks represent significant differences between dietary treatments ($p \leq 0.05$).

physiological condition of the fishes. In the case of cinnamaldehyde, despite the absence of differences in the growth of Nile tilapia (*Oreochromis niloticus*) when supplementing the diets with this active principle (1 and 2 mL/kg) reported by Amer et al. (71), many studies have demonstrated its potential in improving growth performance in different fish species, such as Nile tilapia, grass carp (*Ctenopharyngodon idella*), tongue sole (*Cynoglossus semilaevis*), and fat greenling (*Hexagrammos otakii*) (72–75).

Regarding somatic indices, the fact that there were no significant differences in Fulton's condition factor among dietary treatments suggested that the tested combination of pungent spices did not compromise the overall body condition of gilthead seabream juveniles (76). Furthermore, the HSI was also not altered, in line with the absence of inflammation upon histological evaluation and the absence of differences in the activity of hepatic metabolic stress and oxidative stress enzymes, which confirmed the good health condition of the liver. On the other hand, PVFI was significantly decreased by more than 20% in fish fed the $SPICY_{0.1\%}$ diet. In this regard, the diminishment of the perivisceral fat levels usually has a positive effect on the consumer's perception (i.e., visual impact, more pleasant smell) and extends the shelf-life of the edible fraction (77, 78). Despite the reduced PVFI, the proximate macronutrient composition and fatty acid profile of the liver and fillet in gilthead seabream fed the $SPICY_{0.1\%}$ diet were very conserved.

When supplementing the diet with the combination of pungent spices at 0.15%, the hepatic levels of DHA and n-3 PUFAs significantly increased. These results are highly remarkable, since n3-PUFAs, and especially DHA, are essential to ensure optimal fish growth, development, and reproduction, and to enhance their immune response (4). Regarding the fillet, the DHA/EPA ratio was significantly increased in fish fed the $SPICY_{0.1\%}$ diet in

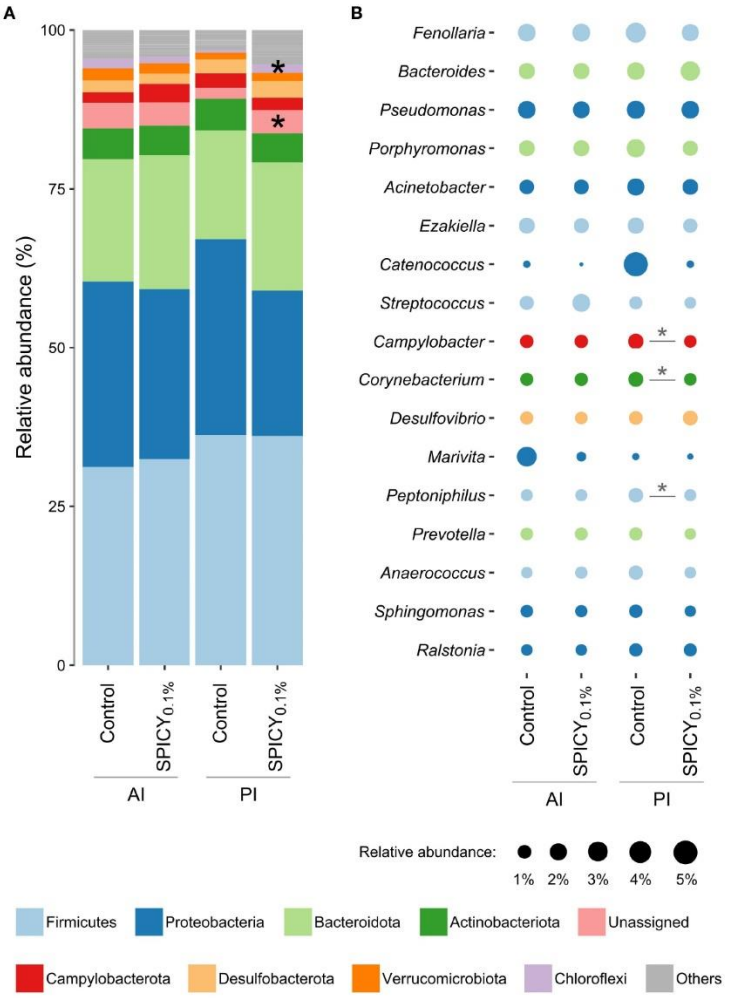

FIGURE 3
Relative abundances of gut bacterial taxa in the anterior (AI) and posterior intestine (PI) in gilthead seabream (n = 12 fish per dietary group) fed the control and the basal diet supplemented with a mixture of pungent spices (capsicum, black pepper, ginger, and cinnamaldehyde) at a dietary inclusion level of 0.1% ($SPICY_{0.1\%}$). Data are expressed at **(A)** phylum and **(B)** genus levels (excluding unassigned genera). Taxa appearance in the figures is in order of decreasing abundance (from bottom to top in the bar graph, and inversely in the bubble plot). Taxa with an abundance <1% are classified as others in the bar graph and not represented in the bubble plot. Asterisks represent significant differences between dietary treatments ($p \leq 0.05$).

comparison to the control group, reaching values closer to those expected in gilthead seabream juveniles fed conventional diets without fish oil replacement (79, 80). This was associated with a slight numerical increase in DHA and decrease in EPA levels in the fillet of fish fed the $SPICY_{0.1\%}$ diet. However, the reason why the combination of pungent spices increased the DHA/EPA ratio is presently unclear. In the previous study testing the same combination of spices in gilthead seabream (20), DHA/EPA ratio in fillet was not significantly affected, and both DHA and EPA were numerically increased by the combination of pungent spices (although DHA had a higher numerical increase and, hence, DHA/EPA was slightly raised). The main effect previously described was a significant increase in the n-3 PUFA levels in fillet and a numerical reduction in n-6 PUFA in seabream fed a diet supplemented with 0.15% of the spicy additive (20; Patent Number WO/2022/117810). Since gilthead seabream has a restricted ability to synthesize DHA and EPA (4), it is unlikely that the observed differences are caused by changes in LC-PUFA biosynthesis pathways. Supporting this, the expression of the genes encoding the enzymes that elongate PUFAs (*elovl4*, *elovl5*, and probably *elovl1*; 81) was not upregulated in fish fed the $SPICY_{0.1\%}$ diet, although the higher increase in hepatic levels of DHA was observed in fish fed the $SPICY_{0.15\%,}$ diet, for which gene expression was not assayed. Another possibility is that the increase in the levels of n-3 PUFAs could be caused by a higher capacity of absorption and assimilation of n-3 PUFAs (specifically DHA) from the diet, conferred by the combination of pungent spices, and related to the increased bile-salt-activated lipase activity, as further discussed below. However, a more likely hypothesis is that these fatty acids were better conserved and deposited in fish tissues. There is good evidence suggesting that animal fats can promote LC-PUFA sparing, associated with their high SFA content, which are preferentially used as metabolic energy sources (82, 83). On the other hand, spices, including the ones used in the combination tested herein, are well-known promotors of fat oxidation in mammals (84). Hence, the combination of pungent spices could have further potentiated the oxidation of dietary SFAs and MUFAs for energy purposes and the sparing of LC-PUFAs, with its resulting increased tissue deposition. Furthermore, in line with the present results, DHA has been shown to be more efficiently retained than EPA in Atlantic salmon tissues (85), which has been explained by the higher complexity of DHA catabolism as compared to EPA, since DHA oxidation requires an extra step involving the peroxisomes (86).

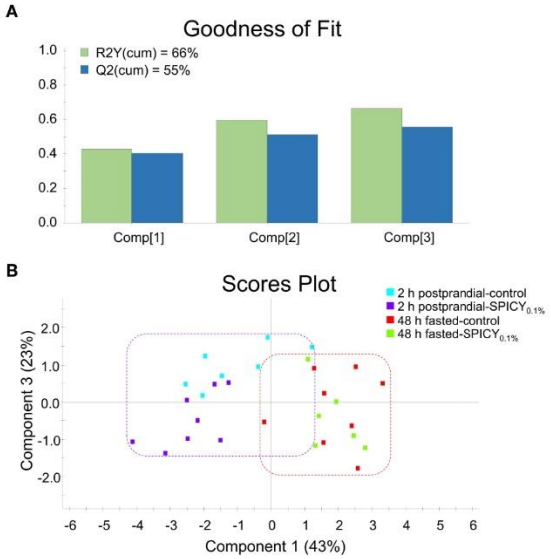

FIGURE 4
(A) Goodness of fit of the PLS-DA model. **(B)** Score plot for two-dimensional PLS-DA representing individual distribution between the component 1 and 3 of the model based on hepatic biomarker expression values with $p \leq 0.1$ (Student's t-test) among diets. Samples were obtained from the liver of 48-h fasted and 2-h postprandial gilthead seabream (n = 8 fish per dietary group) fed the control and the basal diet supplemented with a mixture of pungent spices (capsicum, black pepper, ginger, and cinnamaldehyde) at a dietary inclusion level of 0.1% ($SPICY_{0.1\%}$).

Under the current experimental conditions, the supplementation of the diet with the combination of pungent spices at 0.1% reduced the FCR, in spite of the absence of significant differences in feed intake and lipid and protein ADCs. A similar decrease in FCR was reported in the study of Morais et al. (20) when supplementing gilthead seabream diets with 0.1% and 0.15% of this additive during winter. While none of the aforementioned studies testing the effect of capsicum in fish diets described an amelioration of FCR (56, 58–60), some of the works with black pepper or ginger and many of those with cinnamaldehyde supplementation did (63, 65, 66, 68, 70, 72, 74, 75). Therefore, the decrease in FCR in fish fed the $SPICY_{0.1\%}$ diet might be partly attributed to the active principles of ginger and black pepper and to cinnamaldehyde or to the synergetic effect of the four spices present in the tested product. One of the possible mechanisms by which the combination of pungent spices decreased FCR values may lie, at least partly, in the induction of the bile-salt-activated lipase in the AI, as its activity was higher when supplementing the diets with the tested combination.

When evaluating the histological organization of the liver and AI, none of the fish presented an excessive fat accumulation (score of 5—steatosis; Figure 1), nor were there signs of inflammation or physiological alterations, the presence of which normally indicates disorders associated with unbalanced dietary conditions (87). The most effective dose of the tested additive for reducing hepatic lipid deposits was 0.1%, while in the intestine, both inclusion levels followed a similar dynamic in terms of regulation of intracellular fat deposits. In this sense, it is well-established in mammals that several different spices have an effect on the regulation of body adiposity through different mechanisms (18, 19). One of these mechanisms is the stimulation of lipid digestion, which may occur through two different pathways, as discussed in Platel and Srinivasan (18). The first is by stimulating the activity of digestive pancreatic and intestinal enzymes, whereas the second pathway is associated with the potential of the spices to induce a higher secretion of BAs from the liver into the bile, and subsequently to the intestine. Bile acids allow fat emulsification and provide a higher action surface for lipases through the formation of micelles, and being necessary for the activation of bile-salt-activated lipase (88). In the current experiment, we did not report statistical variation on lipid ADC

values among experimental groups, which might be related to the relatively high water temperature (22.5°C ± 0.5°C), not imposing a major challenge for lipid digestibility. Nevertheless, the numerical increase in the levels of BAs (T-CA, T-CDCA, and their sum) in the AI of fish fed the $SPICY_{0.1\%}$ diet and the higher activity of the bile-salt-activated lipase at both dietary inclusion levels indicated a stimulatory effect towards hepatic production and/or secretion of BAs by the tested combination of spices. To our knowledge, this is currently the only study that has evaluated the fish BA profile under dietary supplementation with any of the spices herein tested. Drawing on the existing mammalian literature, some studies on rats have reported an induced synthesis and secretion of BAs into bile after dietary supplementation with capsaicin (the pungent active principle of capsicum) or ginger, the latter also increasing the bile flowrate. By contrast, dietary supplementation with piperine (the active principle of black pepper) and with cinnamon (the main source of cinnamaldehyde) did not show a stimulatory effect on BA synthesis and secretion (18, 19). However, when the four spices were combined in a mixture, along with others, and supplemented in rat diets, the secretion of BAs and bile flowrate markedly increased, even though the higher increase was shown when the mixture did not contain cinnamon (89). In the current study, the apparently increased secretion of BAs may be associated with a higher synthesis of BAs in the liver, considering the significantly upregulated expression of cholesterol 7-alpha-monooxygenase (*cyp7a1*) in fish fed the $SPICY_{0.1\%}$ diet. Indeed, CYP7A1 is the

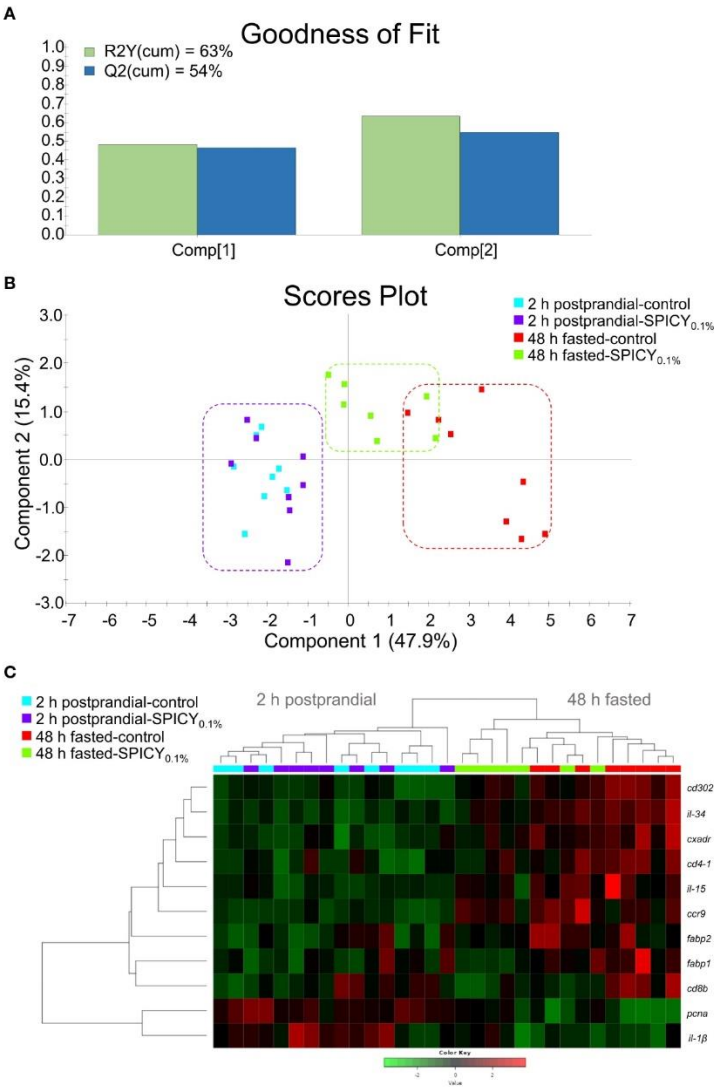

FIGURE 5
(A) Goodness of fit of the PLS-DA model. **(B)** Scores plot for two-dimensional PLS-DA representing individual distribution between the two components of the model based on intestinal biomarker expression values with $p \leq 0.1$ (Student's t-test) among diets. **(C)** Heatmap plotting hierarchical clustering of intestinal biomarker expression values (color key scale) with $p \leq 0.1$. Samples were obtained from the anterior intestine of 48-h fasted and 2-h postprandial gilthead seabream (n = 8 fish per dietary group) fed the control and the basal diet supplemented with a mixture of pungent spices (capsicum, black pepper, ginger, and cinnamaldehyde) at a dietary inclusion level of 0.1% ($SPICY_{0.1\%}$).

first rate-limiting enzyme in the classic pathway of the biosynthesis of BAs from cholesterol.

Considering the results on growth and feeding performance, DHA/EPA ratio of the fillet, vacuolization levels in the liver and AI, the activity of bile-salt-activated lipase, and the BA profile, the best dietary inclusion level of the combination of pungent spices under the present experimental conditions was 0.1%. Hence, samples from the control and $SPICY_{0.1\%}$ diets fed fish were selected for analyzing the gene expression patterns of the liver and AI, and the gut microbial communities. Assuming a higher synthesis and secretion of BAs in fish fed the $SPICY_{0.1\%}$ diet, these BAs might be responsible for the 2-h postprandial hepatic downregulation of lipoprotein lipase (*lpl*) and upregulation of fatty acid synthase (*fasn*). In this regard, we previously carried out an assay feeding gilthead seabream juveniles with the same basal diet used in this study but supplemented with a blend of BAs (31), and the expression patterns of *lpl* and *fasn* followed similar trends to the ones of the current work. Regarding LPL, this enzyme hydrolyzes the triacylglycerides from chylomicrons and very low-density lipoproteins circulating in the plasma into glycerol and free fatty acids (90). In this way, LPL facilitates the posterior incorporation of these fatty acids into the tissues, which are stored as an energetic reservoir in the form of triacylglycerides. Thus, *lpl* downregulation may be in line with the decreased PVFI and lower levels of fat deposits in the liver. On the other hand, FASN is a key lipogenic enzyme involved in *de novo* fatty acid synthesis, which catalyzes the conversion of malonyl-CoA and acetyl-CoA into palmitic (C16:0) acid (90). Furthermore, Dorn et al. (91) suggested a correlation between *fasn* expression and hepatocellular lipid accumulation. Thus, the upregulation of *fasn* could be a counter-regulatory mechanism to maintain a balance on the hepatic fat deposits and on the levels of fatty acids regarding their lower incorporation caused by reduced expression of *lpl* (31). In this sense, many studies in fish have reported an inverse regulation between the expression patterns of *lpl* and *fasn* in the liver (92–94, among others). It might be worthwhile to further examine the mechanisms underlying the transcription of these two biomarkers of lipid metabolism in fish from a deeper molecular perspective. Interestingly, in the current study, the supplementation of the diet with the combination of pungent spices also led to a hepatic upregulation of the *de novo* lipogenic biomarkers stearoyl-CoA desaturase 1b (*scd1b*) and elongation of very long chain fatty acids 6 (*elovl6*). SCD1 metabolizes palmitic and stearic (C18:0) acid into palmitoleic (C16:1 n-7) and oleic (C18:1 n-9) acid, respectively (95), while ELOVL6 elongates SFAs and MUFAs with 12, 14, and 16 carbons (96). Assuming that activity and gene expression matched, the upregulation of *elovl6* and *scd1b* may be part of the mechanism, in which *fasn* is involved, to stabilize hepatic fatty acid levels in response to their lower incorporation linked to *lpl* downregulation and/or be a strategy to prevent accumulation of palmitic (C16:0) acid triggered by *fasn* upregulation. The expression changes in the abovementioned biomarkers of lipid metabolism were accompanied by the downregulation of peroxisome proliferator-activated receptor β (*pparβ*) in fish fed the $SPICY_{0.1\%}$ diet. The role of PPARβ in lipid metabolism is not yet fully elucidated, and the change reported in this work was not very pronounced given its low expression levels. However, PPARβ may stimulate the expression of genes involved in fatty acid oxidation, and its ligands are likely fatty acids (97, 98), so a possibility is that *pparβ* is downregulated due to the presumed lower hepatic accumulation of fatty acids linked to *lpl* downregulation, which would also match the lower PVFI observed in fish fed the $SPICY_{0.1\%}$ diet.

Regarding the gut microbial composition, several studies have highlighted that the most dominant bacterial phyla of the gut microbial communities in gilthead seabream are Firmicutes, Proteobacteria, Bacteroidota, and Actinobacteriota (44, 99), in line with the results obtained in the present work. Under current conditions, the gut microbial community composition was very conserved, with only an increase in the relative abundance of Chloroflexi in the PI of fish fed the $SPICY_{0.1\%}$ diet. This is a widespread and metabolically diverse phylum of bacteria that has been reported as part of fish microbiota (44, 100, 101), but whose role remains unknown. No differences were found in the values of the F/B ratio, which is a well-documented factor whose changes have been used as a biomarker of intestinal dysbiosis in fishes (102). Our F/B values remain within the results reported for farmed fish, which vary widely depending on the rearing conditions and experimental diets utilized (103, 104). On the other hand, the absence of differences in beta diversity (Weighted UniFrac) indicated that all samples were similar in terms of their phylogenetic features, and therefore, the addition of pungent spices to the diet did not pose a threat in terms of dysbiosis. Regarding alpha diversity metrics, there were no differences in estimated richness (Chao1 and ACE indices) or phylogenetic diversity (Faith index), but there was an increased value of Simpson's diversity index in the PI of fish fed the $SPICY_{0.1\%}$ diet with respect to the control group. Both the Shannon and Simpson's indices are estimators of species richness and evenness, but while the former puts more weight on species richness, the latter puts more weight on species evenness (51). Therefore, the variation reported with Simpson's Index is more likely due to the differential abundances of species among dietary treatments. Such changes in abundance may be attributed to the potential antimicrobial effect on some bacterial strains of the spices present in the additive (105–108). Specifically, in the current study, there was a reduction in relative abundance of the genera *Campylobacter*, *Corynebacterium*, and *Peptoniphilus* in the PI using the pungent spices supplement at a dietary inclusion level of 0.1%. On the other hand, such changes in abundance may also be related to the presumed higher secretion of bile stimulated by the spices and/or to the higher levels of BAs found in the intestine of fish fed the $SPICY_{0.1\%}$ diet. Furthermore, when supplementing the diets with the combination of pungent spices in the present work, there was an increase in the relative abundance of unassigned genera belonging to the family Lachnospiraceae (Supplementary Table S9), some members of whom possess bile acids-inducible genes, which encode for enzymes involved in the metabolism of primary BAs into secondary BAs (109). In addition, *Prevotella*, which is a non-bile-acid-resistant genus, tended to be present at a lower relative

abundance in fish fed the $SPICY_{0.1\%}$ diet than the control group ($p = 0.051$), which can be seen as another evidence of an increase in the secretion of bile acids.

As clearly reflected by the PLS-DA representation, the overall gene expression profile from the intestine of fed animals (2 h postprandial) did not show any significant differences among dietary groups (Supplementary Table S11). Only the expressions of interleukin-1 beta (*il-1β*) and C–C chemokine receptor type 9 (*ccr9*) showed a substantial increase in fish fed the $SPICY_{0.1\%}$ diet ($p < 0.1$). IL-1β is a pro-inflammatory cytokine produced by several cell types in response to different processes, such as the activation of pattern recognition receptors (PRRs) by pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), among others. Additionally, IL-1β is responsible for a cascade of effects on different members of this cytokine family, leading to signal transduction and activation of the nuclear factor (NF)-kB pathway (110). Furthermore, CCR9 is a cell marker found in a wide range of immune cells (B cells, T cells, monocytes, macrophages, and dendritic cells) that drives their migration to the gut-associated lymphoid tissue (GALT), where these cells may play a regulatory role in inflammation (111). Considering the absence of signs of intestinal inflammation under histological analysis and the absence of expression changes on other pro-inflammatory cytokines (*tnf-α*, *il-6*, *il-8*, *il-15*, or *il-34*) and on the measured PRRs, the upregulation of *il-1β* and *ccr9* may be attributed to a mild innate immune system priming. Since CCR9 has both pro- and anti-inflammatory functions (111), it is difficult to know whether the upregulation of *ccr9* was also associated with the pro-inflammatory effect that IL-1β may have on the intestine, or it was part of an anti-inflammatory response to maintain homeostasis counteracting IL-1β-induced pro-inflammatory response. In any case, as mentioned above, no signs of inflammation or physiological disorders were observed with any of the dietary treatments under current experimental conditions, meaning that during the feeding period, the supplementation of pungent spices did not compromise the intestinal health of the fish.

Regarding the gene expression of biomarkers of inflammation in 48-h fasted fish, an anti-inflammatory effect of the tested additive was indicated by the downregulation of interleukin-15 (*il-15*), interleukin-34 (*il-34*), cluster of differentiation 4-1 (*cd4-1*), cluster of differentiation 8 beta (*cd8b*), and CD302 antigen (*cd302*). While the role of IL-15 and IL-34 in the fish immune response is yet unclear, many studies have suggested that, as in mammals, these cytokines may be associated with an inflammatory response (110). In mammals, IL-15 induces the proliferation of B cells, T cells, and natural killer (NK) cells (112), and IL-34 stimulates the proliferation and differentiation of monocytes and macrophages and can promote the proliferation of $CD8^+$ T regulatory cells (113). Thus, the downregulation of both cytokines may be in line with the lower expression of the cell markers *cd4* and *cd8b*, which are commonly found on the surface of different T-cell subtypes (114). Similarly, the C-type lectin receptor CD302 is expressed in monocytes, macrophages, granulocytes, and dendritic cells and is involved in cell adhesion and migration and in endocytosis and phagocytosis (115). Some *in vitro* assays using cells from ayu (*Plecoglossus altivelis*) and Nile tilapia have also unraveled the phagocytic and bactericidal activity of CD302 (116, 117). In this sense, the downregulation of *il-15*, *il-34*, *cd4-1*, *cd8b*, and *cd302* may be related to an anti-inflammatory effect of the tested combination of pungent spices. This has been seen previously when each of the spices was tested individually in mammals (107, 118) and is also in line with the anti-inflammatory effect that ginger and cinnamaldehyde apparently have in the intestine of rohu fish and bacteria-infected zebrafish (*Danio rerio*), respectively (68, 119). Although black pepper and capsicum have also been reported to act as immunostimulants in fish, such as rainbow trout and rohu fish, respectively (65, 120, 121), their effects on inflammation have not yet been elucidated.

In addition to the potential anti-inflammatory response, the dietary supplementation with the combination of pungent spices may also have an effect on intestinal epithelial integrity. In particular, there was an upregulation of proliferating cell nuclear antigen (*pcna*), which may suggest a higher cell proliferation (122) and, in line with it, a downregulation of the coxsackievirus and adenovirus receptor homolog (*cxadr*), whose expression has been inversely correlated with the rate of cell proliferation (123). Based on these discussed results, a possibility is that such enhanced functionality may modulate the potential entry of PAMPs through the enterocytes, which could be the cause of the lower expression of the PRR *cd302* by the leukocytes of the GALT. Consequently, this would lead to a lower immune response as indicated by the lower expression of *cd4-1* and *cd8b* cell markers and reduced secretion of the pro-inflammatory cytokines IL-15 and IL-34. Hence, the reduced levels of expression of pro-inflammatory biomarkers may be the consequence of a reduced exposure to foreign molecules due to the increased permeability induced by *pcna* upregulation rather than an orchestrated activation of an anti-inflammatory response. Regardless of the cause of the downregulation of *il-15*, *il-34*, *cd4-1*, *cd8b*, and *cd302*, the results suggest that the tested additive enhanced the health status of the intestine under short-term fasting conditions, since the $SPICY_{0.1\%}$ diet prevented intestinal inflammation that can affect the fish intestinal motility and deregulate digestion and absorption of nutrients, leading to a lower utilization of the feed and to physiological disorders (124). In this sense, some strategies currently used in aquaculture, such as the replacement of fish oil or the use of high-fat diets, have been correlated with inflammation and excessive lipid accumulation in digestive tissues and with a lower non-specific immunity and disease resistance (16, 125, 126). Therefore, the tested combination of pungent spices may be used as a potential tool to aid in the prevention of intestinal inflammation, enhance peristalsis, and optimize nutrient digestion and absorption. However, further studies are needed to confirm the anti-inflammatory effect in the intestine of the combination of spices tested herein. Overall, to complete the detailed understanding of the mechanisms underlying the above-mentioned pathways, the invaluable help of omics sciences (i.e., RNA-seq or microarray analyses) may be needed in order to further track the gene expression of key biomarkers, such as some anti-inflammatory cytokines (TGF-β, IL-4/13A, and IL-4/13B).

Although there was no suggestion of potential anti-inflammatory effect of the combination of pungent spices until feeding cessation, this was probably because the transit of feed along

the gastrointestinal tract had a greater impact on gene expression than the differential ingredient formulation of the experimental diets themselves. Regardless, based on our above-discussed results, we recommend that the incorporation of the combination of pungent spices in the diet be continuous whenever the purpose is to promote lipid metabolism to reduce body fat content and improve the health status of the fish, accompanied by an increase in fish growth and feed utilization. In this sense, the regulatory effect that the combination of spices had on the expression of hepatic biomarkers related to lipid metabolism practically disappeared after the 48-h fasting period (Supplementary Table S10), except for the upregulation of sterol regulatory element-binding proteins 1 (*srebp1*) and peroxiredoxin 5 (*prdx5*). SREBP1 is a membrane transcription factor that has been related to lipid homeostasis through the regulation of *de novo* lipogenesis and fatty acid synthesis in fish liver (81), which may be activated after feed deprivation to maintain a balance on the levels of hepatic fatty acids. Albeit the function of PRDX5 in fish still needs to be further studied, recent works in mammals have elucidated its role in preventing adipogenesis by maintaining the intracellular redox balance and avoiding fat deposition through inhibition of fatty acid synthesis and acceleration of their oxidation (127, 128). Thus, the decreased fatty acid production triggered by *prdx5* upregulation in the liver of 48-h fasted fish fed the $SPICY_{0.1\%}$ diet may be in line with the downregulation in the intestine of the liver-type fatty acid-binding protein (*fabp1*) and the intestinal fatty acid-binding protein (*fabp2*) (Supplementary Table S11), both involved in fatty acid uptake, transport, and metabolism (129).

5 Conclusions

This study showed that under the regimen of a high saturated fat diet (with poultry fat as the major lipid source), the supplementation of the feed with 0.1% of a product containing a combination of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde improved the growth performance and FCR in gilthead seabream. In addition, the $SPICY_{0.1\%}$ diet reduced the levels of fat deposits in the visceral cavity, liver, and intestine and increased the DHA/EPA ratio in the fillet to a range closer to the levels commonly found in fillet of farmed gilthead seabream fed a conventional fish-oil-based diet. Moreover, the $SPICY_{0.1\%}$ diet was able to increase the activity of the bile-salt-activated lipase and regulate the gene expression of biomarkers of lipid metabolism. In this sense, the upregulation of *cyp7a1*, which is involved in the synthesis of primary BAs, which may stimulate lipid digestion, and the downregulation of *lpl*, which hydrolyzes plasmatic triacylglycerides releasing free fatty acids that can be incorporated into the tissues, was noteworthy. There was no great impact in the structure and composition of the gut microbial communities, albeit the relative abundance of the phylum Chloroflexi increased, while the relative abundances of the genera *Campylobacter*, *Corynebacterium*, and *Peptoniphilus* decreased. In addition, when supplementing the basal diet with the combination of pungent spices at 0.1%, there was a downregulation of the PRR *cd302*, the immune cell markers *cd4-1* and *cd8b*, and the pro-inflammatory cytokines *il-15* and *il-34* in 48-h fasted fish. On the other hand, the supplementation of the diet with the pungent spices at an inclusion level of 0.15% also had a positive effect on growth performance, in the reduction in lipid vacuolization within the enterocytes, and in the activity of the bile-salt-activated lipase in the intestine. Moreover, the $SPICY_{0.15\%}$ diet showed higher levels of DHA and total n-3 PUFAs in the liver than the control group. Therefore, the supplementation of fish feeds containing reduced levels of fish oil, especially when fish oil is substituted by oils containing high levels of SFAs, with the combination of pungent spices, may provide a functional solution to optimize the usage of fish oil as a strategic ingredient in the aquafeed industry, even though the optimal inclusion level might need to be optimized depending on the species and diet composition.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was approved by Ethical Committee of the Institute of Agrifood Research and Technology and the Generalitat of Catalunya. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SM and EG conceptualized the research. EG designed the nutritional assay. AR, IS, PH, and JP processed the samples. AR, PH, JC-G, JP, MV, JP-S, and EG analyzed the data. AR, KA, DF, JC-G, JP, JP-S, SM, and EG interpreted the results. AR wrote the original draft of the manuscript. EG supervised the study and the writing of the original draft and acquired the funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

JJP and SM are current employees of Lucta S.A.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1222173/full#supplementary-material

References

1. Campoy C, Escolano-Margarit MV, Anjos T, Szajewska H, Uauy R. Omega 3 fatty acids on child growth, visual acuity and neurodevelopment. *Br J Nutr* (2012) 107:S85–106. doi: 10.1017/S0007114512001493

2. Delgado-Lista J, Perez-Martinez P, Lopez-Miranda J, Perez-Jimenez F. Long chain omega-3 fatty acids and cardiovascular disease: a systematic review. *Br J Nutr* (2012) 107:S201–13. doi: 10.1017/S0007114512001596

3. Gerber M. Omega-3 fatty acids and cancers: a systematic update review of epidemiological studies. *Br J Nutr* (2012) 107:S228–39. doi: 10.1017/S0007114512001614

4. Glencross BD. Exploring the nutritional demand for essential fatty acids by aquaculture species. *Rev Aquac* (2009) 1:71–124. doi: 10.1111/J.1753-5131.2009.01006.X

5. Trushenski JT, Boesenberg J, Kohler CC. Influence of grow-out feed fatty acid composition on finishing success in Nile tilapia. *N Am J Aquac* (2009) 71:242–51. doi: 10.1577/A08-051.1

6. Kenari AA, Mozanzadeh MT, Pourgholam R. Effects of total fish oil replacement to vegetable oils at two dietary lipid levels on the growth, body composition, haemato-immunological and serum biochemical parameters in caspian brown trout (*Salmo trutta caspius* Kessler, 1877). *Aquac Res* (2011) 42:1131–44. doi: 10.1111/j.1365-2109.2010.02701.x

7. Mourente G. Bell JG. Partial replacement of dietary fish oil with blends of vegetable oils (rapeseed, linseed and palm oils) in diets for European sea bass (*Dicentrarchus labrax* L.) over a long term growth study: effects on muscle and liver fatty acid composition and effectiveness of a fish oil finishing diet. *Comp Biochem Physiol B Biochem Mol Biol* (2006) 145:389–99. doi: 10.1016/j.cbpb.2006.08.012

8. Moretti VM, Corino C. "Omega-3 and trans fatty acids". In: Nollet LML, Toldra F, editors. *Handbook of processed meats and poultry analysis*. Crystal Bay: CRC Press (2008). p. 249–88.

9. Turchini GM, Torstensen BE, Ng WK. Fish oil replacement in finfish nutrition. *Rev Aquac* (2009) 1:10–57. doi: 10.1111/j.1753-5131.2008.01001.x

10. Campos I, Matos E, Maia MR, Marques A. Valente LM. Partial and total replacement of fish oil by poultry fat in diets for European seabass (*Dicentrarchus labrax*) juveniles: Effects on nutrient utilization, growth performance, tissue composition and lipid metabolism. *Aquaculture* (2019) 502:107–20. doi: 10.1016/j.aquaculture.2018.12.004

11. Lorente-Cebrián S, Costa AG, Navas-Carretero S, Zabala M, Martínez JA, Moreno-Aliaga MJ. Role of omega-3 fatty acids in obesity, metabolic syndrome, and cardiovascular diseases: a review of the evidence. *J Physiol Biochem* (2013) 69:633–51. doi: 10.1007/s13105-013-0265-4

12. Alvarez MJ, Diez A, Lopez-Bote C, Gallego M, Bautista JM. Short-term modulation of lipogenesis by macronutrients in rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Br J Nutr* (2000) 84:619–28. doi: 10.1017/S0007114500001951

13. Morais S, Pratoomyot J, Taggart JB, Bron JE, Guy DR, Bell JG, et al. Genotype-specific responses in Atlantic salmon (*Salmo salar*) subject to dietary fish oil replacement by vegetable oil: a liver transcriptomic analysis. *BMC Genom* (2011) 12:1–17. doi: 10.1186/1471-2164-12-255

14. Morais S, Silva T, Cordeiro O, Rodrigues P, Guy DR, Bron JE, et al. Effects of genotype and dietary fish oil replacement with vegetable oil on the intestinal transcriptome and proteome of Atlantic salmon (*Salmo salar*). *BMC Genom* (2012) 13:1–21. doi: 10.1186/1471-2164-13-448

15. Olsen RE, Myklebust R, Ringø E, Mayhew TM. The influences of dietary linseed oil and saturated fatty acids on caecal enterocytes in Arctic char (*Salvelinus alpinus* L.): a quantitative ultrastructural study. *Fish Physiol Biochem* (2000) 22:207–16. doi: 10.1023/A:1007879127182

16. Iwashita Y, Suzuki N, Yamamoto T, Shibata JI, Isokawa K, Soon AH, et al. Supplemental effect of cholyltaurine and soybean lecithin to a soybean meal-based fish meal-free diet on hepatic and intestinal morphology of rainbow trout. *Oncorhynchus mykiss*. *Fish Sci* (2008) 74:1083–95. doi: 10.1111/j.1444-2906.2008.01628.x

17. Zhang Y, Feng H, Liang XF, He S, Lan J, Li L. Dietary bile acids reduce liver lipid deposition via activating farnesoid X receptor, and improve gut health by regulating gut microbiota in Chinese perch (*Siniperca chuatsi*). *Fish Shellfish Immunol* (2022) 121:265–75. doi: 10.1016/j.fsi.2022.01.010

18. Platel K, Srinivasan K. Digestive stimulant action of spices: a myth or reality? *Indian J Med Res* (2004) 119:167–79.

19. Srinivasan K. Spices as influencers of body metabolism: an overview of three decades of research. *Food Res Int* (2005) 38:77–86. doi: 10.1016/j.aquaculture.2020.735291

20. Morais S, Gonçalves RA, Aires T, Magalhães S. The potential of spices to support the reduction of fish oil in gilthead seabream diets. *Aquafeed: Adv Process Formulation* (2022) 14:57–60.

21. Salomón R, Furones MD, Reyes-López FE, Tort L, Firmino JP, Quintela JC, et al. Phytogenics from sage and lemon verbena promote growth, systemic immunity and disease resistance in Atlantic salmon (*Salmo salar*). *Front Mar Sci* (2022) 9:828497. doi: 10.3389/fmars.2022.828497

22. Salomón R, Firmino JP, Reyes-López FE, Andree KB, González-Silvera D, Esteban MA, et al. The growth promoting and immunomodulatory effects of a medicinal plant leaf extract obtained from *Salvia officinalis* and *Lippia citriodora* in gilthead seabream (*Sparus aurata*). *Aquaculture* (2020) 524:735291. doi: 10.1016/j.aquaculture.2020.735291

23. Bakke AM, Glover C, Krogdahl Å. Grosell M, Farrell AP, Brauner CJ, editors. *Fish physiology: The multifunctional gut of fish*. London: Academic Press (2010). p. 57–110.

24. Naya-Català F, do Vale Pereira G, Piazzon MC, Fernandes AM, Calduch-Giner JA, Sitjà-Bobadilla A, et al. Cross-talk between intestinal microbiota and host gene expression in gilthead sea bream (*Sparus aurata*) juveniles: insights in fish feeds for increased circularity and resource utilization. *Front Physiol* (2021) 12:748265. doi: 10.3389/fphys.2021.748265

25. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* (1951) 193:265–75. doi: 10.1016/S0021-9258(19)52451-6

26. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* (1957) 226:497–509. doi: 10.1016/S0021-9258(18)64849-5

27. Dubois M, Gilles KA, Hamilton JK, Rebers PT, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem* (1956) 28:350–6. doi: 10.1021/ac60111a017

28. Association of Official Analytical Chemists. *Official methods of analysis of the association of official analytical chemists. 15th ed.* Washington, DC: AOAC (1990).

29. Ramos-Júdez S, Estévez A, González-López WÁ, Duncan N. Lipid and fatty acid composition of muscle, liver, ovary, and peritoneal fat in wild flathead grey mullet (*Mugil cephalus*) according to ovarian development. *Theriogenology* (2022) 198:317–26. doi: 10.1016/j.theriogenology.2022.12.046

30. Cheng ZJ, Hardy RW. Apparent digestibility coefficients of nutrients and nutritional value of poultry by-product meals for rainbow trout *Oncorhynchus*

mykiss measured in *vivo* using settlement. *J World Aquac Soc* (2002) 33:458–65. doi: 10.1111/j.1749-7345.2002.tb00025.x

31. Ruiz A, Andree KB, Sanahuja I, Holhorea PG, Calduch-Giner JA, Morais S, et al. Bile salt dietary supplementation promotes growth and reduces body adiposity in gilthead seabream (*Sparus aurata*). *Aquaculture* (2023) 566:739203. doi: 10.1016/j.aquaculture.2022.739203

32. Bergmeyer HU, Bernt E. "Lactate dehydrogenase: UV-assay with pyruvate and NADH". In: Bergmeyer HU, editor. *Methods of enzymatic analysis*. New York: Academic Press (1974). p. 574–9.

33. Bergmeyer HU, Bernt E. "Glutamate-oxaloacetate transaminase: UV-assay, manual method". In: Bergmeyer HU, editor. *Methods of enzymatic analysis*. New York: Academic Press (1974). p. 727–33.

34. Bergmeyer HU, Bernt E. "Glutamate-pyruvate transaminase: UV-assay, manual method". In: Bergmeyer HU, editor. *Methods of enzymatic analysis*, vol. . p . New York: Academic Press (1974). p. 752–8.

35. McCord JM, Fridovich I. Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). *J Biol Chem* (1969) 244:6049–55. doi: 10.1016/S0021-9258(18)63504-5

36. Aebi H. "Catalase". In: Bergmeyer HU, editor. *Methods of enzymatic analysis*. New York: Academic Press (1974). p. 673–84.

37. Carlberg I, Mannervik B. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *J Biol Chem* (1975) 250:5475–80. doi: 10.1016/S0021-9258(19)41206-4

38. Solé M, Potrykus J, Fernández-Díaz C, Blasco J. Variations on stress defences and metallothionein levels in the Senegal sole, *Solea Senegalensis*, during early larval stages. *Fish Physiol Biochem* (2004) 30:57–66. doi: 10.1007/s10695-004-6786-6

39. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* (1976) 72:248–54. doi: 10.1016/0003-2697(76)90527-3

40. Solovyev M, Gisbert E. Influence of time, storage temperature and freeze/thaw cycles on the activity of digestive enzymes from gilthead sea bream (*Sparus aurata*). *Fish Physiol Biochem* (2016) 42:1383–94. doi: 10.1007/s10695-016-0226-2

41. Herrero-Encinas J, Huerta A, Blanch M, Pastor JJ, Morais S, Menoyo D. Impact of dietary supplementation of spice extracts on growth performance, nutrient digestibility and antioxidant response in broiler chickens. *Animals* (2023) 13:250. doi: 10.3390/ani13020250

42. Meyerholz DK, Beck AP. Fundamental concepts for semiquantitative tissue scoring in translational research. *ILAR J* (2018) 59:13–7. doi: 10.1093/ilar/ily025

43. Gisbert E, Skalli A, Campbell J, Solovyev MM, Rodríguez C, Dias J, et al. Spray-dried plasma promotes growth, modulates the activity of antioxidant defenses, and enhances the immune status of gilthead sea bream (*Sparus aurata*) fingerlings. *J Anim Sci* (2015) 93:278–86. doi: 10.2527/jas.2014-7491

44. Ruiz A, Andree KB, Furones D, Holhorea PG, Calduch-Giner JÀ, Viñas M, et al. Modulation of gut microbiota and intestinal immune response in gilthead seabream (*Sparus aurata*) by dietary bile salt supplementation. *Front Microbiol* (2023) 14:1123716. doi: 10.3389/fmicb.2023.1123716

45. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* (2016) 13:581–3. doi: 10.1038/nmeth.3869

46. Smith PE, Waters SM, Gómez Expósito R, Smidt H, Carberry CA, McCabe MS. Synthetic sequencing standards: a guide to database choice for rumen microbiota amplicon sequencing analysis. *Front Microbiol* (2020) 11:606825. doi: 10.3389/fmicb.2020.606825

47. McKnight DT, Huerlimann R, Bower DS, Schwarzkopf L, Alford RA, Zenger KR. Methods for normalizing microbiome data: an ecological perspective. *Methods Ecol Evol* (2019) 10:389–400. doi: 10.1111/2041-210X.13115

48. Masek T, Vopalensky V, Suchomelova P, Pospisek M. Denaturing RNA electrophoresis in TAE agarose gels. *Anal Biochem* (2005) 336:46–50. doi: 10.1016/j.ab.2004.09.010

49. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* (2001) 25:402–8. doi: 10.1006/meth.2001.1262

50. Faith DP. Conservation evaluation and phylogenetic diversity. *Biol Conserv* (1992) 61:1–10. doi: 10.1016/0006-3207(92)91201-3

51. Kim BR, Shin J, Guevarra RB, Lee JH, Kim DW, Seol KH, et al. Deciphering diversity indices for a better understanding of microbial communities. *J Microbiol Biotechnol* (2017) 27:2089–93. doi: 10.4014/jmb.1709.09027

52. Chang Q, Luan Y, Sun F. Variance adjusted weighted UniFrac: a powerful beta diversity measure for comparing communities based on phylogeny. *BMC Bioinform* (2011) 12:1–14. doi: 10.1186/1471-2105-12-118

53. Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecol* (2001) 26:32–46. doi: 10.1111/j.1442-9993.2001.01070.pp.x

54. White JR, Nagarajan N, Pop M. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. *PloS Comput Biol* (2009) 5:e1000352. doi: 10.1371/journal.pcbi.1000352

55. Liu C, Cui Y, Li X, Yao M. *microeco*: an R package for data mining in microbial community ecology. *FEMS Microbiol Ecol* (2021) 97:fiaa255. doi: 10.1093/femsec/fiaa255

56. Wassef EA, Chatzifotis S, Sakr EM, Saleh NE. Effect of two natural carotenoid sources in diets for gilthead seabream, *Sparus aurata*, on growth and skin coloration. *J Appl Aquac* (2010) 22:216–29. doi: 10.1080/10454438.2010.497741

57. Yilmaz S, Ergün S. Effect of red pepper (*Capsicum annum*) on pigmentation of blue streak hap (*Labidochromis caeruleus*). *Isr J Aquac* (2011) 63:633–8.

58. Yilmaz S, Ergün S, Soytas N. Enhancement of growth performance and pigmentation in red *Oreochromis mossambicus* associated with dietary intake of astaxanthin, paprika, or capsicum. *Isr J Aquac* (2013) 65:825-31. doi: 10.46989/001c.20666

59. Yanar M, Büyükçapar HM, Yanar Y. Effects of hot and sweet red peppers (*Capsicum annuum*) as feed supplements on pigmentation, sensory properties and weight gain of rainbow trout (*Onchorhynchus mykiss*). *Ann Anim Sci* (2016) 16:825–34. doi: 10.1515/aoas-2016-0011

60. Yigit NO, Ozdal AM, Bahadir Koca S, Ozmen O. Effect on pigmentation, growth and liver histopathology of red pepper (*Capsicum annuum*) added in different ratios to jewel cichlid (*Hemichromis guttatus*) diet. *Aquac Res* (2021) 52:5579–84. doi: 10.1111/are.15433

61. Talebi M, Khara H, Zoriehzahra J, Ghobadi S, Khodabandelo A, Mirrasooli E. Study on effect of red bell pepper on growth, pigmentation and blood factors of rainbow trout (*Oncorhynchus mykiss*). *World J Zool* (2013) 8:17–23. doi: 10.5829/idosi.wjz.2013.8.1.7136

62. El-Houseiny W, Khalil AA, Abd-Elhakim YM, Badr HA. The potential role of turmeric and black pepper powder diet supplements in reversing cadmium-induced growth retardation, ATP depletion, hepatorenal damage, and testicular toxicity in Clarias gariepinus. *Aquaculture* (2019) 510:109–21. doi: 10.1016/j.aquaculture.2019.05.045

63. Stoev T, Zhelyazkov G. Effect of black pepper (*Piper nigrum*) extract on productive traits, economic efficiency and blood biochemical parameters of rainbow trout (*Oncorhynchus mykiss* W.). *Agric Sci Technol* (2021) 13:266–71. doi: 10.15547/ast.2021.03.042

64. Wojno M, Mandas A, Kwasek K, Dabrowski K. The effect of dietary supplements of black pepper piper nigrum and turmeric curcuma longa extracts on dietary amino acid utilization and growth performance in common carp. *N Am J Aquac* (2021) 83:155–64. doi: 10.1002/naaq.10179

65. Ullah A, Azmat H, Masood Z, Arooj F, Hussain SM, Malik A. Impact of dietary leaf extracts of black pepper *Piper nigrum* L. on the growth, hematological and immunological parameters of *Labeo rohita* (Hamilton, 1822) cultured in glass aquaria. *Braz J Biol* (2021) 83:e246825. doi: 10.1590/1519-6984.246825

66. Talpur AD, Ikhwanuddin M, Bolong AMA. Nutritional effects of ginger (*Zingiber officinale* Roscoe) on immune response of Asian sea bass, *Lates calcarifer* (Bloch) and disease resistance against *Vibrio harveyi*. *Aquaculture* (2013) 400:46–52. doi: 10.1016/j.aquaculture.2013.02.043

67. Brum A, Pereira SA, Owatari MS, Chagas EC, Chaves FCM, Mouriño JLP, et al. Effect of dietary essential oils of clove basil and ginger on Nile tilapia (*Oreochromis niloticus*) following challenge with *Streptococcus agalactiae*. *Aquaculture* (2017) 468:235–43. doi: 10.1016/j.aquaculture.2016.10.020

68. Sukumaran V, Park SC, Giri SS. Role of dietary ginger *Zingiber officinale* in improving growth performances and immune functions of *Labeo rohita* fingerlings. *Fish Shellfish Immunol* (2016) 57:362–70. doi: 10.1016/j.fsi.2016.08.056

69. Fazelan Z, Vatnikov YA, Kulikov EV, Plushikov VG, Yousefi M. Effects of dietary ginger (*Zingiber officinale*) administration on growth performance and stress, immunological, and antioxidant responses of common carp (*Cyprinus carpio*) reared under high stocking density. *Aquaculture* (2020) 518:734833. doi: 10.1016/j.aquaculture.2019.734833

70. Mohammadi G, Rashidian G, Hoseinifar SH, Naserabad SS, Van Doan H. Ginger (*Zingiber officinale*) extract affects growth performance, body composition, haematology, serum and mucosal immune parameters in common carp (*Cyprinus carpio*). *Fish Shellfish Immunol* (2020) 99:267–73. doi: 10.1016/j.fsi.2020.01.032

71. Amer SA, Metwally AE, Ahmed SA. The influence of dietary supplementation of cinnamaldehyde and thymol on the growth performance, immunity and antioxidant status of monosex Nile tilapia fingerlings (*Oreochromis niloticus*). *Egypt J Aquat Res* (2018) 44:251–6. doi: 10.1016/j.ejar.2018.07.004

72. Abd El-Hamid MI, Ibrahim SM, Eldemery F, El-Mandrawy SA, Metwally AS, Khalifa E, et al. Dietary cinnamaldehyde nanoemulsion boosts growth and transcriptomes of antioxidant and immune related genes to fight *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*). *Fish Shellfish Immunol* (2021) 113:96–105. doi: 10.1016/j.fsi.2021.03.021

73. Zhou Y, Jiang WD, Zhang JX, Feng L, Wu P, Liu Y, et al. Cinnamaldehyde improves the growth performance and digestion and absorption capacity in grass carp (*Ctenopharyngodon idella*). *Fish Physiol Biochem* (2020) 46:1589–601. doi: 10.1007/s10695-020-00813-9

74. Wang Y, Wang Q, Xing K, Jiang P, Wang J. Dietary cinnamaldehyde and *Bacillus subtilis* improve growth performance, digestive enzyme activity, and antioxidant capability and shape intestinal microbiota in tongue sole, *Cynoglossus semilaevis*. *Aquaculture* (2021) 531:735798. doi: 10.1016/j.aquaculture.2020.735798

75. Gu Y, Han J, Wang W, Zhan Y, Wang H, Hua W, et al. Dietary cinnamaldehyde enhances growth performance, digestion, immunity, and lipid metabolism in juvenile fat greenling (*Hexagrammos otakii*). *Aquac Nutr* (2022) 2022:2132754. doi: 10.1155/2022/2132754

76. Sutton SG, Bult TP, Haedrich RL. Relationships among fat weight, body weight, water weight, and condition factors in wild Atlantic salmon parr. *Trans Am Fish Soc* (2000) 129:527–38. doi: 10.1577/1548-8659(2000)129<0527:RAFWBW>2.0.CO;2

77. Hsieh RJ, Kinsella JE. Oxidation of polyunsaturated fatty acids: mechanisms, products, and inhibition with emphasis on fish. *Adv Food Nutr Res* (1989) 33:233–341. doi: 10.1016/S1043-4526(08)60129-1

78. Grigorakis K. Compositional and organoleptic quality of farmed and wild gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) and factors affecting it: a review. *Aquaculture* (2007) 272:55–75. doi: 10.1016/j.aquaculture.2007.04.062

79. Benedito-Palos L, Navarro JC, Kaushik S, Pérez-Sánchez J. Tissue-specific robustness of fatty acid signatures in cultured gilthead sea bream (*Sparus aurata* L.) fed practical diets with a combined high replacement of fish meal and fish oil. *J Anim Sci* (2010) 88:1759–70. doi: 10.2527/jas.2009-2564

80. Lenas DS, Triantafillou DJ, Chatziantoniou S, Nathanailides C. Fatty acid profile of wild and farmed gilthead sea bream (*Sparus aurata*). *J Verbrauch Leb* (2011) 6:435–40. doi: 10.1007/s00003-011-0695-2

81. Xie D, Chen C, Dong Y, You C, Wang S, Monroig O, et al. Regulation of long-chain polyunsaturated fatty acid biosynthesis in teleost fish. *Prog Lipid Res* (2021) 82:101095. doi: 10.1016/j.plipres.2021.101095

82. Henderson RJ. Fatty acid metabolism in freshwater fish with particular reference to polyunsaturated fatty acids. *Arch Anim Nutr* (1996) 49:5–22. doi: 10.1080/17450399609381859

83. Rombenso AN, Trushenski JT, Drawbridge M. Saturated lipids are more effective than others in juvenile California yellowtail feeds—Understanding and harnessing LC-PUFA sparing for fish oil replacement. *Aquaculture* (2018) 493:192–203. doi: 10.1016/j.aquaculture.2018.04.040

84. Westerterp-Plantenga M, Diepvens K, Joosen AM, Bérubé-Parent S, Tremblay A. Metabolic effects of spices, teas, and caffeine. *Physiol Behav* (2006) 89:85–91. doi: 10.1016/j.physbeh.2006.01.027

85. Rosenlund G, Torstensen BE, Stubhaug I, Usman N, Sissener NH. Atlantic salmon require long-chain n-3 fatty acids for optimal growth throughout the seawater period. *J Nutr Sci* (2016) 5:e19. doi: 10.1017/jns.2016.10

86. Tocher DR, Betancor MB, Sprague M, Olsen RE, Napier JA. Omega-3 long-chain polyunsaturated fatty acids, EPA and DHA: bridging the gap between supply and demand. *Nutrients* (2019) 11:89. doi: 10.3390/nu11010089

87. Gisbert E, Ortiz-Delgado JB, Sarasquete C. Nutritional cellular biomarkers in early life stages of fish. *Histol Histopathol* (2008) 23:1525–39. doi: 10.14670/HH-23.1525

88. Romano N, Kumar V, Yang G, Kajbaf K, Rubio MB, Overturf K, et al. Bile acid metabolism in fish: disturbances caused by fishmeal alternatives and some mitigating effects from dietary bile inclusions. *Rev Aquac* (2020) 12:1792–817. doi: 10.1111/raq.12410

89. Platel K, Rao A, Saraswathi G, Srinivasan K. Digestive stimulant action of three Indian spice mixes in experimental rats. *Nahrung* (2002) 46:394–8. doi: 10.1002/1521-3803(20021101)46:6<394::AID-FOOD394>3.0.CO;2-D

90. Salmerón C. Adipogenesis in fish. *J Exp Biol* (2018) 221:jeb161588. doi: 10.1242/jeb.161588

91. Dorn C, Riener MO, Kirovski G, Saugspier M, Steib K, Weiss TS, et al. Expression of fatty acid synthase in nonalcoholic fatty liver disease. *Int J Clin Exp Pathol* (2010) 3:505–14.

92. Tian J, Wen H, Zeng LB, Jiang M, Wu F, Liu W, et al. Changes in the activities and mRNA expression levels of lipoprotein lipase (LPL), hormone-sensitive lipase (HSL) and fatty acid synthetase (FAS) of Nile tilapia (*Oreochromis niloticus*) during fasting and re-feeding. *Aquaculture* (2013) 400:29–35. doi: 10.1016/j.aquaculture.2013.01.032

93. Peng S, Shi Z, Gao Q, Zhang C, Wang J. Dietary n-3 LC-PUFA s affect lipoprotein lipase (LPL) and fatty acid synthase (FAS) activities and mRNA expression during vitellogenesis and ovarian fatty acid composition of female silver pomfret (*Pampus argenteus*) broodstock. *Aquac Nutr* (2017) 23:692–701. doi: 10.1111/anu.12436

94. Huang H, Zhang Y, Cao M, Xue L, Shen W. Effects of fasting on the activities and mRNA expression levels of lipoprotein lipase (LPL), hormone-sensitive lipase (HSL) and fatty acid synthetase (FAS) in spotted seabass. *Lateolabrax maculatus. Fish Physiol Biochem* (2018) 44:387–400. doi: 10.1007/s10695-017-0442-4

95. Ntambi JM, Miyazaki M. Regulation of stearoyl-CoA desaturases and role in metabolism. *Prog Lipid Res* (2004) 43:91–104. doi: 10.1016/s0163-7827(03)00039-0

96. Matsuzaka T, Shimano H. Elovl6: a new player in fatty acid metabolism and insulin sensitivity. *J Mol Med* (2009) 87:379–84. doi: 10.1007/s00109-009-0449-0

97. Wang YX, Lee CH, Tiep S, Ruth TY, Ham J, Kang H, et al. Peroxisome-proliferator-activated receptor δ activates fat metabolism to prevent obesity. *Cell* (2003) 113:159–70. doi: 10.1016/S0092-8674(03)00269-1

98. Kidani Y, Bensinger SJ. Liver X receptor and peroxisome proliferator-activated receptor as integrators of lipid homeostasis and immunity. *Immunol Rev* (2012) 249:72–83. doi: 10.1111/j.1600-065X.2012.01153.x

99. Tapia-Paniagua ST, Balebona MDC, Firmino JP, Rodriguez C, Polo J, Moriñigo MA, et al. The effect of spray-dried porcine plasma on gilthead seabream (*Sparus aurata*) intestinal microbiota. *Aquac Nutr* (2020) 26:801–11. doi: 10.1111/anu.13039

100. Liu ZY, Yang HL, Hu LH, Yang W, Ai CX, Sun YZ. Dose-dependent effects of histamine on growth, immunity and intestinal health in juvenile grouper (*Epinephelus coioides*). *Front Mar Sci* (2021) 8:685720. doi: 10.3389/fmars.2021.685720

101. Nikouli E, Kormas KA, Jin Y, Olsen Y, Bakke I, Vadstein O. Dietary lipid effects on gut microbiota of first feeding Atlantic salmon (*Salmo salar* L.). *Front Mar Sci* (2021) 8:665576. doi: 10.3389/fmars.2021.665576

102. Mougin J, Joyce A. Fish disease prevention via microbial dysbiosis-associated biomarkers in aquaculture. *Rev Aquac* (2022) 15:579–94. doi: 10.1111/raq.12745

103. Hossain MS, Dai J, Qiu D. European eel (*Anguilla Anguilla*) GI tract conserves a unique metagenomics profile in the recirculation aquaculture system (RAS). *Aquac Int* (2021) 29:1529–44. doi: 10.1007/s10499-021-00692-8

104. Du J, Liu QH, Liu JS, Zhang KQ, Huang WF. Structural and functional comparisons of the environmental microbiota of pond and tank environments at different locations for the commercial aquaculture of American shad. *Lett Appl Microbiol* (2022) 75:51–60. doi: 10.1111/lam.13709

105. Friedman M. Chemistry, antimicrobial mechanisms, and antibiotic activities of cinnamaldehyde against pathogenic bacteria in animal feeds and human foods. *J Agric Food Chem* (2017) 65:10406–23. doi: 10.1021/acs.jafc.7b04344

106. Beristain-Bauza SDC, Hernández-Carranza P, Cid-Pérez TS, Ávila-Sosa R, Ruiz-López II, Ochoa-Velasco CE. Antimicrobial activity of ginger (*Zingiber officinale*) and its application in food products. *Food Rev Int* (2019) 35:407–26. doi: 10.1080/87559129.2019.1573829

107. Takooree H, Aumeeruddy MZ, Rengasamy KR, Venugopala KN, Jeewon R, Zengin G, et al. A systematic review on black pepper (*Piper nigrum* L.): from folk uses to pharmacological applications. *Crit Rev Food Sci Nutr* (2019) 59:S210–43. doi: 10.1080/10408398.2019.1565489

108. Bhatti SA, Hussain MH, Mohsin MZ, Mohsin A, Zaman WQ, Guo M, et al. Evaluation of the antimicrobial effects of *Capsicum*, *Nigella sativa*, *Musa paradisiaca* L., and *Citrus limetta*: A review. *Front Sustain Food Syst* (2022) 6:1043823. doi: 10.3389/fsufs.2022.1043823

109. Vital M, Rud T, Rath S, Pieper DH, Schlüter D. Diversity of bacteria exhibiting bile acid-inducible 7α-dehydroxylation genes in the human gut. *Comput Struct Biotechnol J* (2019) 17:1016–9. doi: 10.1016/j.csbj.2019.07.012

110. Zou J, Secombes CJ. The function of fish cytokines. *Biology* (2016) 5:23. doi: 10.3390/biology5020023

111. Pathak M, Lal G. The regulatory function of $CCR9^+$ dendritic cells in inflammation and autoimmunity. *Front Immunol* (2020) 11:536326. doi: 10.3389/fimmu.2020.536326

112. Van Heel DA. Interleukin 15: its role in intestinal inflammation. *Gut* (2006) 55:444–5. doi: 10.1136/gut.2005.079335

113. Baghdadi M, Umeyama Y, Hama N, Kobayashi T, Han N, Wada H, et al. Interleukin-34, a comprehensive review. *J Leukoc Biol* (2018) 104:931–51. doi: 10.1002/JLB.MR1117-457R

114. Beck BH, Peatman E. *Mucosal health in aquaculture. 1st ed*. Cambridge, MA: Academic Press (2015).

115. Kato M, Khan S, d'Aniello E, McDonald KJ, Hart DN. The novel endocytic and phagocytic C-Type lectin receptor DCL-1/CD302 on macrophages is colocalized with F-actin, suggesting a role in cell adhesion and migration. *J Immunol* (2007) 179:6052–63. doi: 10.4049/jimmunol.179.9.6052

116. Chen SX, Ma HL, Shi YH, Li MY, Chen J. Molecular and functional characterization of a novel CD302 gene from ayu (*Plecoglossus altivelis*). *Fish Shellfish Immunol* (2016) 55:140–8. doi: 10.1016/j.fsi.2016.05.022

117. Zhang Z, Li Q, Huang Y, Jiang B, Li X, Huang M, et al. Molecular characterization of a novel C-type lectin receptors (CD302) in Nile tilapia (*Oreochromis niloticus*) and its functional analysis in host defense against bacterial infection. *Aquac Rep* (2022) 27:101405. doi: 10.1016/j.aqrep.2022.101405

118. Jungbauer A, Medjakovic S. Anti-inflammatory properties of culinary herbs and spices that ameliorate the effects of metabolic syndrome. *Maturitas* (2012) 71:227–39. doi: 10.1016/j.maturitas.2011.12.009

119. Faikoh EN, Hong YH, Hu SY. Liposome-encapsulated cinnamaldehyde enhances zebrafish (*Danio rerio*) immunity and survival when challenged with *Vibrio vulnificus* and *Streptococcus agalactiae*. *Fish Shellfish Immunol* (2014) 38:15–24. doi: 10.1016/j.fsi.2014.02.024

120. Shamsaie Mehrjan M, Hosseini Shekarabi SP, Mahjob Zardest M, Mohammadi N. Effect of dietary supplementation of red chili pepper (*Capsicum annuum*) powder on growth parameters, survival rate, hematological indices and some immunity responses of rainbow trout (*Oncorhynchus mykiss*) fry. *ISFJ* (2020) 29:131–40.

121. Firouzbakhsh F, Haghparast S, Memarzadeh MR. Study on the effects of red pepper (*Capsicum annuum*) extract on immune responses and resistance of rainbow trout (*Oncorhynchus mykiss*) juveniles against *Yersinia ruckeri*. *Iran J Fish Sci* (2021) 20:1573–88.

122. Iatropoulos MJ, Williams GM. Proliferation markers. *Exp Toxicol Pathol* (1996) 48:175–81. doi: 10.1016/S0940-2993(96)80039-X

123. Raschperger E, Thyberg J, Pettersson S, Philipson L, Fuxe J, Pettersson RF. The coxsackie-and adenovirus receptor (CAR) is an in *vivo* marker for epithelial tight junctions, with a potential role in regulating permeability and tissue homeostasis. *Exp Cell Res* (2006) 312:1566–80. doi: 10.1016/j.yexcr.2006.01.025

124. Serna-Duque JA, Esteban MÁ. Effects of inflammation and/or infection on the neuroendocrine control of fish intestinal motility: A review. *Fish Shellfish Immunol* (2020) 103:342–56. doi: 10.1016/j.fsi.2020.05.018

125. Tan P, Dong X, Mai K, Xu W, Ai Q. Vegetable oil induced inflammatory response by altering TLR-NF-κB signalling, macrophages infiltration and polarization in adipose tissue of large yellow croaker (*Larimichthys crocea*). *Fish Shellfish Immunol* (2016) 59:398–405. doi: 10.1016/j.fsi.2016.11.009

126. Jin M, Pan T, Cheng X, Zhu TT, Sun P, Zhou F, et al. Effects of supplemental dietary L-carnitine and bile acids on growth performance, antioxidant and immune ability, histopathological changes and inflammatory response in juvenile black seabream (*Acanthopagrus schlegelii*) fed high-fat diet. *Aquaculture* (2019) 504:199–209. doi: 10.1016/j.aquaculture.2019.01.063

127. Kim MH, Park SJ, Kim JH, Seong JB, Kim KM, Woo HA, et al. Peroxiredoxin 5 regulates adipogenesis-attenuating oxidative stress in obese mouse models induced by a high-fat diet. *Free Radic Biol Med* (2018) 123:27–38. doi: 10.1016/j.freeradbiomed.2018.05.061

128. Kim MH, Seong JB, Huh JW, Bae YC, Lee HS, Lee DS. Peroxiredoxin 5 ameliorates obesity-induced non-alcoholic fatty liver disease through the regulation of oxidative stress and AMP-activated protein kinase signaling. *Redox Biol* (2020) 28:101315. doi: 10.1016/j.redox.2019.101315

129. Gajda AM, Storch J. Enterocyte fatty acid-binding proteins (FABPs): different functions of liver and intestinal FABPs in the intestine. *Prostaglandins Leukot Essent Fatty Acids* (2015) 93:9–16. doi: 10.1016/j.plefa.2014.10.001

CHAPTER 4

Supplementation of gilthead seabream (*Sparus aurata*) diets with spices as a functional strategy to control excess adiposity through lipid, cholesterol and bile acid metabolism, and to induce an immunomodulatory intestinal regulation

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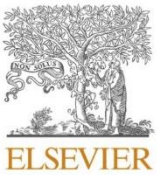

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Supplementation of gilthead seabream (*Sparus aurata*) diets with spices as a functional strategy to control excess adiposity through lipid, cholesterol and bile acid metabolism, and to induce an immunomodulatory intestinal regulation

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ABSTRACT

Reductions in fish oil content in aquafeeds can generate physiological and immunological disorders in farmed fish, associated to low dietary levels of n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFAs) and increased levels of n-6 PUFAs. Based on mammalian literature, spices could potentially counteract some of these negative effects. To explore this hypothesis, this work intended to offer a holistic insight on the effect of supplementing a diet (44% crude protein, 18% crude fat, 21.4 MJ kg^{-1} gross energy) containing low (3%) fish oil and poultry fat as the main oil source (43% of added oils) with 0.2% of an encapsulated combination of spice oleoresins (SO) including turmeric, capsicum, black pepper, and ginger in gilthead seabream (*Sparus aurata*, 44.1 ± 4.1 g). At the end of the trial (90 days), fish fed with the SO diet displayed lower levels of perivisceral and hepatic fat, as well as reduced accumulation of lipid deposits, assessed histologically, in liver and intestine. These findings were consistent with an increased activity of bile salt-activated lipase in the anterior intestine and the regulation of gene expression (qPCR) of several biomarkers related to lipid metabolism, including several key metabolic sensors and nuclear factors (*hnf4a* and *srebp2* at 2 h after feeding and *lxrα*, *fxr*, *pparγ*, *pparβ*, *sirt1* in 48 h-fasted fish) that coordinate the expression of a network of genes (many of them significantly affected herein) regulating lipid, cholesterol and bile acid homeostasis. Moreover, the combination of SO had an immunomodulatory effect in the intestine which reached its climax in 48 h fasted-fish with the down-regulation of the pro-inflammatory cytokines *tnf-α*, *il-12β*, *il-15*, *il-34* and cell markers *cd4–1*, *cd8b*, and *ccr11*, suggesting a potential anti-inflammatory response. No major effects were noted on fish growth and feed performance, on the oxidative status of the fish (although there were some subtle indications of reduced hepatic ROS levels). When assessing the gut microbial communities of gilthead seabream (16S rRNA gene sequencing on Illumina MiSeq) there were no differences in alpha and beta diversity between both experimental diets in the anterior and posterior intestines, except for an increase in the relative abundance of *Bacteroides*, *Desulfovibrio*, *Candidatus* Arthromitus, and *Ralstonia*. In conclusion, supplementation of diets with the tested combination of SO may be an effective strategy to prevent lipid accumulation and inflammation in fish fed diets containing reduced fish oil levels.

1. Introduction

The incorporation of fish oil (FO) into aquafeeds has made aquaculture the largest consumer sector of this ingredient (*ca.* 73% of FO production) (FAO, 2022). Topping the second position is direct human consumption of FO (16%) due to its increasing use for nutraceutical

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applications (Shepherd and Bachis, 2014). The importance of FO in the aquafeed industry lies in the fact that it is the main source of omega 3 long-chain polyunsaturated fatty acids (n-3 LC-PUFAs). Indeed, incorporation of n-3 PUFAs into feeds does not only improve the health of farmed fish, but also its quality and the health benefits for the consumer (Tocher, 2015). There are different mechanisms of action of LC-PUFAs, derived from the production of eicosanoids which are involved in extracellular actions at the level of tissue, to their direct action as intracellular ligands of transcription factors which regulate the expression of a multitude of gene pathways (Tocher, 2015). Among others, the expression of farnesoid X receptor (*fxr*), liver X receptor α (*lxrα*), sterol regulatory element-binding protein-1c (*srebp-1c*), and the family of peroxisome proliferator-activated receptors (*ppar*'s) have been shown to be activated by LC-PUFAs (Sampath and Ntambi, 2005; Zhao et al., 2004). Fish studies have demonstrated that n-3 LC-PUFAs are able to activate β-oxidation, modulate fat digestion and mobilization, and inhibit lipogenesis and *de novo* fatty acid synthesis (Turchini et al., 2009). Furthermore, LC-PUFAs can also act as an energy reservoir and have key structural roles as constituents of phospholipids in cellular membranes (Tocher, 2015).

On the other hand, the limited availability and insufficient production of FO have driven up the economic value of this ingredient in recent decades (Naylor et al., 2021). Thus, the rapid globalization and intensification of the aquaculture sector urgently require a reduction in FO usage, which have forced the aquafeed sector to look for other economically viable and environmentally sustainable ingredients. Some of the most tested conventional ingredients have been plant-based oils. However, many of them, such as soybean, corn, safflower, cottonseed, and sunflower oils, have much lower levels of n-3 PUFAs and higher levels of n-6 PUFAs than fish oil, unbalancing the n-6/n-3 ratio (Brown and Hart, 2010). Terrestrial animal rendered oils contain less n-6 LC-PUFAs than plant-based oils, causing a lower n-6/n-3 ratio imbalance (Moretti and Corino, 2008). Moreover, terrestrial animal fats, and some plant-based oils (*i.e.*, palm oil, palm kernel oil), have a higher content of saturated fatty acids (SFAs) and/or monounsaturated fatty acids (MUFAs) (Turchini et al., 2009). In this regard, the preferential β-oxidation of SFAs, followed by MUFAs for energy production, has been shown to spare n-3 LC-PUFAs in fish, which is a promising strategy to partly compensate for the reduced levels of n-3 LC-PUFAs of the mentioned ingredients (Rombenso et al., 2022), helping to better conserve the DHA and EPA supplied by the reduced FO levels in fish diets. Consequently, despite generally not having detrimental effects on growth performance, FO reduction has been reported to induce lipid deposition and a pro-inflammatory status in the fish digestive organs (Tan et al., 2016; Torstensen and Tocher, 2010), which can impair intestinal motility and deregulate digestion and absorption of nutrients (Serna-Duque and Esteban, 2020); as well as decreasing non-specific immunity and disease resistance (Tan et al., 2016). Likewise, the decrease in the levels of n-3 LC-PUFAs caused by FO replacement in seafood also compromises consumers' health (Rosenlund et al., 2010). Thus, the blue food sector is at a stage of searching for new nutritional strategies aimed at the judicious use of FO while meeting the nutritional needs of the animal and the consumer.

Spices are vegetal compounds which are traditionally utilized for culinary purposes but, thanks to their wide range of functionalities, can also be used for many therapeutic and prophylactic applications (*e.g.*, prevention and/or reduction of carcinogenesis, diabetes, and obesity) and are considered powerful nutraceuticals (Platel and Srinivasan, 2004; Srinivasan, 2005; Westerterp-Plantenga et al., 2006). In mammalians, many studies have verified the digestive, hypocholesterolemic, and hypolipidemic effects of many spices, accelerating metabolic rate and fat oxidation, as well as their antioxidant, antimicrobial and anti-inflammatory properties (Platel and Srinivasan, 2004; Srinivasan, 2005; Westerterp-Plantenga et al., 2006). One of the mechanisms of the regulation of lipid metabolism and body adiposity involves the stimulation of hepatic production of bile acids (BAs) and their secretion into the bile, promoting a better lipid digestion (Platel and Srinivasan, 2004). A major function of BAs is to allow fat emulsification and the formation of micelles that provide a higher action surface for lipases, as well as being necessary for the functioning of the bile salt-activated lipase (Romano et al., 2020). The secretion of BAs depends not only on the rate of BA synthesis, but also on the reabsorption and/or flow rate of BAs, which in mammalian vertebrates have been noted to increase after the ingestion of several spices (Platel and Srinivasan, 2004). Therefore, microencapsulated spices are promising phytogenics to help prevent the above-mentioned physiological and metabolic disorders and to enhance the general health status of farmed fish (Huyben et al., 2021; Pelusio et al., 2020). The present study aims to assess the effects of supplementing a reduced FO diet with a product based on an encapsulated combination of turmeric, capsicum, black pepper, and ginger oleoresins in fish performance, nutrient digestibility, body adiposity, metabolism, antioxidant capacity, and intestinal health in terms of immunity and gut microbial communities. Gilthead seabream (*Sparus aurata*) was chosen as a model fish for this study due to its importance in the Mediterranean fish farming.

2. Material and methods

2.1. Animal ethics

All procedures involving fish manipulation and tissue sampling complied with the Spanish (law 32/2007 and Royal Decree 1201/2015) and ongoing European legislation (EU2010/63), and were authorized by the Ethical Committee of the Institute of Agrifood Research and Technology (Spain) and the Generalitat of Catalunya (CEEA 219/2020).

2.2. Animals and husbandry conditions

Juveniles of gilthead seabream (initial body weight, BW = 44.1 ± 4.1 g; mean ± standard deviation, SD) were purchased from a commercial fish farm (Piscicultura Marina Mediterranea SL, Andromeda Group, Valencia, Spain) and transported to the facilities of the Institute of Agrifood Research and Technology (IRTA) in La Ràpita (Tarragona, Spain). After an acclimation period of two weeks, fish were randomly distributed in eight tanks of 450 L (30 fish per tank; initial density = 3 kg m^{-3}) connected to an IRTAmar® water recirculation system. This assay took place under natural photoperiod, with constant temperature (22.5 ± 0.5 °C), dissolved oxygen (6.3 ± 0.2 mg L^{-1}) and pH (7.6 ± 0.01), which were measured daily. Salinity (36‰), nitrite (0.16 ± 0.1 mg NO_2^- L^{-1}) and ammonia (0.22 ± 0.08 mg NH_4^+ L^{-1}) levels were monitored every week.

2.3. Diets and feeding trial

Two experimental diets (Table 1; 3 mm pellet size) were manufactured by Sparos Lda. (Portugal) to be isonitrogenous (44% crude protein), isolipidic (18% crude fat) and isoenergetic (21.4 MJ kg^{-1}) as follows. All powder ingredients, including the encapsulated combination of spice oleoresins when added, were mixed in a double-helix mixer (TGC Extrusion model 500 L, France) and ground (<400 mm) in a micropulverizer hammer mill (Hosokawa-Alpine model SH1, Germany). Diets were manufactured with a twin-screw extruder (Clextral model BC45, France) with a screw diameter of 55.5 mm. Extrusion conditions were: feeder rate (80–85 kg/h), screw speed (247–266 rpm), water addition in barrel 1 (345 mL/min), temperature in barrel 1 (32–34 °C), temperature in barrel 2 (59–62 °C) and temperature in barrel 3 (111–114 °C). Extruded pellets were dried in a vibrating fluid bed dryer (TGC Extrusion model DR100, France). After cooling, oils were added by vacuum coating (Dinnissen model PG-10VCLAB, Netherlands). Coating conditions were: pressure (700 mbar), spraying time under vacuum (approximately 90 s), and return to atmospheric pressure (120 s). The first diet was used as the control diet and was based on a standard

Table 1
Ingredient formulation, proximate and fatty acid composition of the experimental diets: a control and a basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO).

Ingredients (%)	Control	SO
Fishmeal Super Prime	7.50	7.50
Fishmeal 60 (62% CP)	5.00	5.00
Fish protein concentrate	2.00	2.00
Feathermeal hydrolysate	5.00	5.00
Porcine blood meal	3.00	3.00
Poultry meal	15.00	15.00
Aminopro NT70 - *C. glutamicum*	4.00	4.00
Corn gluten meal	8.00	8.00
Soybean meal 48 (47% CP)	12.00	12.00
Sunflower meal	5.00	5.00
Wheat meal	10.31	10.31
Whole peas	5.00	5.00
Pea starch (raw)	2.40	2.40
Fish oil	3.02	3.02
Soybean oil	2.35	2.35
Poultry fat	8.04	8.04
Vitamin and mineral premix*	1.00	1.00
Vitamin C35	0.05	0.05
Vitamin E50	0.02	0.02
Betaine HCl	0.20	0.20
Choline chloride 60	0.10	0.10
Antioxidant	0.20	0.20
Sodium propionate	0.10	0.10
Monoammonium phosphate	0.35	0.35
L-Tryptophan	0.15	0.15
DL-Methionine	0.20	0.20
Encapsulated spice oleoresins (Lucta)	–	0.20
Yttrium oxide	0.02	0.02
Proximate composition		
Moisture, %	7.25 ± 0.09	7.18 ± 0.05
Ash, %	6.90 ± 0.13	6.91 ± 0.05
Crude protein, %	44.14 ± 0.05	44.20 ± 0.07
Crude fat, %	18.10 ± 0.04	18.12 ± 0.03
Carbohydrates, %	23.60 ± 0.23	23.58 ± 0.14
Gross energy, MJ kg^{-1}	21.38 ± 1.11	21.45 ± 1.00
Fatty acid profile (% of total fatty acids)		
Saturated fatty acids (SFAs)	27.19 ± 0.40	27.53 ± 0.40
Monounsaturated fatty acids (MUFAs)	36.61 ± 0.73	36.08 ± 0.56
n-6 Polyunsaturated fatty acids (n-6 PUFAs)	26.65 ± 0.06	26.71 ± 0.30
n-3 Polyunsaturated fatty acids (n-3 PUFAs)	9.55 ± 0.39	9.68 ± 0.19
Total PUFAs	36.20 ± 0.45	36.38 ± 0.49

*PREMIX Lda, Portugal. Vitamins (IU or mg * kg $diet^{-1}$): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg * kg $diet^{-1}$): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings.
The complete fatty acid profile of the experimental diets is detailed in Supplementary Table S1. The proximate and fatty acid composition of diets were analysed in duplicate; values are represented as mean ± standard deviation (SD).

commercial seabream feed formulation, but included a higher content of poultry fat while lowering the levels of FO and soybean oil. This ingredient formulation was chosen to maintain relatively high dietary levels of SFAs (27% of total fatty acids; Table 1; Supplementary Table S1) and to control the rise in n-6 PUFA. The experimental diet had the same basal formulation but was supplemented with 0.2% of an encapsulated combination of spice oleoresins (SO), including turmeric, capsicum, black pepper, and ginger (Lucta S. A., Spain; Patent Number WO/2022/117810). The purpose of the encapsulation is to allow the manipulation of the spices during feed preparation, as the pungent substances can be irritable when handled in high concentration but, once included in the feed, the active ingredients are released from the encapsulating fat matrix during the extrusion process. Yttrium oxide (Y_2O_3, Sigma Aldrich, Spain) was added to the diets (0.2 g kg^{-1}) as an inert marker to determine the apparent digestibility coefficients (ADCs) of macronutrients.

At the beginning of the trial, four tanks were randomly assigned to each dietary treatment. Diets were distributed twice a day in 12 meals spread over an hour (one every five minutes) with automatic feeders (Arvo-Tec T Drum 2000, Finland) for 90 days. Once a month, fish were anesthetized with buffered tricaine methanesulfonate (MS-222, Sigma-Aldrich, Spain; 100 mg L^{-1}) for monitoring growth in terms of BW and standard length (SL). Each day, uneaten pellets were recovered from the bottom of the tank, dried (100 °C) and weighed to guarantee that feed was offered in excess and to calculate the daily feed intake. For assessing ADCs of lipids and proteins, faeces were collected by means of a sedimentation column 10–12 h after removal of uneaten feed during three consecutive days.

2.4. Fish performance parameters

After the 90 days-feeding trial, fish were fasted for 48 h, anesthetized (100 mg L^{-1} MS-222) and their final body weight (BW_f) and standard length (SL_f) were individually measured. The following growth and feed performance indicators were calculated:

$$\text{Specific growth rate (SGR; \%day}^{-1}) = 100 \times ln\left[\text{BW}_f\ (\text{g}) - ln\ \text{BW}_i\ (\text{g})\right]/\text{time (days)}.$$

$$\text{Thermal growth coefficient (TGC)} = 1000 \times \left(\text{BW}_f\ (\text{g})^{1/3} - \text{BW}_i\ (\text{g})^{1/3}\right)/(\text{Temperature (°C)} \times \text{day}).$$

$$\text{Fulton's condition factor (K)} = 100 \times \text{BW}_f\ (\text{g})/\text{SL}_f\ (\text{g})^3.$$

$$\text{Feed intake (FI, g fish}^{-1}) = \text{total feed intake per tank (g)}/\text{number of fish per tank (g)}.$$

$$\text{Feed conversion ratio (FCR)} = \text{total feed intake per tank (g)}/\text{fish biomass increase per tank (g)}.$$

Then, six fish per tank (24 per dietary treatment) were netted and euthanized with an overdose of MS-222 (300 mg L^{-1}) and the liver and perivisceral fat were gently extracted and individually weighed for calculating the following somatic indices:

$$\text{Perivisceral fat index (PVFI; \%)} = \text{perivisceral fat weight (g)}/\text{BW}_f\ (\text{g}).$$

$$\text{Hepatosomatic index (HSI; \%)} = \text{liver weight (g)}/\text{BW}_f\ (\text{g}).$$

2.5. Tissue sampling

At the end of the trial, eight fish from each tank (32 per treatment) were euthanized (including those sacrificed for calculating the PVFI and the HSI). Then, the fillet and the liver of three fish per tank were dissected and frozen at −20 °C for proximate and fatty acid composition analyses. A piece of the liver (*ca.* 1.5–2 cm^2) and a piece of anterior intestine (AI; 4 cm) from three individuals per tank (12 fish per diet) were fixed in 10% neutral buffered formalin (pH = 7.2) and stored at 4 °C until further histological analysis. The AI was selected because the digestion and absorption rate of fat is higher in this region (Bakke et al., 2010). The rest of the liver was divided in pieces and stored at −80 °C for assessing hepatic antioxidant stress and metabolic biomarkers (4 replicates per diet). For studying the bile acid (BA) profile, the walls of the gallbladders of four fish per tank were ruptured with a blunt scalpel, and the content of all fish from the same tank were pooled together (4 replicates per diet) and stored at −80 °C. A piece of the liver and a piece of

AI (*ca.* 1 and 2 cm^2 respectively) of two specimens per tank (8 per dietary treatment) were separately immersed in 5 volumes of RNAlater (Sigma-Aldrich, USA), incubated at 4 °C overnight and frozen at −80 °C for further transcriptomic analysis. A section of AI (*ca.* 4 cm long-cut from the pyloric caeca) and posterior intestine (PI; *ca.* 4 cm long-cut from the anus backwards) from three fish per tank (12 per treatment) were dissected and opened lengthwise. The mucosal content was gently scraped with a round edge spatula and immediately frozen at −80 °C for further microbial analysis. The described tissues were collected from fish that had been fasted for 48 h (Naya-Català et al., 2021), in order to ensure sample stability and avoid contamination by allochthonous bacteria.

To restore the non-fasting physiology of fish, the remaining fish were fed for three days, and then, two hours after the last feeding, ten fish per tank (40 per treatment) were randomly selected and euthanized (300 mg L^{-1}, MS-222). The digestive tract of four fish per tank was divided in two regions: i) the stomach and pyloric caeca, and ii) the AI, and stored at −80 °C for measuring the activity of pancreatic digestive enzymes. To assay the BA profile of the AI in 2 h postprandial-fish, the luminal content of four fish per tank was stripped with tweezers into one tube (4 replicates per diet) and immediately frozen at −80 °C. A piece of the liver and of AI from two fish per tank (8 per dietary treatment) were dissected and conserved as previously described for studying the temporal effect of the diet on hepatic and intestinal gene expression. Before euthanasia, four fish from each tank (16 fish per diet) were anesthetized with MS-222 (100 mg L^{-1}) and *ca.* 1 mL of blood was extracted from the caudal vein with heparinized syringes and centrifugated (1600 ×*g*, 10 min, 4 °C) for collection of plasma, which was frozen at −80 °C.

2.6. Proximate composition, fatty acid profile, and macronutrient ADCs

A pool of three liver pieces per tank were homogenized together (IKA T25 digital ULTRA-TURRAX, IKA Works, USA) and processed, whereas the three fillets per tank were individually processed. The total content of protein, lipids, carbohydrates and ash, and the fatty acid profile of livers and fillets were determined as described in the work of Ruiz et al. (2023a). In brief, crude fat was quantified gravimetrically after extraction in chloroform/methanol (2:1) and evaporation of the solvent under a stream of nitrogen followed by vacuum desiccation overnight (Folch et al., 1957). Then, transmethylated fatty acids from total lipids were extracted, purified, and finally quantified by gas-liquid chromatography on a Thermo Trace GC (Thermo Fisher, Spain) coupled to a TRACE™ TR-FAME GC Column (Thermo Scientific, Spain), using heneicosylic acid (21:0) as internal standard (ref. H5,149, Sigma-Aldrich, Spain) (Skalli et al., 2020). Crude protein and carbohydrate contents were determined following the methodology described by Dumas (1826) and DuBois et al. (1956), respectively. Ash contents were determined by keeping the sample at 500 to 600 °C for 24 h in a muffle furnace (Association of Official Analytical Chemists (AOAC), 1990) and water content was estimated by sample drying at 120 °C for 24 h. All chemical analyses were performed in triplicate.

The nutritional quality of the lipid fraction from fillets, based on the percentages of saturated fatty acids (SFAs; lauric C12:0, myristic C14:0, palmitic C16:0, and stearic C18:0 acids), monounsaturated fatty acids (MUFAs; oleic C18:1 n-9 acid), and polyunsaturated fatty acids (PUFAs; gamma-linolenic C18:3 n-6, alpha-linolenic C18:3 n-3, eicosapentaenoic C20:5 n-3, and docosahexaenoic C22:6 n-3 acids), was estimated with the following indices described in Chen and Liu (2020):

$$\text{Index of atherogenicity (IA)} = [(C12{:}0 + (4 \times C14{:}0) + C16{:}0)] / (\Sigma\text{MUFAs} + \Sigma n-6\text{PUFAs} + \Sigma n-3\text{PUFAs}).$$

$$\text{Index of thrombogenicity (IT)} = (C14{:}0 + C16{:}0 + C18{:}0) / [(0.5 \times \Sigma\text{MUFAs}) + (0.5 \times \Sigma n-6\text{ PUFAs}) + (3 \times \Sigma n-3\text{ PUFAs}) + (\Sigma n-3\text{ PUFAs} / \Sigma n-6\text{ PUFAs})].$$

$$\text{Hypocholesterolemic/hypercholesterolemic fatty acids ratio (h/H)} = (C18{:}1n-9 + C18{:}3n-6 + C18{:}3n-3 + C20{:}5n-3 + C22{:}6n-3) / (C12{:}0 + C14{:}0 + C16{:}0).$$

The concentration of Y_2O_3 in the diets and faeces was determined with an Agilent 7700 ICP-MS (Agilent Technologies, USA) in order to assess protein and lipid ADCs (Cheng and Hardy, 2002):

$$\text{ADC of nutrient (\%)} = 100 \times [1 - (\%Y_2O_3 \text{ in diet} / \%Y_2O_3 \text{ in faeces}) \times (\%\text{nutrient in faeces} / \%\text{nutrient in diet})].$$

2.7. Histological analyses

Small segments of the fixed liver and AI (*ca.* 0.5–1 cm^2) were dehydrated in ethanol solutions of graded concentrations, cleared with xylene, and embedded in paraffin. Samples were sectioned (4 μm) by means of a microtome (Leica RM2155, Leica Microsystems, Germany), stained with hematoxylin and eosin, and dried sections were examined under light microscopy (Leica DM LB, Leica Microsystems). Inflammation and accumulation of fat deposits in both tissues were semi-quantitatively evaluated from 1 to 5 following the classification described by Ruiz et al. (2023a) under blinded conditions by two different observers (Meyerholz and Beck, 2018).

2.8. Oxidative stress and hepatic metabolism biomarkers

Homogenates of three liver pieces (*ca.* 60 mg each) per tank were resuspended in 5 volumes *v/w* of buffer (150 mM KCl, 1 mM EDTA, pH 7.4) and after centrifugation (9000 ×*g*, 30 min, 4 °C), supernatants were collected. The enzymatic activities of catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD) were measured according to Aebi (1974), Carlberg and Mannervik (1975), and McCord and Fridovich (1969). A commercial kit (ref. 19,160, Sigma-Aldrich, USA) was used to determine SOD activity, at λ = 240 nm, with H_2O_2 (50 mM) as substrate. Thiobarbituric acid-reactive substances (TBARs) were used as an indicator of lipid peroxidation (LPO) levels (Fernández-Alacid et al., 2021). Total antioxidant capacity (TAC) was measured following the manufacturer's instructions of a commercial kit (ref. MAK187, Sigma-Aldrich, USA). All oxidative stress biomarkers were normalized to soluble protein content (Bradford, 1976), except for SOD activity, which was expressed as % of enzyme inhibition.

To evaluate hepatic metabolism, pools of three pieces of liver of 100 mg per tank were homogenized in 9 volumes of a buffer (pH = 7.6) composed of 1:1 *v/v* of a lysis solution (1.24 mM Triton X-100, 1 mM EDTA, 1 mM $NaHCO_3$) and a stabilizer solution (3.7 mM EDTA, 5 mM β-mercaptoethanol). Then, homogenates were centrifuged (5000 ×*g*, 10 min, 4 °C) and supernatants collected. Lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) activities were quantified with commercial kits (ref. 41,222, ref. 41,222, ref.

41,272; Spinreact, Spain) based on the methodology of Bergmeyer and Bernt (1974a, 1974b, 1974c).

All described measurements were run in triplicate at 25 °C by UV/Vis spectrophotometry (Infinite M200 Plate Reader, Tecan Switzerland) and analysed with the Magellan™ software (v6, Tecan).

2.9. Plasmatic biochemical parameters

Cholesterol, triacylglycerides (TAGs), albumin, total proteins, AST, ALT, and alkaline phosphatase (ALP) in plasma were measured using an UV/Vis spectrophotometer (Laboratorio Echevarne, Barcelona, Spain). Total globulin in plasma was calculated as the subtraction between total protein and albumin.

2.10. Pancreatic digestive enzymes

Four samples of stomach and pyloric caeca, and of AI were separately homogenized in 5 volumes *v/w* of ice-cold distilled water, centrifuged (3300 ×*g*, 3 min, 4 °C), and supernatants were collected, aliquoted and frozen at −80 °C (Gisbert et al., 2009). The activities of the main pancreatic digestive enzymes (α-amylase, total alkaline proteases, and bile salt-activated lipase) were assayed as described by Solovyev and Gisbert (2016) and samples were handled as described by the former authors to prevent their degradation. The activity of all enzymes was determined at 25 °C in triplicate per sample using a spectrophotometer (Infinite M200 Plate Reader, Tecan Switzerland).

2.11. Bile acid profile

Bile acid quantification was performed following the procedures described by Herrero-Encinas et al. (2020) with modifications. Gallbladder bile samples were first diluted 1/2000 in water and 100 μL of the water-diluted bile extracted with 400 μL of acetonitrile (ACN) containing internal standard (chenodeoxycholic acid-d_4, CDCA-d4). After vortexing, centrifugated supernatants were diluted 1/10 in H_2O:ACN (1:1 *v/v*) and directly injected in the liquid chromatograph-mass spectrometer (LC-MS). Intestinal digesta samples were firstly lyophilized and homogenized on a TissueLyzer II (QIAGEN, Germany). Then, 20 mg of homogenate were extracted with 800 μL of H_2O:ACN (1:1 *v/v*) including the internal standard for 15 min, centrifuged (15,000 ×*g*, 10 min, 4 °C) and the supernatants diluted again in H_2O:ACN (1/1000). Ultra-performance liquid chromatography-mass spectrometry (ACQUITY UPLC I-Class connected to Xevo-G2 QTof MS, Waters Corp.) was carried out, using response comparison against calibration curves generated with BA standards for BA quantification, with the aid of the QuanLynx v4.2 software for operations and quantification (Waters Corp.; USA).

2.12. Extraction of DNA and raw sequence processing

Intestinal DNA was extracted from *ca.* 250 mg of the scraped intestinal samples with the DNeasy PowerSoil Pro Kit (ref. 47,016, QIAGEN, Germany). The protocol followed for this kit contains a step for sample homogenization and chemical and mechanical cell lysis through bead-beating. Amplification for library generation of the region V3-V4 of the 16S rRNA gene was carried out according to the 16S Metagenomic Sequencing Library Preparation guide (Illumina, 2013). An initial amplification step was performed using the specific 341F/805R primers (5'-CCTACGGGNGGCWGCAG-3′, 5'-GACTACHVGGGTATCTAATCC-3′) and overhang adapters attached, with 12.5 ng DNA as initial input. The PCR programme consisted of an initial step of DNA denaturation (95 °C, 30 min), followed by 25 cycles of 30 s at 95 °C, 30s at 55 °C and 30 s at 72 °C, and finally 5 more minutes at 72 °C to complete elongation. Then, a second PCR with the same programme but only 8 cycles was used to add Illumina sequencing adapters and dual-index barcodes. After each PCR, templates were clean-up with Ampure XP beads (Beckman Coulter, USA) and to verify the size of the final library product, 1 μL of a 1:50 dilution was run on a Bioanalyzer DNA 1000 Chip (Agilent Technologies, USA). Then, amplified DNA from all samples was pooled at equimolar concentrations and sequenced on a Illumina MiSeq platform (MiSeq System, Illumina, USA) with 2 × 300 bp paired-end runs. Two "mock" samples with a known bacterial composition were amplified and sequenced as positive controls, while no-template PCRs were also included and sequenced as negative controls.

Data analysis was carried out with a workflow based on the R package *dada2* (v1.16; Callahan et al., 2016). In brief, all reads with a Phred quality score < 28 or with an expected error > 2 were excluded from the analysis. After merging of paired-end reads, the sequences with an overlap length < 12 nucleotides, with more than 0 mismatches, or identified as chimeras, were also removed. Finally, the SILVA database (v138.1) was used as a reference library for bacterial taxonomy classification of amplicon sequence variants (ASVs), and those ASVs with a bootstrapping confidence <80% were classified as unassigned (Smith et al., 2020). The ASVs which were classified as mitochondria, chloroplasts, or classified within a kingdom different than Bacteria were also discarded from the analysis. According to rarefaction curves (Supplementary Fig. S1), the number of reads per sample were rarefied to the minimum sample depth (53,085 reads) using the R package *vegan* (v2.6–4); and normalized by total sum scaling (McKnight et al., 2019). Raw sequencing data is available in the Sequence Read Archive (SRA) of NCBI under Bioproject accession number PRJNA915342 and PRJNA985270.

2.13. Analyses of gene expression biomarkers from the liver and anterior intestine

The TRI Reagent (Sigma-Aldrich, USA) and QIAGEN RNeasy Mini Kit (ref. 74,106, QIAGEN, Germany) were respectively used for extracting RNA from the liver and AI ($n = 8$ per experimental condition). An initial input of 500 ng of RNA were used for cDNA synthesis by means of the High-Capacity cDNA Archive Kit (Applied Biosystems, USA) using random decamers and 500 ng of total RNA in a final volume of 100 μl. Reverse transcription (RT) reactions were incubated for 10 min at 25 °C and for 2 h at 37 °C. Negative control reactions were run without the enzyme.

A panel of 44 genes for the liver (Table 2) and AI (Table 3) were simultaneously amplified by real-time quantitative PCR using specific primer sequences (Supplementary Table S2 and Supplementary Table S3) with a CFX96 Connect Real-Time PCR Detection System (Bio-Rad, USA) as described by Ruiz et al. (2023a). Briefly, diluted RT reactions (× 6) were used for qPCR assays in a 25-μl volume, in combination with SYBR Green Master Mix (Bio-Rad, Hercules, CA, United States) and specific primers at a final concentration of 0.9 μM. The PCR amplification program consisted of an initial step of DNA polymerase activation and cDNA denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 15 s at 95 °C, and an annealing/extension step of 60 s at 60 °C. To improve data reproducibility, all the pipetting operations were performed using an EpMotion 5070 Liquid Handling Robot (Eppendorf, Germany). The efficiency of PCRs (>92%) was checked, and the specificity of reactions was verified by analyzing the melting curves (ramping rates of 0.5 °C/10 s over a temperature range of 55–95 °C) and linearity of serial dilutions of RT reactions ($r^2 > 0.98$). Fluorescence data acquired during the extension phase were normalized with the delta-delta Ct method (Livak and Schmittgen, 2001), following standard calculation and normalization procedures (Benedito-Palos et al., 2014; Bermejo-Nogales et al., 2014; Pérez-Cordón et al., 2014). In brief, *beta-actin* was used as a housekeeping gene, after testing its expression stability (GeNorm software; M score = 0.21), and all values in the liver and AI were referenced to the expression levels of *grp-170* and *hes1-b* of fish fed the control diet respectively.

Table 2
PCR-array layout for gene expression profile of the liver in gilthead seabream (*Sparus aurata*) fed experimental diets.

Function	Gene	Symbol	GenBank
Fatty acids, cholesterol & phospholipid metabolism	Fatty acid synthase	*fasn*	JQ277708
	Elongation of very long chain fatty acids 1	*elovl1*	JX975700
	Elongation of very long chain fatty acids 4	*elovl4*	JX975701
	Elongation of very long chain fatty acids 5	*elovl5*	AY660879
	Elongation of very long chain fatty acids 6	*elovl6*	JX975702
	Fatty acid desaturase 2	*fads2*	AY055749
	Stearoyl-CoA desaturase 1a	*scd1a*	JQ277703
	Stearoyl-CoA desaturase 1b	*scd1b*	JQ277704
	Cholesterol 7-alpha-monooxygenase	*cyp7a1*	KX122017
	Phospholipid transfer protein	*pltp*	XM_030418561
	Adipose triglyceride lipase	*atgl*	JX975711
	Hepatic lipase	*hl*	EU254479
	Lipoprotein lipase	*lpl*	AY495672
Lipases	85 kDa calcium-independent phospholipase A2	*pla2g6*	JX975708
Transcription factors & nuclear receptors	Hepatocyte nuclear factor 4 alpha	*hnf4a*	FJ360721
	Sterol regulatory element-binding proteins 1	*srebp1*	JQ277709
	Sterol regulatory element-binding protein 2	*srebp2*	XM_030408996
	Farnesoid X receptor	*fxr*	XM_030426192
	Liver X receptor α	*lxrα*	FJ502320
	Peroxisome proliferator-activated receptor α	*pparα*	AY590299
	Peroxisome proliferator-activated receptor β	*pparβ*	AY590301
	Peroxisome proliferator-activated receptor γ	*pparγ*	AY590304
Oxidative metabolism & energy sensing	Carnitine palmitoyltransferase 1A	*cpt1a*	JQ308822
	Hydroxyacyl-CoA dehydrogenase	*hadh*	JQ308829
	Fatty acid translocase/CD36	*fat/cd36*	XM_030440140
	Fatty acid binding protein, heart	*h-fabp*	JQ308834
	Citrate synthase	*cs*	JX975229
	NADH-ubiquinone oxidoreductase chain 2	*nd2*	KC217558
	NADH-ubiquinone oxidoreductase chain 5	*nd5*	KC217559
	Cytochrome c oxidase subunit I	*coxi*	KC217652
	Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1α*	JX975264
	Sirtuin1	*sirt1*	KF018666
	Sirtuin2	*sirt2*	KF018667
	Catalase	*cat*	JQ308823
	Uncoupling protein 1	*ucp1*	FJ710211
	Glutathione peroxidase 1	*gpx1*	DQ524992
	Glutathione peroxidase 4	*gpx4*	AM977818
	Peroxiredoxin 3	*prdx3*	GQ252681
	Peroxiredoxin 5	*prdx5*	GQ252683
Antioxidant defense	Superoxide dismutase [Cu-Zn]	*cu-zn-sod / sod1*	JQ308832
	Superoxide dismutase [Mn]	*mn-sod / sod2*	JQ308833
	Glucose-regulated protein, 170 kDa	*grp-170*	JQ308821
	Glucose-regulated protein, 94 kDa	*grp-94*	JQ308820
	Glucose-regulated protein, 75 kDa	*grp-75*	DQ524993

Table 3
PCR-array layout for gene expression profile of the intestine in gilthead seabream (*Sparus aurata*) fed experimental diets.

Function	Gene	Symbol	GenBank
Epithelial integrity	Proliferating cell nuclear antigen	*pcna*	KF857335
	Transcription factor HES-1-B	*hes1-b*	KF857344
	Krüppel-like factor 4	*klf4*	KF857346
	Claudin-12	*cldn12*	KF861992
	Claudin-15	*cldn15*	KF861993
	Cadherin-1	*cdh1*	KF861995
	Cadherin-17	*cdh17*	KF861996
	Tight junction protein ZO-1	*tjp1*	KF861994
	Desmoplakin	*dsp*	KF861999
	Gap junction Cx32.2 protein	*cx32.2*	KF862000
	Coxsackievirus and adenovirus receptor homolog	*cxadr*	KF861998
Nutrient transport	Intestinal-type alkaline phosphatase	*alpi*	KF857309
	Liver type fatty acid-binding protein	*fabp1*	KF857311
	Intestinal fatty acid-binding protein	*fabp2*	KF857310
	Ileal fatty acid-binding protein	*fabp6*	KF857312
Mucus production	Mucin 2	*muc2*	JQ277710
	Mucin 13	*muc13*	JQ277713
Interleukins	Tumor necrosis factor-alpha	*tnf-α*	AJ413189
	Interleukin-1 beta	*il-1β*	AJ419178
	Interleukin-6	*il-6*	EU244588
	Interleukin-7	*il-7*	JX976618
	Interleukin-8	*il-8*	JX976619
	Interleukin-10	*il-10*	JX976621
	Interleukin-12 subunit beta	*il-12β*	JX976624
	Interleukin-15	*il-15*	JX976625
	Interleukin-34	*il-34*	JX976629
Cell markers	Cluster of differentiation 4–1	*cd4–1*	AM489485
	Cluster of differentiation 8 beta	*cd8b*	KX231275
	C-C chemokine receptor type 3	*ccr3*	KF857317
	C-C chemokine receptor type 9	*ccr9*	KF857318
	C-C chemokine receptor type 11	*ccr11*	KF857319
	C-C chemokine CK8 / C-C motif chemokine 20	*ck8/ ccl20*	GU181393
	Macrophage colony-stimulating factor 1 receptor 1	*csf1r1*	AM050293
Ig production	Immunoglobulin M	*igm*	JQ811851
	Immunoglobulin T membrane-bound form	*igt-m*	KX599201
Pattern recognition receptors (PRRs)	Galectin-1	*lgals1*	KF862003
	Galectin-8	*lgals8*	KF862004
	Toll-like receptor 2	*tlr2*	KF857323
	Toll-like receptor 5	*tlr5*	KF857324
	Toll-like receptor 9	*tlr9*	AY751797
	CD209 antigen-like protein D	*cd209d*	KF857327
	CD302 antigen	*cd302*	KF857328
	Macrophage mannose receptor 1	*mrc1*	KF857326
	Fucolectin	*fcl*	KF857331

2.14. Statistical analyses

After verifying normal distribution (Shapiro-Wilk test) and homoscedasticity (Levene's test) of data, a Student's *t*-test was performed for testing significant differences between dietary groups ($P \leq 0.05$). When data was non-parametric, a Wilcoxon rank-sum test was performed. Regarding gene expression biomarkers, a two-way ANOVA was used for evaluating interaction between diet and feeding time (2 h postprandial or 48 h fasted). The *P* value was set to 0.05 to determine significant differences between dietary groups, whereas $P \leq 0.1$ was considered as a tendency for biomarker expressions (Ganesh and Cave, 2018). No multiple correction adjustment was used. Using the gene expression values that displayed a $P \leq 0.1$, a partial least squares-discriminant analysis (PLS-DA) was constructed with the software EZifno (v3.0, Umetrics, Sweden). Cluster separation was assessed by calculating Hotelling's T^2

statistic. Points with a T^2 above 95% confidence limit were considered as outliers and discarded. A good quality of the model was ensured through fitness [R2Y(*cum*)] and prediction ability [QY(*cum*)]. Each PLS-DA model was validated with a permutations-test in the R package *ropls* (v1.22.0), to guarantee that there was no over-fitting (Supplementary Fig. S2 and Supplementary Fig. S3).

Concerning gut microbiota, significant differences between dietary groups in alpha diversity metrics (indices of Chao1, ACE, Shannon, Simpson, and Faith's phylogenetic diversity) were determined by Wilcoxon test ($P \leq 0.05$). As a beta diversity index, the unweighted and weighted UniFrac distances were used to estimate similarities among samples considering the phylogenetic relationships of their ASVs (Lozupone et al., 2011). A permutational multivariate analysis of variance (PERMANOVA) was performed to check significant differences in beta diversity ($P \leq 0.05$). Differential abundances between groups in phyla and genera with a relative abundance ≥1.0% were calculated with the method *Metastats*, adjusting the *P* value by FDR (White et al., 2009). All the described statistics for gut microbiota data were executed with the R package *microeco* (Liu et al., 2021). For statistical analyses of gene expression and gut microbiota, the RStudio version 4.1.2 was used.

3. Results

3.1. Fish performance

At the end of the trial, somatic growth in terms of BW, SL, SGR and TGC, was not affected by the supplementation of SO into the basal diet (Table 4; $P > 0.05$). There were no differences in the values of Fulton's condition factor or HSI between dietary groups ($P > 0.05$). On the other hand, the perivisceral fat levels were reduced by 17% in fish fed the SO diet with respect to the control group ($P = 0.017$). Regarding feeding performance and apparent macronutrient digestibility, the supplementation of the diet did not alter the FI, FCR and ADCs of lipids and proteins ($P > 0.05$).

Table 4
Growth and feed performance indicators, somatic condition indices and apparent digestibility coefficients of macronutrients in gilthead seabream (*Sparus aurata*) fed the control and a basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO).

	Control	SO	*t*-value	*P*-value
Survival (%)	100.00 ± 0.00	100.0 ± 0.00	0.00	1.000
Growth performance				
BW_i (g)	44.05 ± 0.04	44.06 ± 0.04	−0.35	0.736
SL_i (cm)	12.04 ± 0.12	12.17 ± 0.08	−1.80	0.121
BW_f (g)	215.80 ± 1.06	213.84 ± 1.64	2.01	0.092
SL_f (cm)	19.32 ± 0.21	19.42 ± 0.23	−0.64	0.545
SGR (% day^{-1})	1.81 ± 0.01	1.80 ± 0.01	1.76	0.129
TGC	1.22 ± 0.01	1.21 ± 0.01	1.58	0.164
Somatic indexes				
K	3.00 ± 0.11	2.93 ± 0.14	0.79	0.462
HSI (%)	1.84 ± 0.11	1.83 ± 0.15	0.11	0.918
PVFI (%)	3.01 ± 0.28^{b}	2.50 ± 0.14^{a}	3.26	0.017
Feeding performance				
FI (g $fish^{-1}$)	195.89 ± 8.70	197.90 ± 4.33	−0.41	0.693
FCR	1.21 ± 0.05	1.24 ± 0.04	−0.94	0.385
Apparent digestibility coefficients (ADCs)				
Lipid ADC (%)	81.60 ± 0.96	78.77 ± 2.84	1.89	0.108
Protein ADC (%)	79.41 ± 3.60	77.35 ± 3.88	0.78	0.466

Values are represented as mean ± SD (n = 4 tanks per dietary group, considering a tank as the experimental unit) and differences between dietary groups ($P \leq 0.05$) are indicated by different superscript letters. Abbreviations: BW_i: initial body weight; SL_i: initial standard length; BW_f: final body weight; SL_f: final standard length; SGR: specific growth rate; TGC: thermal growth coefficient; K: Fulton's condition factor; HSI: hepatosomatic index; PVFI: perivisceral fat index; FI: feed intake; FCR: feed conversion ratio.

3.2. Proximate composition and fatty acid profile

When supplementing the basal diet with the combination of SO, there was a significant reduction in the levels of total crude lipids in the liver (Table 5; $P = 0.024$). No differences between experimental diets were found for crude protein, carbohydrate, or ash contents in the liver nor fillets ($P > 0.05$). The fatty acid profile of the liver (Table 6) and fillets (Table 7) of gilthead seabream was very conserved under both dietary treatments, even though there was a significant increase in the levels of stearic acid (C18:0) in the liver of fish fed the SO diet ($P = 0.027$). No differences were found for the content of EPA + DHA, DHA/EPA and n-6/n-3 ratios, nor for the values of IA, IT, and h/H among both dietary groups ($P > 0.05$).

3.3. Histomorphological analyses of the liver and anterior intestine

The organisation of the hepatic parenchyma was similar under both dietary treatments. In brief, hepatocytes were polyhedral, with varying degrees of vacuolization in their cytoplasm, and they were arranged in anastomosing plates separated by sinusoidal capillaries leading to central veins. No signs of inflammation nor infiltration of lymphocytes were observed between dietary groups. The number of individuals with high hepatic lipid accumulation (score 4) was lower in fish fed the SO diet compared to the control group (Fig. 1A and B).

The histological organisation of the mucosal layers from the AI was similar under both dietary treatments, with no signs of enteritis or inflammation. The regulatory effect of the SO diet in the accumulation of intestinal fat deposits was very apparent, as the number of individuals with no observable lipid accumulation (score 1) tripled that of the control group (Fig. 1C and D).

3.4. Hepatic metabolism, oxidative stress, and plasmatic biomarkers

Under the current experimental conditions, hepatic biomarkers of metabolism (LDH, AST, ALT) and oxidative stress (SOD, CAT, GR, LPO, TAC) were not altered by the SO diet (Table 8; $P > 0.05$). Similarly, there were no significant differences in plasmatic biomarkers related to metabolism of lipids, proteins, and amino acids between dietary groups (Table 8; $P > 0.05$).

3.5. Activity of pancreatic digestive enzymes

As shown in Table 9, there were no significant differences in the specific activities of total alkaline proteases, α-amylase, and bile salt-activated lipase between diets in the stomach and pyloric caeca samples ($P > 0.05$). On the other hand, the activity of bile salt-activated lipase in the AI increased in gilthead seabream fed the SO diet with respect to the control group ($P = 0.023$), while the activities of total alkaline proteases and α-amylase did not change ($P > 0.05$).

3.6. Composition of bile

The BA profiles of the gallbladder (48 h fasted-fish) and the AI (2 h postprandial-fish) are shown in Table 10. In both organs, two primary BAs in their tauro-conjugated form were detected: the taurocholic acid (T-CA) and the taurochenodeoxycholic acid (T-CDCA). In the gallbladder, similar levels of these BAs were found between dietary groups ($P > 0.05$). In the AI, no significant differences ($P > 0.05$) were displayed between treatments due to the large interindividual variability. However, the mean numerical values of T-CA and T-CDCA concentrations increased by 66.2 and 35.9% respectively, resulting in a total increase of 54.1% in the sum of BAs in the AI in fish fed the SO diet.

Table 5
Proximate composition (%) in dry mass of the liver and fillet of gilthead seabream (*Sparus aurata*) fed the control and a basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO).

	Liver				Fillet			
	Control	SO	*t*-value	*P*-value	Control	SO	*t*-value	*P*-value
Protein	21.72 ± 1.35	24.38 ± 3.79	−1.32	0.234	79.81 ± 0.78	80.45 ± 0.92	−1.06	0.329
Lipid	42.62 ± 3.12[b]	36.79 ± 2.25[a]	3.03	0.024	12.97 ± 1.75	11.08 ± 0.98	1.89	0.108
Carbohydrates	24.39 ± 2.47	25.36 ± 2.86	−0.51	0.626	0.98 ± 0.15	0.96 ± 0.16	0.18	0.861
Ash	2.70 ± 0.19	3.09 ± 0.90	−0.39	0.429	6.96 ± 0.42	7.29 ± 0.25	−1.35	0.226

Values are represented as mean ± SD (n = 4 tanks per dietary group, considering a tank as the experimental unit) and differences between dietary groups ($P \leq 0.05$) are indicated by different superscript letters.

Table 6
Fatty acid profile (mg × g lipid^{-1}) of the liver in gilthead seabream (*Sparus aurata*) fed the control and a basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO).

	Control	SO	*t*-value	*P*-value
Myristic acid (C14:0)	8.61 ± 2.87	7.22 ± 2.17	0.77	0.469
Pentadecylic acid (C15:0)	1.47 ± 0.29	1.39 ± 0.35	0.35	0.737
Palmitic acid (C16:0)	125.93 ± 2.91	125.48 ± 5.24	0.15	0.886
Stearic acid (C18:0)	46.63 ± 2.66[a]	55.76 ± 5.65[b]	−2.92	0.027
Saturated fatty acids (SFAs)	176.52 ± 19.53	192.24 ± 8.36	−1.48	0.189
Palmitoleic acid (C16:1 n-7)	26.20 ± 4.93	26.30 ± 1.60	−0.04	0.970
Vaccenic acid (C18:1 n-7)	34.41 ± 3.06	37.23 ± 5.17	−0.94	0.384
Oleic acid (C18:1 n-9)	238.38 ± 8.25	276.45 ± 34.54	−2.14	0.076
Eicosenoic acid (C20:1 n-9)	4.08 ± 0.39	4.62 ± 1.07	−1.83	0.127
Nervionic acid (C24:1 n-9)	1.76 ± 0.15	2.04 ± 0.45	−1.02	0.357
Monounsaturated fatty acids (MUFAs)	307.14 ± 13.72	346.91 ± 39.29	−1.91	0.105
Linoleic acid (C18:2 n-6)	126.14 ± 6.76	129.07 ± 10.81	−0.41	0.700
Gamma-linolenic acid (C18:3 n-6)	6.94 ± 1.24	9.47 ± 1.60	2.26	0.073
Arachidonic acid (C20:4 n-6)	4.47 ± 0.73	5.88 ± 1.22	−1.98	0.095
n-6 Polyunsaturated fatty acids (n-6 PUFAs)	137.90 ± 5.82	144.42 ± 13.06	−0.91	0.397
Alpha-linolenic acid (C18:3 n-3)	8.48 ± 0.35	8.20 ± 0.91	0.57	0.587
Stearidonic acid (C18:4 n-3)	1.52 ± 0.04	1.15 ± 0.41	1.80	0.123
Eicosatetraenoic acid (C20:4 n-3)	1.67 ± 0.22	2.05 ± 0.33	−1.92	0.104
Eicosapentaenoic acid (C20:5 n-3)	15.17 ± 0.39	16.19 ± 1.83	−1.09	0.317
Docosapentaenoic acid (C22:5 n-3)	7.96 ± 1.10	8.66 ± 1.16	−0.88	0.415
Docosahexaenoic acid (C22:6 n-3)	15.85 ± 2.21	21.03 ± 4.38	−2.11	0.079
n-3 Polyunsaturated fatty acids (n-3 PUFAs)	49.15 ± 4.88	57.29 ± 8.30	−1.70	0.142
Total PUFAs	189.48 ± 5.85	201.71 ± 21.01	−1.12	0.305
DHA/EPA	1.11 ± 0.09	1.29 ± 0.15	−2.06	0.085
EPA + DHA	32.05 ± 0.81	37.22 ± 6.14	−1.67	0.146
n-6/n-3	2.67 ± 0.12	2.54 ± 0.19	1.16	0.291

Non represented fatty acids were not detected in the analysis. Values are represented as mean ± SD (n = 4 tanks per dietary group, considering a tank as the experimental unit) and differences between dietary groups ($P \leq 0.05$) are indicated by different superscript letters.

3.7. Gene expression profile of liver and anterior intestine

A total of 35 out of 44 hepatic markers were shown to be differentially regulated by feeding time (2 h postprandial *vs.* 48 h fasted) and/or diet (control *vs* SO) (Supplementary Table S4; two-way ANOVA, $P < 0.05$). In 2 h postprandial-fish, *fasn*, *elovl6*, *cs* (Student's *t*-test; $P < 0.05$) and, to a lower degree, *scd1b*, *hnf4α* and *srebp2* were up-regulated, whereas *lpl* and *prdx5* were down-regulated ($P < 0.1$) in fish fed with the SO diet. After 48 h of fasting, there was an up-regulation of *elovl4*, *pltp*, *pla2g6*, *fxr*, *lxrα*, *pparβ*, *pparγ*, *cpt1a*, *cs*, *nd2*, *nd5*, *sirt1* and *prdx5* in the SO group ($P < 0.05$). On the other hand, there was a down-regulation of *pgc1α* ($P = 0.046$), *elovl1* and *h-fabp* ($P < 0.1$) in fish fed the SO diet. The PLS-DA model of liver gene expression data was based on three components, which explained [R2Y(*cum*)] 89% and predicted [Q2(*cum*)] 85% of total variance (Supplementary Fig. S4A). Feeding time was the most discriminant factor along component 1 (48% of total variance), and for 48 h fasted-animals a clear differential clustering between dietary groups was evidenced along component 2 (36% of total variance) of the PLS-DA (Fig. 2A) as well as in the heatmap expression pattern (Fig. 2B). Gene expression differences by diet were much less marked in 2 h postprandial-fish, so there was not a clear separation on the PLS-DA model nor on the heatmap.

Feeding time and/or diet was the cause of significant changes on the expression of 32 out of 44 target genes in the AI (Supplementary Table S5; two-way ANOVA, $P < 0.05$). In 2 h postprandial groups, fish fed the SO diet showed an up-regulation of *il-8*, *ccr3*, *lgals1*, *mrc1* ($P < 0.05$) and *il-1β* ($P = 0.052$), and a down-regulation of *tlr9* ($P = 0.036$), *fabp6* and *fcl* ($P < 0.1$). In 48 h fasted-fish, the supplementation of the diet with the combination of SO caused a down-regulation of *tnf-α*, *il-6*, *il-34*, *cd4–1*, *cd8b* ($P < 0.05$), and to a lower degree, *il-10*, *il-12β*, *il-15*, *ccr9*, *ccr11* and *tlr2* ($P < 0.1$). The PLS-DA model on AI expression data was based in two components, with R2Y(*cum*) of 77% and Q2(*cum*) of 68% (Supplementary Fig. S4B), and clearly separated feeding time along component 1, which explained 48% of total variance (Fig. 3A). Clustering was able to discriminate dietary groups in 2 h postprandial-fish but not in 48 h fasted-individuals (Fig. 3A and B).

3.8. Microbial diversity, structure, and composition

After rarefaction (53,085 reads per sample), a total of 2,388,825 reads that clustered into 19,900 ASVs were obtained. In terms of microbial community diversity and structure, there were no differences in the alpha diversity indices (ACE, Shannon, Simpson and Faith's phylogenetic diversity; Fig. 4 and Supplementary Table S6; $P > 0.05$) nor in beta diversity (see PcoA and PERMANOVA on Fig. 5; $P > 0.05$) between dietary treatments, regardless of the region of the intestine considered.

Table 7
Fatty acid profile (mg × g lipid^{-1}) of the fillet, and nutritional quality of the lipid fraction of the fillet in gilthead seabream (*Sparus aurata*) fed the control and a basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO).

	Control	SO	*t*-value	*P*-value
Myristic acid (C14:0)	8.60 ± 1.35	8.68 ± 1.35	−0.08	0.936
Pentadecylic acid (C15:0)	1.21 ± 0.10	1.19 ± 0.15	0.22	0.832
Palmitic acid (C16:0)	129.29 ± 3.49	126.88 ± 4.85	0.81	0.451
Stearic acid (C18:0)	32.41 ± 1.39	32.53 ± 1.21	−0.13	0.901
Lignoceric acid (C24:0)	1.44 ± 0.16	1.41 ± 0.17	0.26	0.806
Saturated fatty acids (SFAs)	173.63 ± 2.79	171.28 ± 7.35	0.60	0.572
Palmitoleic acid (C16:1 n-7)	33.50 ± 2.27	33.04 ± 3.67	0.21	0.838
Oleic acid (C18:1 n-9)	247.55 ± 10.03	250.86 ± 17.06	−0.34	0.749
Eicosenoic acid (C20:1 n-9)	3.39 ± 0.14	3.59 ± 0.43	−0.89	0.410
Nervionic acid (C24:1 n-9)	1.43 ± 0.22	1.62 ± 0.26	−1.12	0.307
Monounsaturated fatty acids (MUFAs)	285.59 ± 12.55	289.11 ± 21.10	−0.29	0.784
Linoleic acid (C18:2 n-6)	143.89 ± 7.49	141.58 ± 6.52	0.47	0.658
Gamma-linolenic acid (C18:3 n-6)	3.26 ± 0.37	3.77 ± 0.89	−1.06	0.331
Arachidonic acid (C20:4 n-6)	5.03 ± 0.43	4.94 ± 0.52	0.27	0.799
n-6 Polyunsaturated fatty acids (n-6 PUFAs)	152.56 ± 8.17	150.29 ± 7.02	0.42	0.688
Alpha-linolenic acid (C18:3 n-3)	9.82 ± 0.78	9.93 ± 1.09	−0.16	0.875
Stearidonic acid (C18:4 n-3)	1.56 ± 0.15	1.75 ± 0.32	−1.08	0.324
Eicosatetraenoic acid (C20:4 n-3)	1.63 ± 0.23	1.46 ± 0.15	1.24	0.262
Eicosapentaenoic acid (C20:5 n-3)	23.87 ± 2.38	23.70 ± 1.95	0.11	0.916
Docosapentaenoic acid (C22:5 n-3)	9.92 ± 1.22	9.34 ± 0.28	0.93	0.390
Docosahexaenoic acid (C22:6 n-3)	29.37 ± 2.75	30.40 ± 2.73	−0.53	0.614
n-3 Polyunsaturated fatty acids (n-3 PUFAs)	77.88 ± 9.41	76.59 ± 3.32	0.26	0.805
Total PUFAs	230.43 ± 15.81	226.87 ± 7.50	0.41	0.698
DHA/EPA	1.23 ± 0.03	1.28 ± 0.05	−1.72	0.137
EPA + DHA	53.24 ± 5.08	54.10 ± 4.55	−0.25	0.809
n-6/n-3	1.97 ± 0.18	1.97 ± 0.14	0.07	0.943
Index of atherogenicity (IA)	0.32 ± 0.02	0.31 ± 0.01	0.47	0.656
Index of thrombogenicity (IT)	0.38 ± 0.03	0.37 ± 0.01	0.26	0.803
Hypocholesterolemic/hypercholesterolemic ratio (h/H)	2.28 ± 0.07	2.35 ± 0.03	−1.58	0.166

Non represented fatty acids were not detected in the analysis. Values are represented as mean ± SD (n = 4 tanks per dietary group, considering a tank as the experimental unit) and differences between dietary groups ($P \leq 0.05$) are indicated by different superscript letters.

The four most dominant phyla were Firmicutes, Proteobacteria, Bacteroidota and Actinobacteriota, which represented 86% of the total microbial population in both AI and PI (Fig. 6). There were no significant differences in the relative abundance of any phyla between both dietary groups (Supplementary Table S7), neither on the Firmicutes/Bacteroidetes (F/B) ratio, that was maintained at 1.60–1.66 in the AI, and at 2.12–2.20 in the PI ($P > 0.05$). At the level of genus, the relative abundance of *Bacteroides*, *Desulfovibrio*, *Candidatus* Arthromitus and *Ralstonia* increased in the AI when supplementing the control diet with the combination of SO (Supplementary Table S8; $P < 0.05$), while no differences were reported in the PI ($P > 0.05$).

4. Discussion

4.1. Fish performance and adiposity

Present results showed that the supplementation of turmeric, capsicum, black pepper, and ginger oleoresins at the tested dietary inclusion level did not compromise the somatic growth and the overall body condition in gilthead seabream. The HSI was not altered, which suggests a good condition of the liver and absence of potential hepatic disorders (Van der Oost et al., 2003), as also confirmed by histological analyses. On the other hand, a marked reduction of the PVFI was observed in fish fed the SO diet. This could be an advantage in terms of the final product acceptance, taking into account that the perivisceral fat diminishes the shelf-life of the edible fraction and has a negative effect on the consumer's perception due to its visual impact and potential changes in organoleptic quality (Grigorakis, 2007). However, despite the reduced PVFI, there were no changes in the proximate composition nor in the fatty acid profile of the fillets. Similarly, the nutritional quality of the fillet lipid fraction (IA, IT and h/H ratio) did not change when supplementing the diet with SO, which remained within the range of values reported for other fish species (Chen and Liu, 2020). Regarding feeding efficiency, the absence of variation in FCR values was consistent with the non-differential lipid and protein ADC values between dietary groups. Additionally, the supplementation of the diet with the combination of SO did not affect feed intake, meaning that the additive did not compromise the palatability and acceptability of the feed to the fish. These results are in agreement with existing studies testing the effect of various spices or plant extracts in different fish species, which despite sometimes not affecting growth and feeding efficiency, or some somatic indices such as K and HSI, can have other physiological benefits in the fish (Firmino et al., 2020; Firmino et al., 2021; Huyben et al., 2021; Salomón et al., 2021).

4.2. Liver condition and lipid metabolism

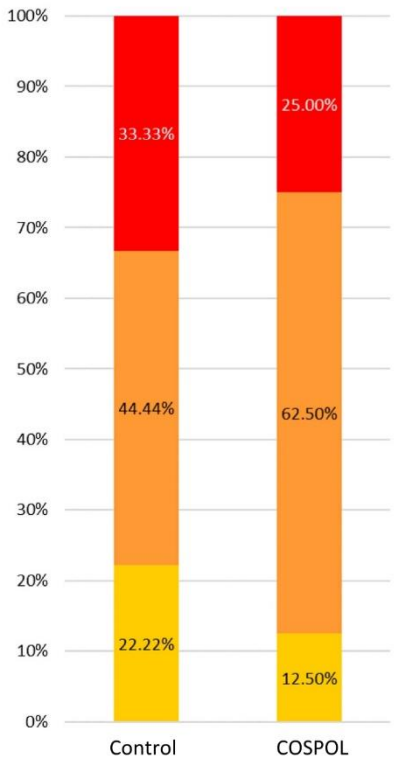
Under the current experimental conditions, dietary supplementation with the combination of SO led to reduced levels of lipids in the liver without markedly affecting its fatty acid profile, except for stearic acid (18:0), which increased in fish fed the SO diet. Regarding histological analyses, no signs of inflammation nor other physiological alterations were observed in the liver and or the intestine, reflecting the lack of disorders usually associated to unbalanced dietary conditions (Gisbert et al., 2008). These results, together with the absence of differences in the HSI and in the measured biomarkers of hepatic function and oxidative stress, indicated a healthy hepatic condition in both groups. Furthermore, none of the observed fish presented an excessive accumulation of fat deposits within the hepatic parenchyma (score 5). Nonetheless, dietary supplementation with SO led to a reduction in the number of specimens with large hepatic lipid accumulation (score 4), which was consistent with the significantly decreased content of crude fat in liver measured in this treatment.

Many studies have shown that dietary supplementation with curcumin, capsaicin, and ginger, individually or together and in combination with black pepper and other spices, can induce BA secretion in mammals

A

Score	Histological description
1	Hepatocytes are small. There are few small vacuoles in the cytoplasm. Central positioning of their nuclei. Very low lipid accumulation.
2	Slightly enlarged hepatocytes. Many vacuoles in the cytoplasm. Nuclei are positioned centrally in most hepatocytes. Low lipid accumulation.
3	All hepatocytes are enlarged. Lots of vacuoles in the cytoplasm or one large vacuole. Nuclei are displaced towards the periphery. Moderate lipid accumulation.
4	Almost all hepatocytes are significantly enlarged. Cytoplasm seems "empty" due to the presence of big vacuoles. Large lipid accumulation.
5	Hepatocytes are significantly enlarged. "Empty" cytoplasm. Some pyknotic nuclei. Some hepatocytes are broken. Areas of necrosis and lymphocytes in vascular tissue. Inflammation in the parenchyma and signs of steatosis.

B



C

Score	Histological description
1	No observable lipid accumulation. Barely visible supranuclear vacuoles.
2	Low lipid accumulation. Some supranucelar vacuoles are visible.
3	Moderate lipid accumulation. Almost all supranuclear vacuoles are visible and some are enlarged.
4	High lipid accumulation. Several supranuclear vacuoles are significantly enlarged.
5	Excessive lipid accumulation. Huge enlargement of all vacuoles.

D

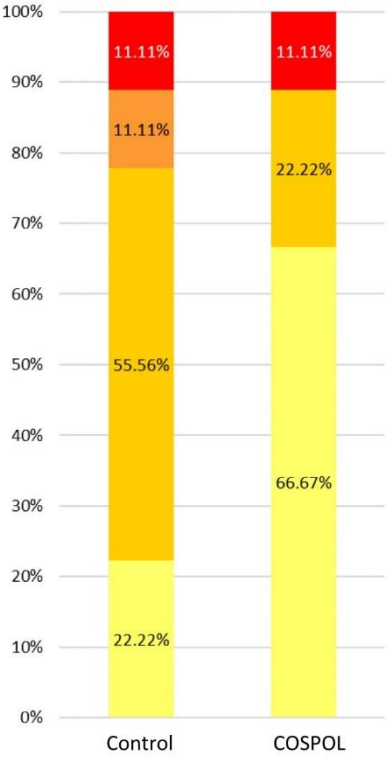


Fig. 1. (A) Semi-quantitative scoring used for evaluating the levels of fat accumulation in the liver of gilthead seabream (*Sparus aurata*). (B) Results (%) of hepatic scoring from gilthead seabream fed the control and a basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO). (C) Semi-quantitative scoring used for evaluating the levels of fat accumulation in the anterior intestine of gilthead seabream. (D) Results (%) of intestinal scoring from gilthead seabream fed the experimental diets.

Table 8
Oxidative stress condition of the liver, activity of antioxidative stress and hepatic enzymes, and plasma parameters in gilthead seabream (*Sparus aurata*) fed the control and a basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO).

	Control	SO	*t*-value	*P*-value
Hepatic oxidative stress				
GR (nmol min^{-1} × mg protein)	3.22 ± 0.73	3.76 ± 0.48	−1.24	0.263
CAT (nmol min^{-1} × mg protein)	96.88 ± 5.62	90.90 ± 12.25	0.89	0.409
SOD (% enzyme inhibition)	56.58 ± 9.23	66.29 ± 4.26	−1.91	0.105
LPO (nmol MDA g^{-1})	15.52 ± 3.66	13.56 ± 2.87	0.84	0.432
Trolox equivalents (nmol μL^{-1})	18.50 ± 1.72	19.74 ± 2.98	−0.72	0.498
Hepatic metabolism				
LDH (mU × mg $protein^{-1}$)	75.49 ± 19.98	65.40 ± 6.47	0.96	0.374
AST (mU × mg $protein^{-1}$)	812.56 ± 180.70	712.55 ± 166.45	0.81	0.447
ALT (mU × mg $protein^{-1}$)	251.12 ± 37.33	285.80 ± 40.36	−1.26	0.254
Blood biochemistry				
Cholesterol (mg dL^{-1})	223.08 ± 9.09	207.94 ± 28.30	1.02	0.348
TAGs (mg dL^{-1})	1072.76 ± 204.38	908.42 ± 151.19	1.29	0.244
Albumin (g L^{-1})	13.35 ± 0.94	14.25 ± 1.97	−0.83	0.441
Total globulins (g L^{-1})	21.73 ± 3.08	22.69 ± 4.45	−0.36	0.735
Total proteins (g L^{-1})	35.08 ± 3.99	36.94 ± 6.41	−0.49	0.640
AST (U L^{-1})	45.43 ± 9.39	62.58 ± 21.50	−1.46	0.194
ALT (U L^{-1})	4.71 ± 0.84	4.94 ± 0.83	−0.39	0.710
ALP (U L^{-1})	182.12 ± 29.71	158.25 ± 16.69	1.40	0.211

Values are represented as mean ± SD ($n = 4$ tanks per dietary group, considering a tank as the experimental unit). Abbreviations: GR: glutathione reductase; CAT: catalase; SOD: superoxide dismutase; LPO: lipid peroxidation; LDH: lactate dehydrogenase; AST: aspartate transaminase; ALT: alanine transaminase; TAGs: triacylglycerides; ALP: alkaline phosphatase.

Table 9
Specific activity (mU × mg $protein^{-1}$) of total alkaline proteases, α-amylase and bile salt-activated lipase in gilthead seabream (*Sparus aurata*) fed the control and a basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO).

	Control	SO	*t*-value	*P*-value
Stomach and pyloric caeca				
Total alkaline proteases	78.52 ± 8.01	101.61 ± 26.53	−1.67	0.147
Alpha-amylase	398.32 ± 51.08	375.97 ± 18.96	0.82	0.443
Bile salt-activated lipase	21.49 ± 6.26	19.52 ± 2.57	0.58	0.582
Anterior intestine				
Total alkaline proteases	133.64 ± 13.45	115.64 ± 21.15	1.44	0.201
Alpha-amylase	402.98 ± 130.44	375.02 ± 100.13	0.34	0.745
Bile salt-activated lipase	50.00 ± 9.57[a]	67.18 ± 6.15[b]	−3.02	0.023

Values are represented as mean ± SD (n = 4 tanks per dietary group, considering a tank as the experimental unit) and differences between groups ($P \leq 0.05$) are indicated by different superscript letters.

(Platel et al., 2002; Platel and Srinivasan, 2004; Srinivasan, 2005). In this study, despite the lack of statistical differences in lipid ADC between dietary groups, the trend to increased levels of BAs, and the significantly higher activity of bile-salt activated lipase in the AI of fish fed the SO diet, suggests that the combination of SO promotes lipid metabolism through a higher synthesis and/or secretion of BAs in fish, having similar effects to mammals. The increased proportion of fish with very low levels of intestinal fat accumulation (score 1) and the reduction of the PVFI in fish fed the SO diet additionally support this hypothesis.

Table 10
Bile acid profile in the gallbladder and the anterior intestine in gilthead seabream (*Sparus aurata*) fed the control and a basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO).

	Control	SO	*t*-value	*P*-value
Gallbladder (mg mL^{-1})				
T-CA	108.23 ± 9.59	113.02 ± 5.77	−0.86	0.425
T-CDCA	47.67 ± 3.69	49.93 ± 6.34	−0.62	0.560
Total BAs	155.90 ± 9.58	162.95 ± 8.83	−1.08	0.321
Anterior intestine (μg mg^{-1})				
T-CA	30.00 ± 15.83	49.85 ± 18.85	−1.61	0.158
T-CDCA	19.94 ± 13.72	27.09 ± 7.12	−0.93	0.391
Total BAs	49.94 ± 29.41	76.95 ± 24.84	−1.40	0.210

Non represented bile acids were not detected in the analysis. Values are represented as mean ± SD (n = 4 tanks per dietary group, considering a tank as the experimental unit). Abbreviations: T-CA: taurocholic acid; T-CDCA: taurochenodeoxycholic acid; BAs: bile acids.

The hepatic gene expression results at 2 h after feeding offer additional support to the hypothesis that spices regulate lipid metabolism, as well as influence BA synthesis and/or reabsorption in fish, in a similar manner as mammals. For instance, fish fed the SO diet showed a postprandial up-regulation of the hepatocyte nuclear factor 4 alpha (*hnf4α*), which plays a central role in the synthesis and conjugation of BAs (Chiang, 2009). In mammals, HNF4α promotes BA production through the transactivation of cholesterol 7-alpha-monooxygenase (*cyp7a1*), the first and rate-limiting enzyme in BA synthesis from cholesterol (Romano et al., 2020). Nonetheless, this was not reflected in changes in *cyp7a1* expression in either of the times sampled. However, the up-regulation of *hnf4α* could be related to other functions rather than BA synthesis, as this gene is also involved in the metabolic regulation of other nutrients, cell proliferation and/or differentiation (Chiang, 2009).

Similar to our previous work where we tested diet supplementation (same formulation as the control diet) with a blend of BAs in gilthead seabream juveniles (Ruiz et al., 2023a), a post-prandial down-regulation of lipoprotein lipase (*lpl*) and up-regulation of fatty acid synthase (*fasn*) were found. Since LPL hydrolyses TAGs from plasmatic chylomicrons and very low-density lipoproteins (VLDLs), releasing free fatty acids which are then incorporated into the tissues (Salmerón, 2018), its down-regulation is in line with the lower levels of fat deposits found in the liver, intestine and visceral cavity (PVFI). The up-regulation of *fasn* may be a counterregulatory mechanism to maintain a balance on the levels of hepatic fatty acids through *de novo* fatty acid synthesis (Salmerón, 2018), in response to their lower incorporation and/or higher hepatic oxidation. The SO diet also triggered the postprandial up-regulation of other *de novo* lipogenic biomarkers, such as the elongation of very long chain fatty acids 6 (*elovl6*) and stearoyl-CoA desaturase 1b (*scd1b*). One of the reactions catalysed by ELOVL6 is the conversion of palmitic acid (C16:0), the end product of FASN, into stearic acid (C18:0) (Sampath and Ntambi, 2005). On the other hand, the up-regulation of *scd1b*, which metabolises palmitic and stearic acids into palmitoleic (C16:1 n-7) and oleic (C18:1 n-9) acids, was apparently not enough to counteract the increase of stearic acid levels in the liver. The supplementation of the diet with the combination of SO also induced an up-regulation of the sterol regulatory element-binding protein 2 (*srebp2*). This transcription factor is known in fish (and mammals) for its important role in cholesterol homeostasis (Leaver et al., 2008; Zhu et al., 2020). Thus, although its role in lipid metabolism in fish needs to be further studied, the up-

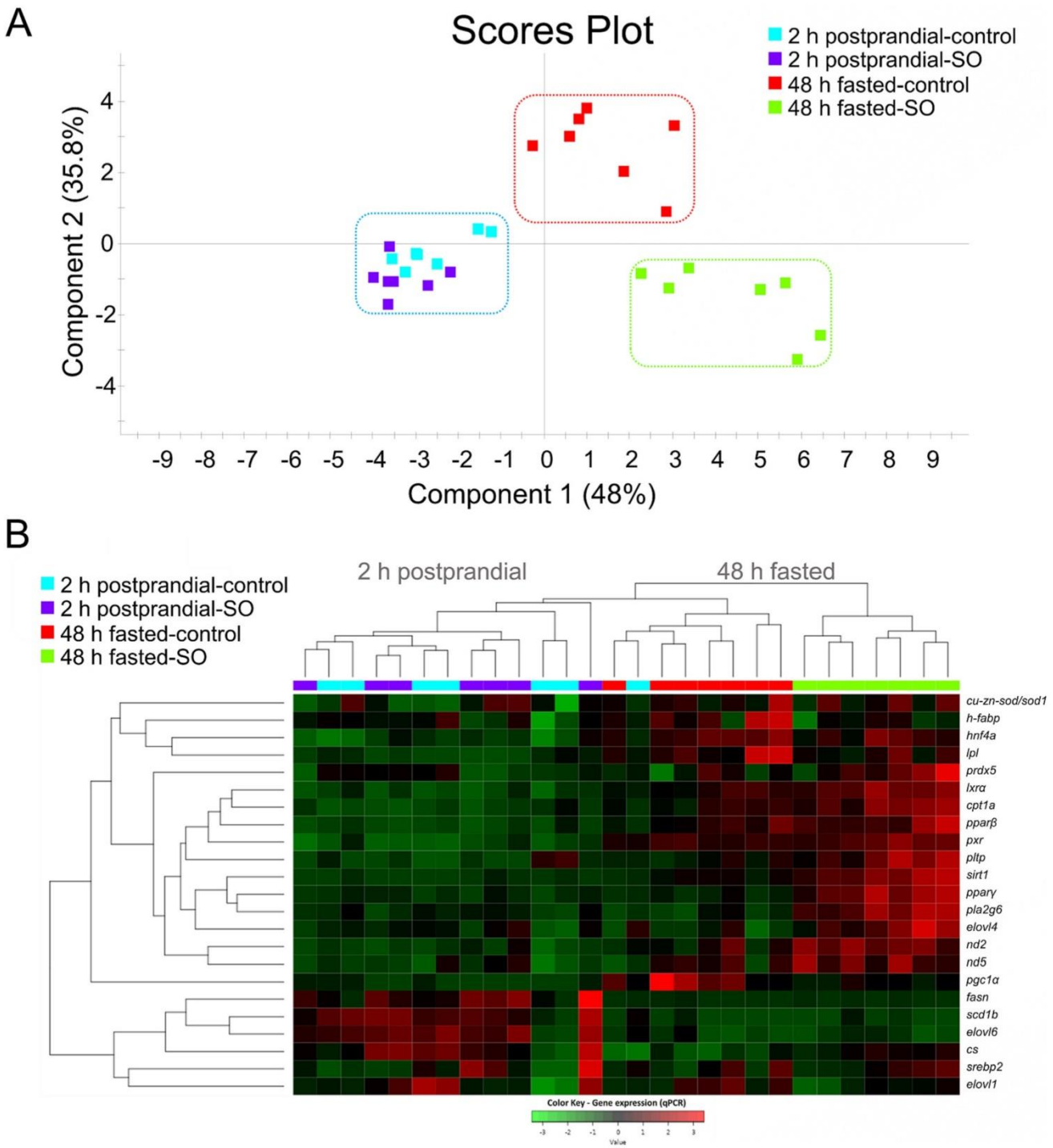


Fig. 2. (A) Scores plot for two-dimensional PLS-DA representing individual distribution between the two components of the model based on hepatic expression values (qPCR) of genes with $P \leq 0.1$ (Student's *t*-test). (B) Heatmap plotting hierarchical clustering of the same hepatic markers expression values. Samples were obtained from the liver of 48 h fasted- and 2 h postprandial- gilthead seabream (*Sparus aurata*; $n = 8$ fish per dietary group) fed the control and the basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO). Expression values of genes are available at Supplementary Table S4.

regulation of *srebp2* may balance the levels of cholesterol (which, among other roles, is the precursor of BAs). Finally, fish fed the SO diet also showed a postprandial up-regulation of citrate synthase (*cs*), which catalyses the conversion of acetyl-CoA and oxalacetate into citrate in the citric acid cycle (Akram, 2014). This is likely associated with a higher lipid oxidation rate, as this is one of the commonly described effects of spices' supplementation in mammalian diets (Westerterp-Plantenga et al., 2006). In this sense, a higher energy production coming from NADH + H^+ and $FADH_2$ released from the citric acid cycle would be expected. However, the expression of the electron transport chain

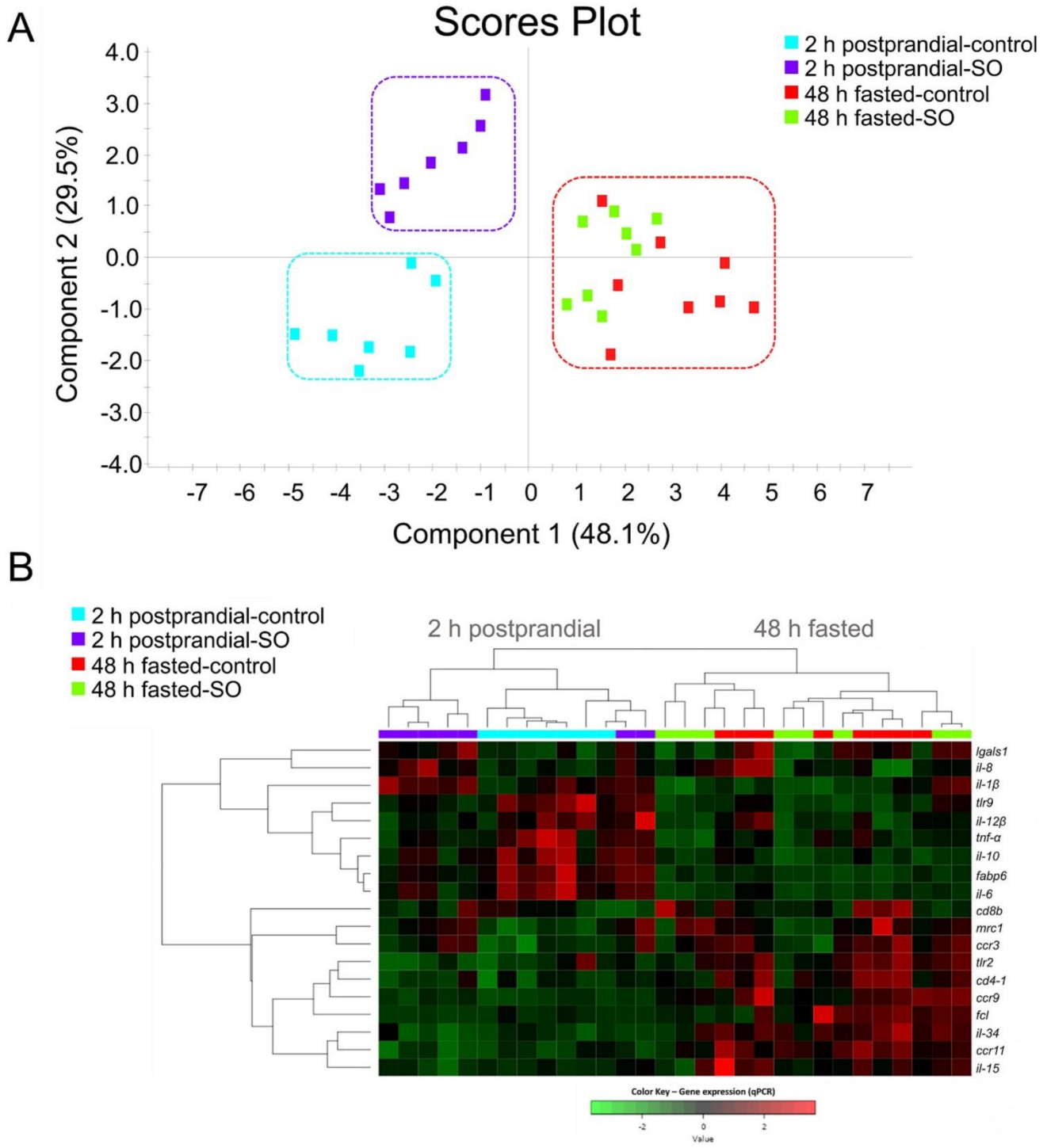


Fig. 3. (A) Scores plot for two-dimensional PLS-DA representing individual distribution between the two components of the model based on intestinal expression values (qPCR) of genes with $P \leq 0.1$ (Student's t-test). (B) Heatmap plotting hierarchical clustering of the same intestinal markers expression values. Samples were obtained from the anterior intestine of 48 h fasted- and 2 h postprandial- gilthead seabream (*Sparus aurata*; n = 8 fish per dietary group) fed the control and the basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO). Expression values of genes are available at Supplementary Table S5.

enzymes NADH-ubiquinone oxidoreductase chain 2 (*nd2*), NADH-ubiquinone oxidoreductase chain 5 (*nd5*), and cytochrome *c* oxidase subunit I (*coxi*) were not affected in this sampling point.

The PLS-DA model stated that differences among dietary groups in the hepatic expression profiles were more marked in 48 h fasted fish, indicating that these differences were not driven by the postprandial nutrient metabolization and storage carried out by this organ, rather than by the catabolism of nutrients (especially lipids) to produce energy in a fasting state. Having said that, the differential pattern of gene expression further supports that spices can significantly influence lipid,

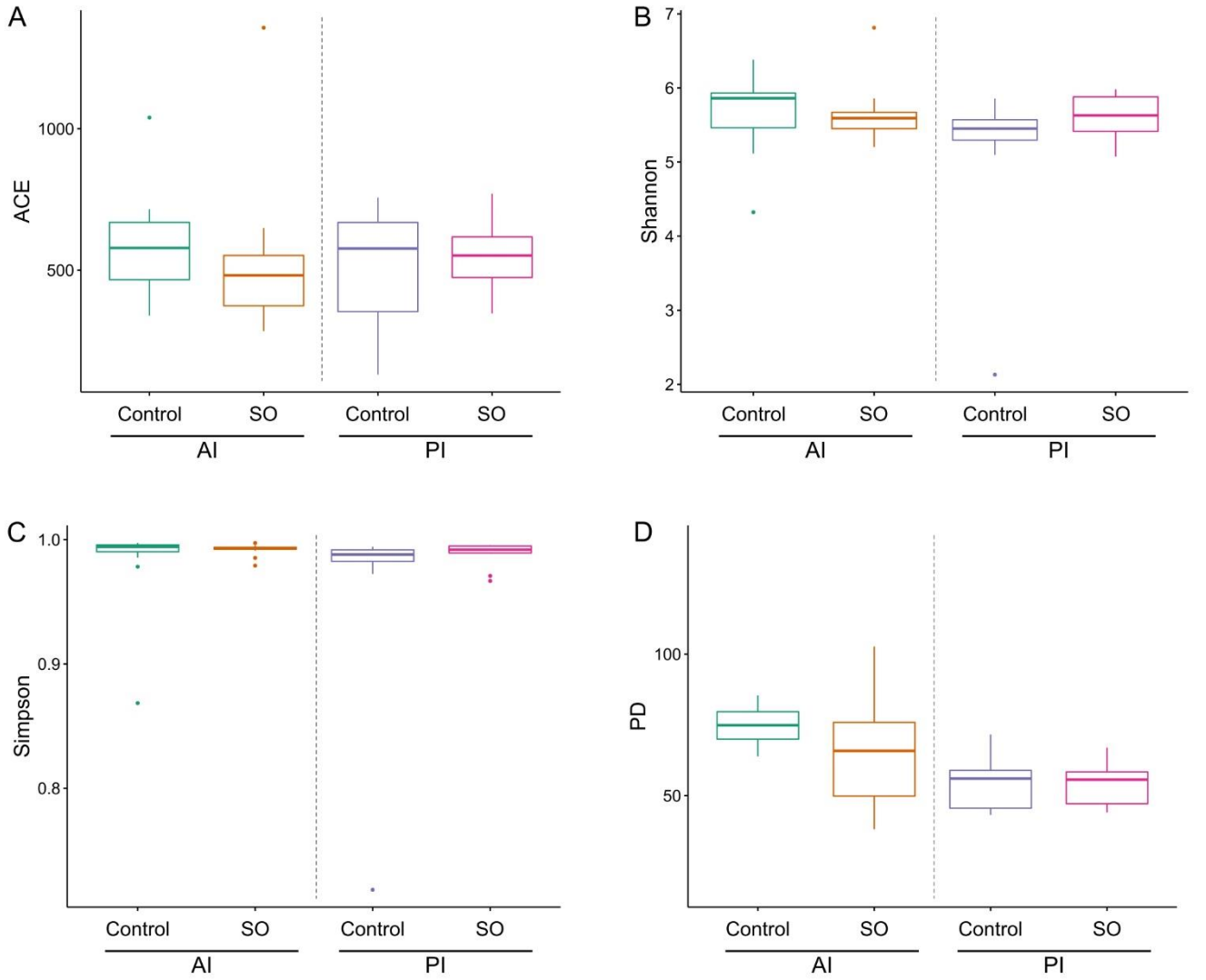


Fig. 4. Microbial alpha diversity of the anterior (AI) and posterior intestine (PI) in gilthead seabream (*Sparus aurata*; $n = 12$ fish per dietary group) fed the control and a basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO): (A) ACE index, (B) Shannon index, (C) Simpson's index, and (D) Faith's phylogenetic diversity (PD). There were no significant differences among dietary groups.

cholesterol, and BA metabolism in fish, although it is still challenging to pinpoint the exact mechanisms of action. After 48 h fasting, fish fed the SO diet showed a down-regulation of elongation of very long chain fatty acids 1 (*elovl1*), and an up-regulation of elongation of very long chain fatty acids 4 (*elovl4*) and phospholipid transfer protein (*pltp*) with respect to those fed the control diet. Both *elovl1* and *elovl4* are involved in *de novo* fatty acid synthesis (Xie et al., 2021), while PLTP is involved in lipoprotein metabolism and reverse cholesterol transport, and its activity has been positively correlated with the content of cholesterol and TAGs in plasma (Albers et al., 2012). Therefore, their regulation could be a mechanism to attempt to restore the lipid content in the liver, further induced by the fasting condition of fish. There was also an up-regulation of *fxr* in the liver of fasting fish from the SO treatment. In mammals, FXR is stimulated by elevated levels of BAs returning to the hepatocytes and transactivates a small heterodimer partner (*shp*), which represses *cyp7a1* transcription by interacting with α-fetoprotein transcription factor and HNF4α (Romano et al., 2020). In addition, *lxrα* was up-regulated in fish fed the SO diet. In mammals, LXR is stimulated by high levels of oxysterols (derivatives of cholesterol) and functions as a heterodimer with the retinoid X receptor (RXR), forming an important transcriptional regulator of lipid and cholesterol metabolism, as well as inflammation (Romano et al., 2020). In zebrafish, LXR has similarly been shown to be involved in the regulation of cholesterol and lipid homeostasis (Archer et al., 2008). Some of the genes that are transcriptionally regulated by LXR include *fasn*, *scd1* and *lpl*, which were significantly regulated in the liver of SO-fed fish, and hence this could be a key regulatory element through which SO exert lipotropic effects. The up-regulation of *lxrα* is probably associated to the up-regulation of the peroxisome proliferator-activated receptor γ (*pparγ*), since PPARγ can induce the expression of *lxrα*. Indeed, in line with the present findings, several studies in mammals -most of them *in vitro*- have reported that curcumin activates a PPARγ–LXRα–ABCA1 pathway (Panahi et al., 2018). The ATP binding cassette transporter A1 (ABCA1) is a transmembrane protein that mediates the transport of cholesterol and phospholipids to lipid-poor apolipoproteins (apo-A1 and apo-E), so it is critical for the synthesis of high-density lipoproteins (HDLs) and regulation of cholesterol efflux. PPARγ is also involved in differentiation of adipocytes, as well as regulating glucose homeostasis and having an anti-inflammatory role in mammals (Walczak and Tontonoz, 2002).

Another member of the PPAR family whose function has been less elucidated, the peroxisome proliferator-activated receptor β (PPARβ), was also up-regulated in 48 h fasted-fish from the SO treatment. Among

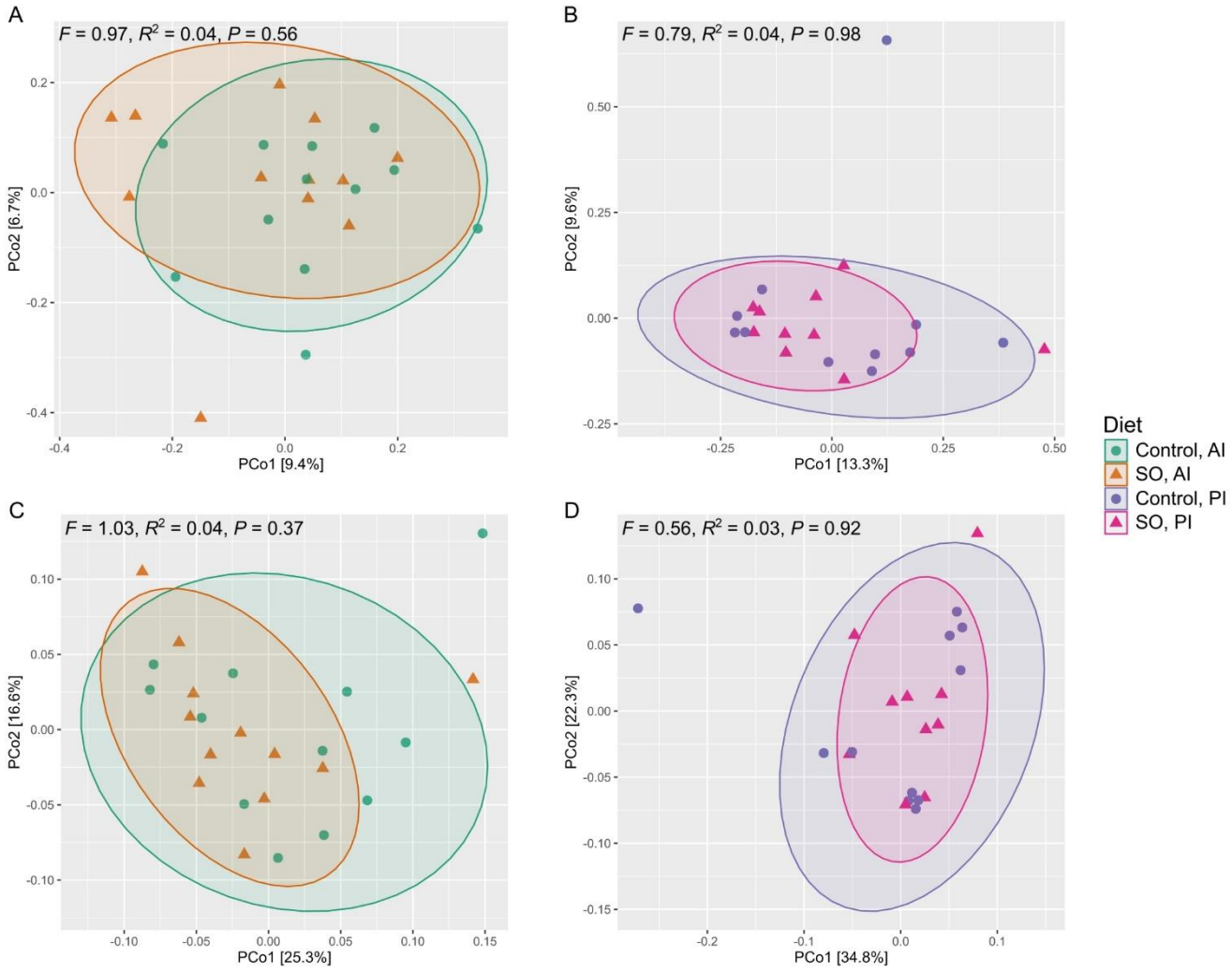


Fig. 5. Gut microbial structure in gilthead seabream (*Sparus aurata*; n = 12 fish per dietary group) fed the control and a basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO); represented by principle coordinate analysis (PCoA) diagrams based on the unweighted UniFrac distances in the (A) anterior (AI) and (B) posterior intestine (PI); and based on the weighted UniFrac distances in the (C) AI and (D) PI, and statistically compared by means of a PERMANOVA. The significant effect of the variables diet and intestinal region and their interaction can be found in Supplementary Fig. S5.

other roles, as in mammals, in fish PPARβ is believed to stimulate the expression of genes involved in fatty acid oxidation (Leaver et al., 2008), which is consistent with the observed up-regulation of carnitine palmitoyltransferase 1 A (*cpt1a*). CPT1A is the rate-limiting enzyme in fatty acid β-oxidation, as it catalyses the transport of long chain fatty acids across the outer membrane of the mitochondria, where β-oxidation takes place (Weil et al., 2013). The up-regulation of *cs*, *nd2* and *nd5* may also be in line with an increased oxidation of fatty acids, as $FADH_2$, NADH + H^+, and acetyl-CoA released from β-oxidation enter the citric acid cycle (Akram, 2014), and subsequently $FADH_2$ and NADH + H^+ pass to the electron transport chain in the inner mitochondrial membrane to generate energy (Nolfi-Donegan et al., 2020). On the other hand, the heart-type fatty acid binding protein (*h-fabp*), which facilitates the transport of fatty acids from the cell membrane into the mitochondrial membrane (Glatz and van der Vusse, 1989), and proliferator-activated receptor gamma coactivator 1 alpha (*pgc1α*), which regulates oxidative metabolism by stimulating mitochondrial biogenesis (Jamwal et al., 2021), were down-regulated in 48 h fasted-fish fed the SO diet. Such results seem to be in contradiction with the expression of the above-mentioned biomarkers related to fatty acid catabolism, especially considering that *pgc1α* can be activated by PPARγ and sirtuin 1 (SIRT1) (Jamwal et al., 2021), which were up-regulated in fish fed the SO diet. SIRT1 is an important regulatory effector that deacetylates a broad range of transcription factors and coregulators, among them PGC1α, LXR, FXR and SREBPs (Schug and Li, 2011). It functions as a metabolic sensor, providing a molecular link between cellular metabolic status and adaptive transcriptional responses, and has been associated with many beneficial roles, several of them in line with the observed results in this study (*i.e.*, reduction of hepatic lipogenesis, oxidative stress and inflammation, stimulation of fatty acid β-oxidation, and maintenance of cholesterol and BA levels) (Schug and Li, 2011; Singh et al., 2018). In fish, SIRT1 activation was also implicated in the hepatic alleviation from lipid deposition (through the regulation of lipogenesis and lipolysis) and oxidative stress (Huang et al., 2021).

An interesting observation was the differential regulation of the expression of peroxiredoxin 5 (*prdx5*) in relation to feeding time, being down-regulated during feeding (2 h postprandially) and up-regulated during fasting in fish that were fed the SO diet. Its increased expression in 48 h fasted-fish could be related to the role of PRDX5 in repressing lipogenesis and lipid accumulation (Kim et al., 2018, 2020). However, considering that the most widely recognized role of PRDX5 is to scavenge reactive oxygen species (ROS) (Kim et al., 2018), another

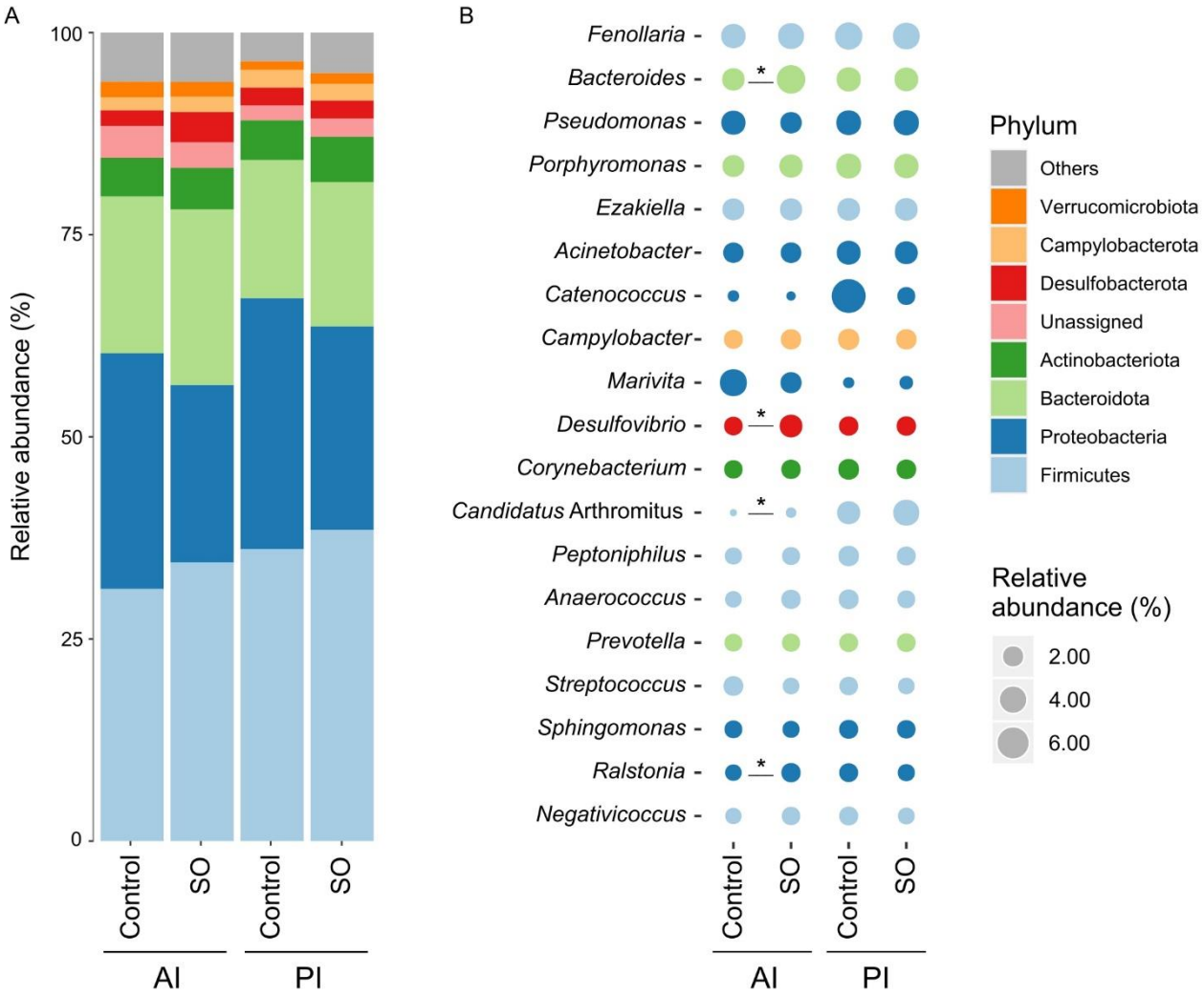


Fig. 6. Relative abundances of gut bacterial taxa in the anterior (AI) and posterior intestine (PI) in gilthead seabream (*Sparus aurata*; n = 12 fish per dietary group) fed the control and a basal diet supplemented with a combination of spice oleoresins from turmeric, capsicum, black pepper, and ginger (SO). Data are expressed at the level of (A) phylum and (B) genus (excluding unassigned genera). Taxa appearance in the figures is in the order of decreasing abundance (from bottom to top in the bar graph, and inversely in the bubble plot). Taxa with an abundance <1% are classified as others in the bar graph and are not represented in the bubble plot. Asterisks represent significant differences between dietary treatments ($P \leq 0.05$).

possibility is that its up-regulation was associated to the higher activity of the electron transport chain, which not only generates ATP but also ROS (Nolfi-Donegan et al., 2020). On the other hand, the down-regulation of *prdx5* in 3 h postprandial-fish fed the SO diet may indicate lower ROS levels, possibly due to the antioxidant properties of the spices herein tested, especially curcumin and capsaicin (Srinivasan, 2005). In addition, there was also an up-regulation of 85 kDa calcium-independent phospholipase A2 (*pla2g6*) in SO-fed fish after 48 h fasting, which could be beneficial for repairing the cellular membranes and protecting them against ROS-induced lipid peroxidation (Kinsey et al., 2007).

4.3. Intestinal health status: immunological status and microbial communities

Temporal dynamics of liver and intestine gene expression profiles were different. While in the liver the differences among dietary groups were less marked in the postprandial state than during the fasting period, in the intestine the clustering in two different dietary groups was more evident in 2 h postprandial-fish. This was probably due to the fact that differences in the intestine were derived from the immediate and continuous effect that each diet had in the intestine during the feed transit associated to nutrient absorption and digestion on the regulation of the intestinal gene markers (especially on those related to intestinal immunity).

The tested combination of SO induced a postprandial pattern of regulation in many intestinal biomarkers (*il-8*, *ccr3*, *lgals1*, *tlr9*, *mrc1*; $P \leq 0.05$) similar to that observed in our previous work feeding gilthead seabream with the same basal diet but supplemented with BAs (Ruiz et al., 2023b). In this case, the up-regulation of interleukin-8 (*il-8*) may hint a higher production of IL-8 triggered by the up-regulation of the pattern recognition receptor (PRR) macrophage mannose receptor 1 (*mrc1*), inducing a proinflammatory response (Gazi and Martinez-Pomares, 2009). Such an inflammatory response is in line with the up-regulation of C-C chemokine receptor type 3 (*ccr3*), which elicits the migration and activation of eosinophils (Heath et al., 1997). Under the current experimental conditions, there was also a tendency towards the up-regulation of interleukin-1 beta (*il-1β*), which is a well-studied pro-inflammatory cytokine that in fish induces a cascade of effects on different members of this cytokine family, causing signal transduction

and activation of the nuclear factor (NF)-kB pathway (Zou and Secombes, 2016). The gene expression regulation of other effector molecules such as galectin-1 (*lgals1*) and toll-like receptor 9 (*tlr9*), respectively up-regulated and down-regulated in the SO treatment, enable balancing the inflammatory response to avoid collateral damage from excessive inflammatory activity. In this sense, LGALS1 can reduce the production of pro-inflammatory cytokines (Seropian et al., 2018), while TLR9 induces the synthesis of pro-inflammatory cytokines (Kumagai et al., 2008). In addition, LGALS1 maintains the homeostasis of immune cells and the integrity of the intestinal epithelium (Muglia et al., 2011), while TLR9 recognizes unmethylated bacterial CpG motifs (Kumagai et al., 2008), so *lgals1* up-regulation and *tlr9* down-regulation could also be a response to reduced exposure to pathogen associated molecular patterns (PAMPs), related to changes in gut microbiota as will be further described below. This is supported by the trend for down-regulation of the PRR fucolectin (*fcl*), which is a fucose-binding lectin that induces a rapid immune response against pathogen infections (Shao et al., 2018). On the other hand, the down-regulation of the ileal fatty acid-binding protein (*fabp6*) could be linked to a plausible higher BA transport rate, as FABP6 transports BAs from the apical to the baso-lateral domain of the enterocytes and then returns to the apical side to repeat the cycle (Durník et al., 2022).

The effect of the SO supplement on the fish intestinal immunity was more pronounced after a 48 h fasting-period, leading to a down-regulation of 11 immune-related biomarkers (*tnf-α*, *il-6*, *il-34*, *cd4–1*, *cd8b*; $P < 0.05$; and *il-10*, *il-12β*, *il-15*, *ccr9*, *ccr11* and *tlr2*; $P < 0.1$) in fish fed the SO diet with respect to the control group. In this sense, some studies in fishes have demonstrated that the immune response can be enhanced under short-term starvation periods (Sakyi et al., 2021; Yengkokpam et al., 2013). This result was in line with the down-regulation of interleukin-10 (*il-10*), which is known to suppress immune responses in fish and mammals (Zou and Secombes, 2016). Paradoxically, while IL-10 is an anti-inflammatory cytokine, several pro-inflammatory cytokines, such as tumor necrosis factor-alpha (*tnf-α*), interleukin-12 subunit beta (*il-12β*), interleukin-15 (*il-15*) and interleukin-34 (*il-34*), were also down-regulated under the influence of the SO diet. The pleiotropic cytokine IL-12 is involved in innate and adaptive immune defense against pathogens and has been shown to induce the expression of *tnf-α* in grouper (*Epinephelus coioides*) and olive flounder (*Paralichthys olivaceus*) (Wang et al., 2023; Zou and Secombes, 2016). In grouper the subunit beta of IL-12 (IL-12β) alone was also able to induce *tnf-α* up-regulation to a lesser extent (Zou and Secombes, 2016), which is consistent with *tnf-α* and *il-12β* being similarly regulated in this study. In mammals, IL-12 also up-regulates other inflammatory cytokines, including interleukin-6 (*il-6*) and *il-15* (Liu et al., 2005), and therefore *il-12β* expression could also be driving the down-regulation of these two genes in the present study. TNF-α is also a pleiotropic cytokine expressed in wide range of immune cells (mainly activated monocytes and macrophages, as well as B cells, T cells, dendritic cells, granulocytes), fibroblasts and epithelial cells, which is involved in the regulation of several biological pathways, including cell proliferation and differentiation, apoptosis, inflammation, and immune responses after stimulation by PAMPs (Sakai et al., 2021; Sharif et al., 2020). In fish, the role of IL-6 has been mainly correlated with macrophage growth and proliferation and stimulation of immunoglobulin production in B cells, like immunoglobulin M (IgM; whose expression did not change under current conditions) (Chen et al., 2012; Wei et al., 2018). Additionally, several studies in teleosts have reported an up-regulation of *il-6* after stimulation with different PAMPs or infection with fish pathogens (Chen et al., 2012; Wei et al., 2018, among others). Although the role of IL-15 in fish immune response has not yet been thoroughly elucidated, this pro-inflammatory cytokine in mammals stimulates proliferation of B cells, T cells and NK cells, as well as activation of neutrophils and T cells, immunoglobulin production and inhibition of apoptosis (Van Heel, 2006). There was also a down-regulation of *il-34*, which in mammals is known to induce proliferation and differentiation of monocytes and macrophages, and stimulates proliferation of $CD8^+$ T regulatory cells (Baghdadi et al., 2018). Thus, the down-regulation of the cell marker cluster of differentiation 4–1 (*cd4*), cluster of differentiation 8 beta (*cd8b*), and C-C chemokine receptor type 9 (*ccr9*) may be linked to the down-regulation of *tnf-α*, *il-6*, *il-15* and *il-34*. In fish, CD4 and CD8 are generally found on the surface of different T cell subtypes (Beck and Peatman, 2015). In mammals, the C-C chemokine receptor CCR9 is found in B cells, T cells, monocytes, macrophages and dendritic cells, and drives cell migration to the gut-associated lymphoid tissue (GALT), where these immune cells play a regulatory role in inflammation (Pathak and Lal, 2020). Since IL-6 and CCR9 can have both pro- and anti-inflammatory functions (Zou and Secombes, 2016; Pathak and Lal, 2020), it is difficult to hypothesize whether their down-regulation was also associated to an anti-inflammatory effect or, conversely, was part of an inflammatory response to counteract the effect of pro-inflammatory cytokines, together with *il-10* down-regulation. On the other hand, there was also a down-regulation of the C-C chemokine receptor type 11 (*ccr11*), which is a G protein-coupled receptor whose expression and functions remain unknown in fish (Zou et al., 2015). In mammals, CCR11 scavenges different chemokines belonging to the CXC (C-X-C motif chemokine ligand) and CC (C-C motif chemokine ligand) superfamilies, including CXCL13, CCL19, CCL21 and CCL25 (Mollica Poeta et al., 2019; Zou et al., 2015). Interestingly, CCL25 is the exclusive cognate ligand of CCR9 in mammals (Pathak and Lal, 2020). As this ligand/receptor system seems to be conserved in fish and to maintain a similar functional activity to higher vertebrates (Galindo-Villegas et al., 2013), in this study the down-regulation of *ccr9* and *ccr11* may respond to a lower secretion of CCL25 by gut epithelial cells (Pathak and Lal, 2020). Taking these results together, the down-regulation of *tnf-α*, *il-6*, *il-10*, *il-12β*, *il-15*, *il-34*, *cd4–1*, *cd8b*, *ccr9* and *ccr11* point towards a lower cytokine production and proliferation of immune cells in 48 h fasted-fish fed the SO diet, which may be related to a lower recognition of PAMPs as indicated by the down-regulation of the PRR toll-like receptor 2 (*tlr2*). Ligands binding to TLR2 induce an innate immune response through a MYD88-dependent pathway, which leads to production of ROS and pro-inflammatory cytokines (Sepehri et al., 2016). Therefore, it seems plausible that under the current experimental conditions the tested additive enhanced the health status of the intestine under short-term fasting conditions.

The gut microbial diversity (alpha), structure (beta diversity), and composition (at the level of phylum) were maintained in fish fed the SO diet, and there was no variation in the F/B ratio between dietary treatments, a ratio that is generally used as a biomarker of intestinal dysbiosis in fishes (Mougin and Joyce, 2022). This was also observed in previous studies testing the supplementation of fish diets with micro-encapsulated essential oils (Huyben et al., 2021). Regarding the gut microbial composition at the level of phylum, Firmicutes, Proteobacteria and Bacteroidota represent up to 90% of fish intestinal microbiota in different species (Ghanbari et al., 2015). Although in gilthead seabream their relative proportion can vary with age and the nutritional or genetic background of the fish (Piazzon et al., 2017; Piazzon et al., 2019; Piazzon et al., 2020), several studies have highlighted that the most dominant bacterial phyla of the gut microbial communities in gilthead seabream are Firmicutes, Proteobacteria, Bacteroidota and Actinobacteriota (Tapia-Paniagua et al., 2020; Naya-Català et al., 2021; Ruiz et al., 2023b), in line with the results obtained in the present work. The composition of the gut microbial communities at the level of genus was also very conserved, with only an increase in the relative abundance of *Bacteroides*, *Desulfovibrio*, *Candidatus* Arthromitus, and *Ralstonia* in the AI under SO dietary supplementation. Interestingly, members of the genus *Bacteroides* have many enzymes involved in the transformation of primary BAs into secondary BAs, while *Desulfovibrio* is able to metabolise the taurine released from deconjugated BAs (Chattopadhyay et al., 2022; Hu et al., 2022). Furthermore, both of these Gram-negative genera can promote the growth of bacteria with 7α-dehydroxylase activity, which are necessary for BA metabolism (Hirano and Masuda, 1982; Hu

et al., 2022). Importantly, besides facilitating its reabsorption, the metabolization of BAs into secondary BAs can avoid accumulation of high concentrations of primary BAs and prevent their toxicity (Schubert et al., 2017). Moreover, previous studies in fishes have suggested that the production of short chain fatty acids by *Bacteroides* may induce lipolysis leading to reduced levels of perivisceral fat (Zhou et al., 2018; Ruiz et al., 2023b), which is in agreement with results of the current study. Albeit little is known about the role of *Candidatus* Arthromitus in fish, some studies have shown that this genus has immunostimulatory properties in the intestine (Hedblom et al., 2018). The role of *Ralstonia* in the fish gut also remains unknown, although an increase in its abundance was reported in the study of Dam et al. (2020) in which yellowtail kingfish (*Seriola lalandi*) were fed low digestibility diets. However, in our previous study an increased relative abundance of *Ralstonia* was observed in the AI of gilthead seabream fed a BA supplemented diet, which showed increased lipid digestibility (Ruiz et al., 2023b) as well as, similarly to the present study, a lower accumulation of fat deposits in the visceral cavity and digestive organs, reduced levels of lipids in liver and effects on the expression of biomarkers of lipid metabolism (Ruiz et al., 2023a).

5. Conclusions

Overall, the results presented herein indicate comparable effects of spices in fish as in mammals, with similar beneficial impacts in energy expenditure and fat homeostasis, reduction of body adiposity and improvement in the gut immunological state, through multiple mechanisms. These results suggest that diet supplementation with a combination of spices can be a functional nutritional strategy to help mitigate some of the commonly reported negative effects associated to reductions of fish oil in fish feeds.

Author contributions

Alberto Ruiz: Methodology, Formal analysis, Visualization, Writing - original draft. **Ignasi Sanahuja:** Formal analysis, Writing – review & editing. **Karl B. Andree, Dolors Furones and Jaume Pérez-Sánchez:** Methodology, Visualization, Writing – review & editing. **Paul G. Holhorea, Josep A. Calduch-Giner and Jose J. Pastor**: Data curation, Formal analysis, Writing – review & editing. **Marc Viñas:** Methodology, Writing – review & editing. **Sofia Morais:** Conceptualization, Visualization, Supervision, Writing – review & editing. **Enric Gisbert:** Conceptualization, Visualization, Supervision, Funding acquisition, Project administration, Writing – review & editing. All authors have contributed to the final writing of the paper, and have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Alberto Ruiz reports financial support was provided by Spain Ministry of Science and Innovation. Enric Gisbert reports financial support was provided by Spain Ministry of Science and Innovation. Ignasi Sanahuja reports financial support was provided by Spain Ministry of Science and Innovation. Jose J. Pastor and Sofia Morais reports a relationship with Lucta SA that includes: employment. Sofia Morais has patent #WO/2022/117810 issued to Lucta, SA.

Data availability

As specified in the manuscript, raw sequencing data is available in the Sequence Read Archive (SRA) of NCBI under Bioproject accession number PRJNA915342 and PRJNA985270.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.740378.

References

Aebi, H., 1974. Catalase. In: Bergmeyer, H.U. (Ed.), Methods of enzymatic analysis. Academic Press, New York, pp. 673–684.
Akram, M., 2014. Citric acid cycle and role of its intermediates in metabolism. Cell Biochem. Biophys. 68, 475–478. https://doi.org/10.1007/s12013-013-9750-1.
Albers, J.J., Vuletic, S., Cheung, M.C., 2012. Role of plasma phospholipid transfer protein in lipid and lipoprotein metabolism. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1821, 345–357. https://doi.org/10.1016/j.bbalip.2011.06.013.
Archer, A., Lauter, G., Hauptmann, G., Mode, A., Gustafsson, J.Å., 2008. Transcriptional activity and developmental expression of liver X receptor (*lxr*) in zebrafish. Dev. Dyn. 237, 1090–1098. https://doi.org/10.1002/dvdy.21476.
Association of Official Analytical Chemists (AOAC), 1990. Official Methods of Analysis of the Association of Official Analytical Chemists, fifteenth ed. Association of Official Analytical Chemists, Virginia, USA.
Baghdadi, M., Umeyama, Y., Hama, N., Kobayashi, T., Han, N., Wada, H., Seino, K.I., 2018. Interleukin-34, a comprehensive review. J. Leukoc. Biol. 104, 931–951. https://doi.org/10.1002/JLB.MR1117-457R.
Bakke, A.M., Glover, C., Krogdahl, Å., 2010. Feeding, digestion and absorption of nutrients. In: Grosell, M., Farrell, A.P., Brauner, C.J. (Eds.), Fish Physiology: The Multifunctional Gut of Fish. Academic Press, London, pp. 57–110.
Beck, B.H., Peatman, E., 2015. Mucosal Health in Aquaculture, first ed. Academic Press, Cambridge.
Benedito-Palos, L., Ballester-Lozano, G.F., Pérez-Sánchez, J., 2014. Wide-gene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (*Sparus aurata*). Gene 547, 34–42. https://doi.org/10.1016/j.gene.2014.05.073.
Bergmeyer, H.U., Bernt, E., 1974a. Lactate dehydrogenase: UV-assay with pyruvate and NADH. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic Press, New York, pp. 574–579.
Bergmeyer, H.U., Bernt, E., 1974b. Glutamate-oxaloacetate transaminase: UV-assay, manual method. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic Press, New York, pp. 727–733.
Bergmeyer, H.U., Bernt, E., 1974c. Glutamate-pyruvate transaminase: UV-assay, manual method. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic Press, New York, pp. 752–758.
Bermejo-Nogales, A., Nederlof, M., Benedito-Palos, L., Ballester-Lozano, G.F., Folkedal, O., Olsen, R.E., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2014. Metabolic and transcriptional responses of gilthead sea bream (*Sparus aurata* L.) to environmental stress: New insights in fish mitochondrial phenotyping. Gen. Comp. Endocrinol. 205, 305–315. https://doi.org/10.1016/j.ygcen.2014.04.016.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.
Brown, P.B., Hart, S.D., 2010. Soybean oil and other n-6 polyunsaturated fatty acid-rich vegetable oils. In: Turchini, G.M., Ng, W.K., Tocher, D.R. (Eds.), Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds. CRC Press, Florida, pp. 133–160.
Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.
Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J. Biol. Chem. 250, 5475–5480. https://doi.org/10.1016/S0021-9258(19)41206-4.
Chattopadhyay, I., Gundamaraju, R., Jha, N.K., Gupta, P.K., Dey, A., Mandal, C.C., Ford, B.M., 2022. Interplay between dysbiosis of gut microbiome, lipid metabolism, and tumorigenesis: can gut dysbiosis stand as a prognostic marker in cancer? Dis. Markers 2022, 2941248. https://doi.org/10.1155/2022/2941248.
Chen, J., Liu, H., 2020. Nutritional indices for assessing fatty acids: a mini review. Int. J. Mol. Sci. 21, 5695. https://doi.org/10.3390/ijms21165695.

Chen, H.H., Lin, H.T., Foung, Y.F., Lin, J.H.Y., 2012. The bioactivity of teleost IL-6: IL-6 protein in orange-spotted grouper (*Epinephelus coioides*) induces Th2 cell differentiation pathway and antibody production. Dev. Comp. Immunol. 38, 285–294. https://doi.org/10.1016/j.dci.2012.06.013.

Cheng, Z.J., Hardy, R.W., 2002. Apparent digestibility coefficients of nutrients and nutritional value of poultry by-product meals for rainbow trout *Oncorhynchus mykiss* measured *in vivo* using settlement. J. World Aquacult. Soc. 33, 458–465. https://doi.org/10.1111/j.1749-7345.2002.tb00025.x.

Chiang, J.Y., 2009. Hepatocyte nuclear factor 4α regulation of bile acid and drug metabolism. Expert Opin. Drug Metab. Toxicol. 5, 137–147. https://doi.org/10.1517/17425250802707342.

Dam, C.T.M., Booth, M., Pirozzi, I., Salini, M., Smullen, R., Ventura, T., Elizur, A., 2020. Alternative feed raw materials modulate intestinal microbiota and its relationship with digestibility in Yellowtail Kingfish *Seriola lalandi*. Fishes 5, 2020. https://doi.org/10.3390/fishes5020014.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.T., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350–356. https://doi.org/10.1021/ac60111a017.

Dumas, A., 1826. Ann. Chim. 33, 342.

Durník, R., Šindlerová, L., Babica, P., Jurček, O., 2022. Bile acids transporters of enterohepatic circulation for targeted drug delivery. Molecules 27, 2961. https://doi.org/10.3390/molecules27092961.

FAO, 2022. The State of World Fisheries and Aquaculture 2022. Towards Blue Transformation. FAO, Rome. https://doi.org/10.4060/cc0461en.

Fernández-Alacid, L., Firmino, J.P., Sanahuja, I., Madrid, C., Polo, J., de Borba, M.R., Balsalobre, C., Gisbert, E., Ibarz, A., 2021. Impact of dietary porcine blood by-products in meagre (*Argyrosomus regius*) physiology, evaluated by welfare biomarkers and the antibacterial properties of the skin mucus. Fish Shellfish Immunol. 118, 241–250. https://doi.org/10.1016/j.fsi.2021.09.011.

Firmino, J.P., Vallejos-Vidal, E., Sarasquete, C., Ortiz-Delgado, J.B., Balasch, J.C., Tort, L., Estevez, A., Reyes-López, F.E., Gisbert, E., 2020. Unveiling the effect of dietary essential oils supplementation in *Sparus aurata* gills and its efficiency against the infestation by *Sparicotyle chrysophrii*. Sci. Rep. 10, 17764. https://doi.org/10.1038/s41598-020-74625-5.

Firmino, J.P., Galindo-Villegas, J., Reyes-López, F.E., Gisbert, E., 2021. Phytogenic bioactive compounds shape fish mucosal immunity. Front. Immunol. 12, 695973 https://doi.org/10.3389/fimmu.2021.695973.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509. https://doi.org/10.1016/S0021-9258(18)64849-5.

Galindo-Villegas, J., Mulero, I., García-Alcazar, A., Muñoz, I., Peñalver-Mellado, M., Streitenberger, S., Scapigliati, G., Meseguer, J., Mulero, V., 2013. Recombinant TNFα as oral vaccine adjuvant protects European sea bass against vibriosis: insights into the role of the CCL25/CCR9 axis. Fish Shellfish Immunol. 35, 1260–1271. https://doi.org/10.1016/j.fsi.2013.07.046.

Ganesh, S., Cave, V., 2018. P-values, p-values everywhere! N. Z. Vet. J. 66, 55–56. https://doi.org/10.1080/00480169.2018.1415604.

Gazi, U., Martinez-Pomares, L., 2009. Influence of the mannose receptor in host immune responses. Immunobiology 214, 554–561. https://doi.org/10.1016/j.imbio.2008.11.004.

Ghanbari, M., Kneifel, W., Domig, K.J., 2015. A new view of the fish gut microbiome: advances from next-generation sequencing. Aquaculture 448, 464–475. https://doi.org/10.1016/j.aquaculture.2015.06.033.

Gisbert, E., Ortiz-Delgado, J.B., Sarasquete, C., 2008. Nutritional cellular biomarkers in early life stages of fish. Histol. Histopathol. 23, 1525–1539. https://doi.org/10.14670/HH-23.1525.

Gisbert, E., Giménez, G., Fernández, I., Kotzamanis, Y., Estévez, A., 2009. Development of digestive enzymes in common dentex *Dentex dentex* during early ontogeny. Aquaculture 287, 381–387. https://doi.org/10.1016/j.aquaculture.2008.10.039.

Glatz, J.F., van der Vusse, G.J., 1989. Intracellular transport of lipids. Mol. Cell. Biochem. 88, 37–44. https://doi.org/10.1007/BF00223421.

Grigorakis, K., 2007. Compositional and organoleptic quality of farmed and wild gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) and factors affecting it: a review. Aquaculture 272, 55–75. https://doi.org/10.1016/j.aquaculture.2007.04.062.

Heath, H., Qin, S., Rao, P., Wu, L., LaRosa, G., Kassam, N., Ponath, P.D., Mackay, C.R., 1997. Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. J. Clin. Invest. 99, 178–184. https://doi.org/10.1172/JCI119145.

Hedblom, G.A., Reiland, H.A., Sylte, M.J., Johnson, T.J., Baumler, D.J., 2018. Segmented filamentous bacteria–metabolism meets immunity. Front. Microbiol. 9, 1991. https://doi.org/10.3389/fmicb.2018.01991.

Herrero-Encinas, J., Blanch, M., Pastor, J.J., Mereu, A., Ipharraguerre, I.R., Menoyo, D., 2020. Effects of a bioactive olive pomace extract from *Olea europaea* on growth performance, gut function, and intestinal microbiota in broiler chickens. Poult. Sci. 99, 2–10. https://doi.org/10.3382/ps/pez467.

Hirano, S., Masuda, N., 1982. Enhancement of the 7 alpha-dehydroxylase activity of a gram-positive intestinal anaerobe by *Bacteroides* and its significance in the 7-dehydroxylation of ursodeoxycholic acid. J. Lipid Res. 23, 1152–1158. https://doi.org/10.1016/S0022-2275(20)38052-4.

Hu, H., Shao, W., Liu, Q., Liu, N., Wang, Q., Xu, J., Zhang, X., Weng, Z., Lu, Q., Jiao, L., Chen, C., Sun, H., Jiang, Z., Zhang, X., Gu, A., 2022. Gut microbiota promotes cholesterol gallstone formation by modulating bile acid composition and biliary cholesterol secretion. Nat. Commun. 13, 252. https://doi.org/10.1038/s41467-021-27758-8.

Huang, Y., Wang, S., Meng, X., Chen, N., Li, S., 2021. Molecular cloning and characterization of sirtuin 1 and its potential regulation of lipid metabolism and antioxidant response in largemouth bass (*Micropterus salmoides*). Front. Physiol. 12, 726877 https://doi.org/10.3389/fphys.2021.726877.

Huyben, D., Chiasson, M., Lumsden, J.S., Pham, P.H., Chowdhury, M.A.K., 2021. Dietary microencapsulated blend of organic acids and plant essential oils affects intestinal morphology and microbiome of rainbow trout (*Oncorhynchus mykiss*). Microorganisms 9, 2063. https://doi.org/10.3390/microorganisms9102063.

Illumina, 2013. 16S Metagenomic Sequencing Library Preparation: Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System. Available at: https://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf (accessed 11 November 2022).

Jamwal, S., Blackburn, J.K., Elsworth, J.D., 2021. PPARγ/PGC1α signaling as a potential therapeutic target for mitochondrial biogenesis in neurodegenerative disorders. Pharmacol. Ther. 219, 107705 https://doi.org/10.1016/j.pharmthera.2020.107705.

Kim, M.H., Park, S.J., Kim, J.H., Seong, J.B., Kim, K.M., Woo, H.A., Lee, D.S., 2018. Peroxiredoxin 5 regulates adipogenesis-attenuating oxidative stress in obese mouse models induced by a high-fat diet. Free Radic. Biol. Med. 123, 27–38. https://doi.org/10.1016/j.freeradbiomed.2018.05.061.

Kim, M.H., Seong, J.B., Huh, J.W., Bae, Y.C., Lee, H.S., Lee, D.S., 2020. Peroxiredoxin 5 ameliorates obesity-induced non-alcoholic fatty liver disease through the regulation of oxidative stress and AMP-activated protein kinase signaling. Redox Biol. 28, 101315 https://doi.org/10.1016/j.redox.2019.101315.

Kinsey, G.R., McHowat, J., Beckett, C.S., Schnellmann, R.G., 2007. Identification of calcium-independent phospholipase A2γ in mitochondria and its role in mitochondrial oxidative stress. Am. J. Physiol. Ren. Physiol. 292, F853–F860. https://doi.org/10.1152/ajprenal.00318.2006.

Kumagai, Y., Takeuchi, O., Akira, S., 2008. TLR9 as a key receptor for the recognition of DNA. Adv. Drug Deliv. Rev. 60, 795–804. https://doi.org/10.1016/j.addr.2007.12.004.

Leaver, M.J., Villeneuve, L.A., Obach, A., Jensen, L., Bron, J.E., Tocher, D.R., Taggart, J. B., 2008. Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*). BMC Genomics 9, 1–15. https://doi.org/10.1186/1471-2164-9-299.

Liu, J., Cao, S., Kim, S., Chung, E.Y., Homma, Y., Guan, X., Jimenez, V., Ma, X., 2005. Interleukin-12: an update on its immunological activities, signaling and regulation of gene expression. Curr. Immunol. Rev. 1, 119–137. https://doi.org/10.2174/1573395054065115.

Liu, C., Cui, Y., Li, X., Yao, M., 2021. *Microeco*: an R package for data mining in microbial community ecology. FEMS Microbiol. Ecol. 97, fiaa255. https://doi.org/10.1093/femsec/fiaa255.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Lozupone, C., Lladser, M.E., Knights, D., Stombaugh, J., Knight, R., 2011. UniFrac: an effective distance metric for microbial community comparison. ISME J. 5, 169–172. https://doi.org/10.1038/ismej.2010.133.

McCord, J.M., Fridovich, I., 1969. Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055. https://doi.org/10.1016/S0021-9258(18)63504-5.

McKnight, D.T., Huerlimann, R., Bower, D.S., Schwarzkopf, L., Alford, R.A., Zenger, K.R., 2019. Methods for normalizing microbiome data: an ecological perspective. Methods Ecol. Evol. 10, 389–400. https://doi.org/10.1111/2041-210X.13115.

Meyerholz, D.K., Beck, A.P., 2018. Fundamental concepts for semiquantitative tissue scoring in translational research. ILAR J. 59, 13–17. https://doi.org/10.1093/ilar/ily025.

Mollica Poeta, V., Massara, M., Capucetti, A., Bonecchi, R., 2019. Chemokines and chemokine receptors: new targets for cancer immunotherapy. Front. Immunol. 10, 379. https://doi.org/10.3389/fimmu.2019.00379.

Moretti, V.M., Corino, C., 2008. Omega-3 and trans fatty acids. In: Nollet, L.M.L., Toldra, F. (Eds.), Handbook of Processed Meats and Poultry Analysis. CRC Press, Crystal Bay, pp. 249–288.

Mougin, J., Joyce, A., 2022. Fish disease prevention via microbial dysbiosis-associated biomarkers in aquaculture. Rev. Aquac. 15, 579–594. https://doi.org/10.1111/raq.12745.

Muglia, C., Mercer, N., Toscano, M.A., Schattner, M., Pozner, R., Cerliani, J.P., Gobbi, R. P., Rabinovich, G.A., Docena, G.H., 2011. The glycan-binding protein galectin-1 controls survival of epithelial cells along the crypt-villus axis of small intestine. Cell Death Dis. 2, e163. https://doi.org/10.1038/cddis.2011.44.

Naya-Català, F., do Vale Pereira, G., Piazzon, M.C., Fernandes, A.M., Calduch-Giner, J.A., Sitjà-Bobadilla, A., Conceição, L.E.C., Pérez-Sánchez, J., 2021. Cross-talk between intestinal microbiota and host gene expression in gilthead sea bream (*Sparus aurata*) juveniles: insights in fish feeds for increased circularity and resource utilization. Front. Physiol. 12, 748265 https://doi.org/10.3389/fphys.2021.748265.

Naylor, R.L., Hardy, R.W., Buschmann, A.H., Bush, S.R., Cao, L., Klinger, D.H., Little, D. C., Lubchenco, J., Shumway, S.E., Troell, M., 2021. A 20-year retrospective review of global aquaculture. Nature 591, 551–563. https://doi.org/10.1038/s41586-021-03308-6.

Nolfi-Donegan, D., Braganza, A., Shiva, S., 2020. Mitochondrial electron transport chain: Oxidative phosphorylation, oxidant production, and methods of measurement. Redox Biol. 37, 101674 https://doi.org/10.1016/j.redox.2020.101674.

Panahi, Y., Ahmadi, Y., Teymouri, M., Johnston, T.P., Sahebkar, A., 2018. Curcumin as a potential candidate for treating hyperlipidemia: a review of cellular and metabolic mechanisms. J. Cell. Physiol. 233, 141–152. https://doi.org/10.1002/jcp.25756.

Pathak, M., Lal, G., 2020. The regulatory function of CCR9^{+} dendritic cells in inflammation and autoimmunity. Front. Immunol. 11, 536326 https://doi.org/10.3389/fimmu.2020.536326.
Pelusio, N.F., Rossi, B., Parma, L., Volpe, E., Ciulli, S., Piva, A., D'Amico, F., Scicchitano, D., Candela, M., Gatta, P.P., Bonaldo, A., Grilli, E., 2020. Effects of increasing dietary level of organic acids and nature-identical compounds on growth, intestinal cytokine gene expression and gut microbiota of rainbow trout (*Oncorhynchus mykiss*) reared at normal and high temperature. Fish Shellfish Immunol. 107, 324–335. https://doi.org/10.1016/j.fsi.2020.10.021.
Pérez-Cordón, G., Estensoro, I., Benedito-Palos, L., Calduch-Giner, J.A., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2014. Interleukin gene expression is strongly modulated at the local level in a fish-parasite model. Fish Shellfish Immunol. 37, 201–208. https://doi.org/10.1016/j.fsi.2014.01.022.
Piazzon, M.C., Calduch-Giner, J.A., Fouz, B., Estensoro, I., Simó-Mirabet, P., Puyalto, M., Karalazos, V., Palenzuela, O., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2017. Under control: how a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. Microbiome 5, 1–23. https://doi.org/10.1186/s40168-017-0390-3.
Piazzon, M.C., Naya-Català, F., Simó-Mirabet, P., Picard-Sánchez, A., Roig, F.J., Calduch-Giner, J.A., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2019. Sex, age, and bacteria: how the intestinal microbiota is modulated in a protandrous hermaphrodite fish. Front. Microbiol. 10, 2512. https://doi.org/10.3389/fmicb.2019.02512.
Piazzon, M.C., Naya-Català, F., Perera, E., Palenzuela, O., Sitjà-Bobadilla, A., Pérez-Sánchez, J., 2020. Genetic selection for growth drives differences in intestinal microbiota composition and parasite disease resistance in gilthead sea bream. Microbiome 8, 1–17. https://doi.org/10.21203/rs.3.rs-52276/v1.
Platel, K., Srinivasan, K., 2004. Digestive stimulant action of spices: a myth or reality? Indian J. Med. Res. 119, 167–179.
Platel, K., Rao, A., Saraswathi, G., Srinivasan, K., 2002. Digestive stimulant action of three Indian spice mixes in experimental rats. Nahrung 46, 394–398. https://doi.org/10.1002/1521-3803(20021101)46:6<394::AID-FOOD394>3.0.CO;2-D.
Romano, N., Kumar, V., Yang, G., Kajbaf, K., Rubio, M.B., Overturf, K., Brezas, A., Hardy, R., 2020. Bile acid metabolism in fish: disturbances caused by fishmeal alternatives and some mitigating effects from dietary bile inclusions. Rev. Aquac. 12, 1792–1817. https://doi.org/10.1111/raq.12410.
Rombenso, A.N., Turchini, G.M., Trushenski, J.T., 2022. The omega-3 sparing effect of saturated fatty acids: a reason to reconsider common knowledge of fish oil replacement. Rev. Aquac. 14, 213–217. https://doi.org/10.1111/raq.12593.
Rosenlund, G., Corraze, G., Izquierdo, M., Torstensen, B.E., 2010. The effects of fish oil replacement on nutritional and organoleptic qualities of farmed fish. In: Turchini, G. M., Ng, W.K., Tocher, D.R. (Eds.), Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds. CRC Press, Florida, pp. 487–522.
Ruiz, A., Andree, K.B., Sanahuja, I., Holhorea, P.G., Calduch-Giner, J.A., Morais, S., Pastor, J.J., Pérez-Sánchez, J., Gisbert, E., 2023a. Bile salt dietary supplementation promotes growth and reduces body adiposity in gilthead seabream (*Sparus aurata*). Aquaculture 566, 739203. https://doi.org/10.1016/j.aquaculture.2022.739203.
Ruiz, A., Andree, K.B., Furones, D., Holhorea, P.G., Calduch-Giner, J.À., Viñas, M., Pérez-Sánchez, J., Gisbert, E., 2023b. Modulation of gut microbiota and intestinal immune response in gilthead seabream (*Sparus aurata*) by dietary bile salt supplementation. Front. Microbiol. 14, 1123716. https://doi.org/10.3389/fmicb.2023.1123716.
Sakai, M., Hikima, J.I., Kono, T., 2021. Fish cytokines: current research and applications. Fish. Sci. 87, 1–9. https://doi.org/10.1007/s12562-020-01476-4.
Sakyi, M.E., Cai, J., Ampofo-Yeboah, A., Anokyewaa, M.A., Wang, Z., Jian, J., 2021. Starvation and re-feeding influence the growth, immune response, and intestinal microbiota of Nile tilapia (*Oreochromis niloticus*; Linnaeus 1758). Aquaculture 543, 736959. https://doi.org/10.1016/j.aquaculture.2021.736959.
Salmerón, C., 2018. Adipogenesis in fish. J. Exp. Biol. 221 https://doi.org/10.1242/jeb.161588 jeb161588.
Salomón, R., Furones, M.D., Reyes-López, F.E., Tort, L., Firmino, J.P., Esteban, M.A., Espinosa Ruíz, C., Quintela, J.C., Pinilla-Rosas, J.M., Vallejos-Vidal, E., Gisbert, E., 2021. A bioactive extract rich in triterpenic acid and polyphenols from *Olea europaea* promotes systemic immunity and protects Atlantic salmon smolts against furunculosis. Front. Immunol. 12, 737601 https://doi.org/10.3389/fimmu.2021.737601.
Sampath, H., Ntambi, J.M., 2005. The fate and intermediary metabolism of stearic acid. Lipids 40, 1187–1191. https://doi.org/10.1007/s11745-005-1484-z.
Schubert, K., Olde Damink, S.W., von Bergen, M., Schaap, F.G., 2017. Interactions between bile salts, gut microbiota, and hepatic innate immunity. Immunol. Rev. 279, 23–35. https://doi.org/10.1111/imr.12579.
Schug, T.T., Li, X., 2011. Sirtuin 1 in lipid metabolism and obesity. Ann. Med. 43, 198–211. https://doi.org/10.3109/07853890.2010.547211.
Sepehri, Z., Kiani, Z., Nasiri, A.A., Kohan, F., 2016. Toll-like receptor 2 and type 2 diabetes. Cell. Mol. Biol. Lett. 21, 1–9. https://doi.org/10.1186/s11658-016-0002-4.
Serna-Duque, J.A., Esteban, M.Á., 2020. Effects of inflammation and/or infection on the neuroendocrine control of fish intestinal motility: a review. Fish Shellfish Immunol. 103, 342–356. https://doi.org/10.1016/j.fsi.2020.05.018.
Seropian, I.M., González, G.E., Maller, S.M., Berrocal, D.H., Abbate, A., Rabinovich, G.A., 2018. Galectin-1 as an emerging mediator of cardiovascular inflammation: mechanisms and therapeutic opportunities. Mediat. Inflamm. 2018, 8696543. https://doi.org/10.1155/2018/8696543.
Shao, Y., Che, Z., Xing, R., Wang, Z., Zhang, W., Zhao, X., Jin, C., Li, C., 2018. Divergent immune roles of two fucolectin isoforms in *Apostichopus japonicus*. Dev. Comp. Immunol. https://doi.org/10.1016/j.dci.2018.07.028.
Sharif, P.M., Jabbari, P., Razi, S., Keshavarz-Fathi, M., Rezaei, N., 2020. Importance of TNF-alpha and its alterations in the development of cancers. Cytokine 89, 1–6. https://doi.org/10.1016/j.cyto.2020.155066.
Shepherd, J., Bachis, E., 2014. Changing supply and demand for fish oil. Aquac. Econ. Manag. 18, 395–416. https://doi.org/10.1080/13657305.2014.959212.
Singh, C.K., Chhabra, G., Ndiaye, M.A., Garcia-Peterson, L.M., Mack, N.J., Ahmad, N., 2018. The role of sirtuins in antioxidant and redox signaling. Antioxid. Redox Signal. 28, 643–661. https://doi.org/10.1089/ars.2017.7290.
Skalli, A., Firmino, J.P., Andree, K.B., Salomón, R., Estévez, A., Puig, P., Sabater-Martínez, M., Hechavarria, T., Gisbert, E., 2020. The inclusion of the microalga *Scenedesmus* sp. in diets for rainbow trout, *Onchorhynchus mykiss*, juveniles. Animals 10, 1656. https://doi.org/10.3390/ani10091656.
Smith, P.E., Waters, S.M., Gómez Expósito, R., Smidt, H., Carberry, C.A., McCabe, M.S., 2020. Synthetic sequencing standards: a guide to database choice for rumen microbiota amplicon sequencing analysis. Front. Microbiol. 11, 606825 https://doi.org/10.3389/fmicb.2020.606825.
Solovyev, M., Gisbert, E., 2016. Influence of time, storage temperature and freeze/thaw cycles on the activity of digestive enzymes from gilthead sea bream (*Sparus aurata*). Fish Physiol. Biochem. 42, 1383–1394. https://doi.org/10.1007/s10695-016-0226-2.
Srinivasan, K., 2005. Spices as influencers of body metabolism: an overview of three decades of research. Food Res. Int. 38, 77–86. https://doi.org/10.1016/j.aquaculture.2020.735291.
Tan, P., Dong, X., Mai, K., Xu, W., Ai, Q., 2016. Vegetable oil induced inflammatory response by altering TLR-NF-κB signalling, macrophages infiltration and polarization in adipose tissue of large yellow croaker (*Larimichthys crocea*). Fish Shellfish Immunol. 59, 398–405. https://doi.org/10.1016/j.fsi.2016.11.009.
Tapia-Paniagua, S.T., Balebona, M.D.C., Firmino, J.P., Rodríguez, C., Polo, J., Moriñigo, M.A., Gisbert, E., 2020. The effect of spray-dried porcine plasma on gilthead seabream (*Sparus aurata*) intestinal microbiota. Aquac. Nutr. 26, 801–811. https://doi.org/10.1111/anu.13039.
Tocher, D.R., 2015. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. Aquaculture 449, 94–107. https://doi.org/10.1016/j.aquaculture.2015.01.010.
Torstensen, B.E., Tocher, D.R., 2010. The effects of fish oil replacement on lipid metabolism of fish. In: Turchini, G.M., Ng, W.K., Tocher, D.R. (Eds.), Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds. CRC Press, Florida, pp. 405–438.
Turchini, G.M., Torstensen, B.E., Ng, W.K., 2009. Fish oil replacement in finfish nutrition. Rev. Aquac. 1, 10–57. https://doi.org/10.1111/j.1753-5131.2008.01001.x.
Van der Oost, R., Beyer, J., Vermeulen, N.P., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149. https://doi.org/10.1016/s1382-6689(02)00126-6.
Van Heel, D.A., 2006. Interleukin 15: its role in intestinal inflammation. Gut 55, 444–445. https://doi.org/10.1136/gut.2005.079335.
Walczak, R., Tontonoz, P., 2002. PPARadigms and PPARadoxes: expanding roles for PPARγ in the control of lipid metabolism. J. Lipid Res. 43, 177–186. https://doi.org/10.1016/S0022-2275(20)30159-0.
Wang, X., Zhang, A., Qiu, X., Yang, K., Zhou, H., 2023. The IL-12 family cytokines in fish: Molecular structure, expression profile and function. Dev. Comp. Immunol. 104643 https://doi.org/10.1016/j.dci.2023.104643.
Wei, X., Li, B., Wu, L., Yin, X., Zhong, X., Li, Y., Wang, Y., Guo, Z., Ye, J., 2018. Interleukin-6 gets involved in response to bacterial infection and promotes antibody production in Nile tilapia (*Oreochromis niloticus*). Dev. Comp. Immunol. 89, 141–151. https://doi.org/10.1016/j.dci.2018.08.012.
Weil, C., Lefèvre, F., Bugeon, J., 2013. Characteristics and metabolism of different adipose tissues in fish. Rev. Fish Biol. Fish. 23, 157–173. https://doi.org/10.1007/s11160-012-9288-0.
Westerterp-Plantenga, M., Diepvens, K., Joosen, A.M., Bérubé-Parent, S., Tremblay, A., 2006. Metabolic effects of spices, teas, and caffeine. Physiol. Behav. 89, 85–91. https://doi.org/10.1016/j.physbeh.2006.01.027.
White, J.R., Nagarajan, N., Pop, M., 2009. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. PLoS Comput. Biol. 5, e1000352 https://doi.org/10.1371/journal.pcbi.1000352.
Xie, D., Chen, C., Dong, Y., You, C., Wang, S., Monroig, O., Tocher, D.R., Li, Y., 2021. Regulation of long-chain polyunsaturated fatty acid biosynthesis in teleost fish. Prog. Lipid Res. 82, 101095 https://doi.org/10.1016/j.plipres.2021.101095.
Yengkokpam, S., Debnath, D., Pal, A.K., Sahu, N.P., Jain, K.K., Norouzitallab, P., Baruah, K., 2013. Short-term periodic feed deprivation in *Labeo rohita* fingerlings: effect on the activities of digestive, metabolic and anti-oxidative enzymes. Aquaculture 412, 186–192. https://doi.org/10.1016/j.aquaculture.2013.07.025.
Zhao, A., Yu, J., Lew, J.L., Huang, L., Wright, S.D., Cui, J., 2004. Polyunsaturated fatty acids are FXR ligands and differentially regulate expression of FXR targets. DNA Cell Biol. 23, 519–526. https://doi.org/10.1089/1044549041562267.
Zhou, J.S., Chen, H.J., Ji, H., Shi, X.C., Li, X.X., Chen, L.Q., Du, Z.Y., Yu, H.B., 2018. Effect of dietary bile acids on growth, body composition, lipid metabolism and microbiota in grass carp (*Ctenopharyngodon idella*). Aquac. Nutr. 24, 802–813. https://doi.org/10.1111/anu.12609.
Zhu, T., Corraze, G., Plagnes-Juan, E., Skiba-Cassy, S., 2020. Cholesterol metabolism regulation mediated by SREBP-2, LXRα and miR-33a in rainbow trout (*Oncorhynchus mykiss*) both *in vivo* and *in vitro*. PLoS One 15, e0223813. https://doi.org/10.1371/journal.pone.0223813.
Zou, J., Secombes, C.J., 2016. The function of fish cytokines. Biology 5, 23. https://doi.org/10.3390/biology5020023.
Zou, J., Redmond, A.K., Qi, Z., Dooley, H., Secombes, C.J., 2015. The CXC chemokine receptors of fish: insights into CXCR evolution in the vertebrates. Gen. Comp. Endocrinol. 215, 117–131. https://doi.org/10.1016/j.ygcen.2015.01.004.

CHAPTER 5

Impact of the diet in the gut microbiota after an inter-species microbial transplantation in fish

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Impact of the diet in the gut microbiota after an inter-species microbial transplantation in fish

Alberto Ruiz✉, Enric Gisbert & Karl B. Andree

Inter-species microbial transplantations offer the possibility of transferring species-specific microbes and their associated functionality. As a conceptual approach, an intestinal microbiota transplant (IMT) between two marine carnivorous fish species that thrive in different environmental conditions was conducted: from donor Atlantic salmon (*Salmo salar*) to recipient gilthead seabream (*Sparus aurata*), after obliterating its basal microbiota with an antibiotic treatment. To confirm that the gut microbiota was able to recover after antibiotics without the influence of the diet, a group of gilthead seabream not submitted to the IMT was kept fasted as an internal control. To assess the effect of the diet after the IMT, two groups of gilthead seabream were respectively fed with their typical diet and with Atlantic salmon diet. At 36 days post-IMT, the gut of the individuals fed with their typical diet was dominated by the feed-associated bacteria, while those fed with the salmon diet had developed a unique microbiota from the convergence of the diet, donor, and recipient microbiota. These results suggested that an intestinal microbiota transplantation may be effective if the basal microbiota from the gut is first cleared and a targeted dietary modification is provided to maintain and enrich the novel bacteria species over time.

The gastrointestinal tract is one of the most densely populated ecosystems on our planet where the microbial populations inhabiting it have developed tight relationships with the host over millions of years of co-evolution[1], constituting what is known by many as a holobiont. In this sense, the intestinal microbiota has a major impact on the host health through a wide range of functions, such as feed digestion, nutrient metabolism, energy homeostasis, immune system modulation, barrier function and mucosal integrity, among others[2]. Moreover, an impairment in microbial composition or in the host-microbe interactions (dysbiosis) can lead to digestive and systemic imbalances and diseases[2,3]. These host-microbiota interactions are reciprocal. For instance, the bacterial community can stimulate the immune system by their pathogen-associated molecular patterns (PAMPs) that are recognized through pattern recognition receptors (PRRs) which subsequently activate immune signaling pathways; and in turn the host shapes the microbial composition, regulating the abundance of some bacterial communities considered as potential pathogens[4]. Basic to this, it may be thought that the microbiota of any animal is well-tuned to the host species, but this is not an inviolably established fact. Recent studies differentiate between the core microbiota, which is the one comprising stable, permanent, and usually highly abundant members, which persist regardless time and changing factors, and the non-core microbiota, which is transient and modulable[5]. Thus, understanding the intricate relationships between the gut microbiota and the host and their modulation may provide opportunities to promote and ensure a healthy microbiota as well as evaluate its beneficial effects to the host.

Apart from specific and non-specific host factors, several elements can influence the gut microbiota in humans and animals, such as the environmental conditions (i.e., pH, oxygen, temperatures), the age, the diet, the host's habits, such as physical activity, the presence of diseases and the treatments of those diseases[6–8]. In animal production, as a result of the necessity to reduce antibiotic use, several alternatives to improve animal health through gut microbial modulation have been tested and/or consolidated, such as the application of quorum quenching, anti-microbial peptides, feed additives, among other supplements, including exogenous enzymes, probiotics, and prebiotics, alone or in combination (i.e., synbiotics)[9]. In addition to the above-mentioned strategies, fecal microbiota transplants (FMT) have recently gained attention, as it has the advantage of being a persistent strategy that does not require repeated supply or application[6], and the advent of metagenomic analyses has given to the field more rigor. Numerous applications in humans and animals involving this strategy have been developed over the last decades[1]. The more common and historical use of FMT is for the establishment of a healthy microbiota into

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a diseased individual to improve the host health. For instance, human FMT is a well-proven effective treatment against *Clostridium difficile* infections[10] and clinical trials have also shown promising advances in the treatment of inflammatory bowel diseases, metabolic and neurological disorders, as well as autoimmune diseases[11]. Apart from its therapeutic use in humans, FMT has also been extensively tested with production purposes in the livestock industry. For instance, ruminal transfaunation (transplant of the rumen microbial content) is commonly used to restore digestive and metabolic disorders, and to improve milk production in ruminants[12]. Likewise, inter-species transfaunation of the rumen from bison to cattle have shown to enhance protein digestibility[13]. On the other hand, such successful results have not always been obtained for the swine and poultry industry[1]. In this sense, the fact that the target of the FMT are the stool-associated microorganisms, which are mainly the large-intestinal microbes, rather than the whole-intestinal microbiota, may raise the question "to what extent is FMT effective?"[14]. Fortunately, the ease of handling smaller animals, such as fish, allows the collection of all the microbiota found in the intestine rather than just that from the feces, and as such may be more accurately termed as "Intestinal Microbiota Transplant (IMT)".

To date, some progress has been made regarding gut microbial transplants in teleosts. In particular, recent assays in African turquoise killifish (*Nothobranchius furzeri*), zebrafish (*Danio rerio*), and large yellow croaker (*Larimichthys crocea*), have shown promising results in IMT and FMT as successful strategies to improve host's longevity, growth and reproductive performance, digestive capacities, intestinal health, endocrine resilience against exposure to environmental pollutants, and gut microbial diversity, among others[15–17]. Similarly, the work of Legrand et al.[18] in yellowtail kingfish (*Seriola lalandi*) has shed light on the short-term modulation of the gut microbiota by FMT after an antibiotic treatment. Furthermore, zebrafish has been proposed as a model organism to be colonized by bacteria from human feces to study the interactions between the human and zebrafish microbiota[19,20], even though few taxa were established in the recipient gut. Analogously, when transplanting mouse microbiota into germ-free zebrafish, although the observed phyla resembled those from the donor, their relative abundances were more similar to those of the recipient individuals before transplantation[21]. The former authors stated that these differences in microbial composition were partly imposed by the differential pressures inherent to host-specific gut habitats[21]. This hypothesis was associated to the fact that the above-mentioned vertebrate species have a divergent evolutionary development among others. However, to date there are no works studying the effect from performing an IMT between two different fish species that naturally thrive in different environmental conditions on the gut bacterial communities.

As a conceptual approach, the present study aimed to provide insight into the influence of host and donor gut bacterial communities by carrying out an inter-species IMT using as models two important aquaculture marine fish species[22] that are carnivorous, but they grow at different temperatures: the Atlantic salmon (*Salmo salar*) and the gilthead seabream (*Sparus aurata*). Furthermore, this study also aimed to elucidate the impact of diet in the phylogenetic flux and dynamics of the microbiota over time in terms of bacterial diversity, structure, and composition.

Methods

Fish husbandry and diets

Under current experimental conditions, Atlantic salmon were used as donors of intestinal microbiota, whereas gilthead seabream were chosen as recipients. In particular, Atlantic salmon with an initial body weight (BW_i) of 55.0 ± 0.1 g (mean ± SD) were purchased from SARL SALMO (Gonneville-Le Theil, France), while gilthead seabream with BW_i = 100.2 ± 0.9 g were obtained from Niordseas S.L. (Villajoyosa, Spain), and both were transported by road to the facilities of IRTA, La Ràpita (Tarragona, Spain). Atlantic salmon parrs were smoltified as described in Salomón et al.[23]. Then, salmon smolts were placed in 2000 L-tanks connected to a water recirculation system (IRTAmar™, Spain) and maintained at a water temperature, pH (pH meter 507, Crison Instruments, Barcelona, Spain) and dissolved oxygen (OXI330, Crison Instruments) of 12.1 ± 0.2 °C, 7.4 ± 0.3, and 9.5 ± 0.2 mg/L respectively, under natural photoperiod (8 h light: 16 h darkness) until the beginning of the experiment. Gilthead seabreams were placed in 2000 L-tanks connected to an IRTAmar™ system and water quality parameters were kept at 19.9 ± 2.3 °C, 7.6 ± 0.4, and 6.3 ± 0.6 mg/L. During this period, fish were fed ad libitum with two different feeds; in particular, Atlantic salmon were fed with an experimental diet containing 40% crude protein, 22% crude fat, and 21.6 MJ/kg digestible energy (2–3 mm pellet size; salmon diet)[23], whereas gilthead seabream were fed with an experimental compound feed (3–5 mm pellet size) containing 44% crude protein, 20% lipids, and 18 MJ/kg digestible energy (GSB diet).

Antimicrobial treatment and establishment of baseline criteria

A mixture of antimicrobials (AMs) containing Vancomycin (0.02 g/L), Metronidazole (1.0 g/L), Neomycin (1.0 g/L) and Ampicillin (1.0 g/L) was prepared according to Smith et al.[15], although the concentration for each antibiotic was doubled to compensate for the larger sized fish used herein. The aim of supplying this antimicrobial cocktail to the recipient gilthead seabream prior to the IMT was for eliminating/reducing the host-resident bacterial communities.

To decide the time after the AM treatment at which the intestinal microbiota from the donor salmon should be transplanted into the recipient gilthead seabream the recovery time of the gut-bacterial communities from gilthead seabream after the AMs was evaluated by their growth in TSA (Trypticase Soy Agar) + 0.6% NaCl culture media. Furthermore, this brief assay confirmed the effectiveness of the AM cocktail for elimination of the resident microbiota of the donor fish. For that purpose, three gilthead seabream that had been fasted overnight were hand-netted and anesthetized with buffered tricaine methanesulfonate (MS-222, Sigma-Aldrich, Spain; 100 mg/L). Then, an aliquot of 1 mL of the mixture of AMs was administered by both anal and oral gavage using a 5 mL syringe connected to a cannula (ø = 1.7 mm; ref. 340–6402, Izasa, Spain) (Fig. 1). Orally, the cannula

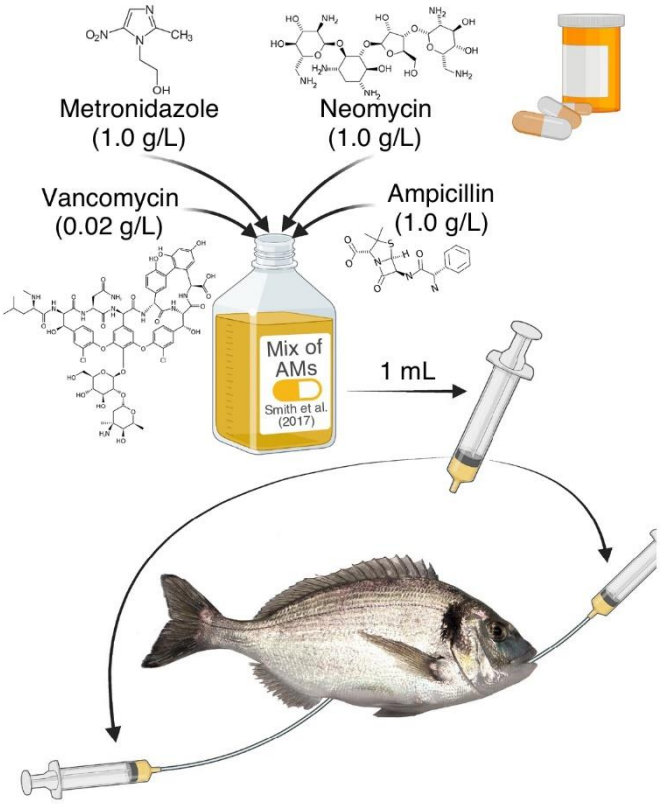

Figure 1. Schematic representation of the composition and administration of the antimicrobial mixture to recipient gilthead seabream (*Sparus aurata*). Created with BioRender.com.

was inserted until the stomach, while anal insertion was *ca.* 2–3 cm towards anterior direction, reaching the mid-posterior intestinal region. After 24 h, the three fish were euthanized in a bucket of water containing an overdose of buffered tricaine methanesulfonate (MS-222, Sigma-Aldrich, Spain; 300 mg/L) and their intestines were extracted, from just after the pyloric caeca to the anus, under sterile conditions. The three intestines were gently stripped with autoclaved tweezers, and the chyme collected from each one was pooled into a 50 mL tube with 10 mL of sterile PBS held on ice. After stripping, the intestines were opened lengthwise and the content from the mucosal layers were insistently but gently scraped with a round edge spatula and pooled into the same 50 mL tubes with the chyme. The remaining intestinal tissue was cut into small pieces of *ca.* 1 cm, placed into 5 mL of sterile PBS in a separate tube, shaken vigorously for 2 min; then the tissue pieces were removed, and the liquid was added to the tube containing the chyme and mucosa collected previously. To homogenize the samples, the content of the tube was transferred to a sterile stomacher bag and triturated at maximum speed for 3 min (Stomacher *Lab-Blender* 400, Seward, United Kingdom) (Fig. 2). An aliquot of 100 µL was spread onto TSA + 0.6% NaCl, which was used as a general medium for the growth of bacteria and fungi, and incubated at 22 °C. Bacterial growth was checked at 24 h. The same protocol was followed with three gilthead seabream that did not receive the mixture of AMs as a control to compare normal bacterial growth in culture media to the AM-treated samples. The process was repeated at 48 h post AM-treatment and growth evaluated after 24 h at 22 °C.

Additionally, to ensure the absence of effects from potential antimicrobial residues in the blood of gilthead seabream, 24 h after the AM gavage three individuals were randomly netted and anesthetized for blood collection via caudal vein with 1 mL heparinized syringes (ref. 303,179, BD Plastik, Canada) and the plasma was separated from the blood by centrifugation (1600×*g*, 10 min, 4 °C). A volume of 100 µL from different plasma dilutions (1:2, 1:10, and 1:20) were placed in equidistant 5 mm diameter-wells made on the surface of Mueller–Hinton media in which a lawn of *E. coli* had previously been spread and the plates were incubated at 22 °C.

A separate group of gilthead seabream (n = 15) were kept fasted during 17 days after the AM administration for testing the effect of the AM treatment on the gut bacterial communities and to record the establishment of the microbiota over time, regardless of the influence of the diet. For this purpose, intestinal samples from gilthead seabream pre-AMs and at 24 h, 8 days and 17 days post-AMs that were continuously fasted during all the period, were collected as described above (chyme + mucosal content + liquid after tissue shaking; Fig. 2) and frozen at − 80 °C until DNA extraction (n = 3 for gilthead seabream pre- and 24 h post-AMs; n = 6 for 8 and 17 days post-AMs since fasting produced very little microbial content).

Intestinal microbiota transplant

For the preparation of the IMT, 30 Altantic salmon (BW = 240.2 ± 19.3 g) were euthanized with an overdose of MS-222 (> 150 mg/L MS-222) and their intestinal content was extracted following the above-described procedure (Fig. 2), but in this case each sample was resuspended in 10 mL of 0.9% PBS and 10% glycerol, rather than only in PBS, following the recommendations of Quaranta et al.[24] for handling practices for microbial transplants. Three randomly chosen samples were stored at − 80 °C for DNA extraction, and the rest of the samples were pooled in groups of five and homogenized together in a stomacher (*Lab-Blender* 400), then mixed all together in a sterile 1 L-bottle by constant shaking at 4 °C. This suspension was filtered through sterile gauze into another bottle for reducing the amount of residual partially digested food that might clog the cannula during administration to the recipient fish. The filtered homogenate was immediately frozen at − 80 °C until IMT.

A total of 50 gilthead seabream (BW = 570.5 ± 58.3 g) were anesthetized with 100 mg/L MS-222 and the mixture of AMs was orally and anally administered as described in Fig. 1. Then, fish were randomly separated into two different 2000-L tanks (n = 25), as each tank would be assigned to a diet (salmon and GSB diets). After 24 h, all the specimens from each tank were netted, anesthetized and the thawed bacterial suspension collected from salmon was brought to ambient temperature and administered via oral and anal gavages (1 mL each route) using a cannula as described above. After the IMT, all gilthead sea breams were returned to their respective tanks. No mortality derived from fish handling and IMT was observed.

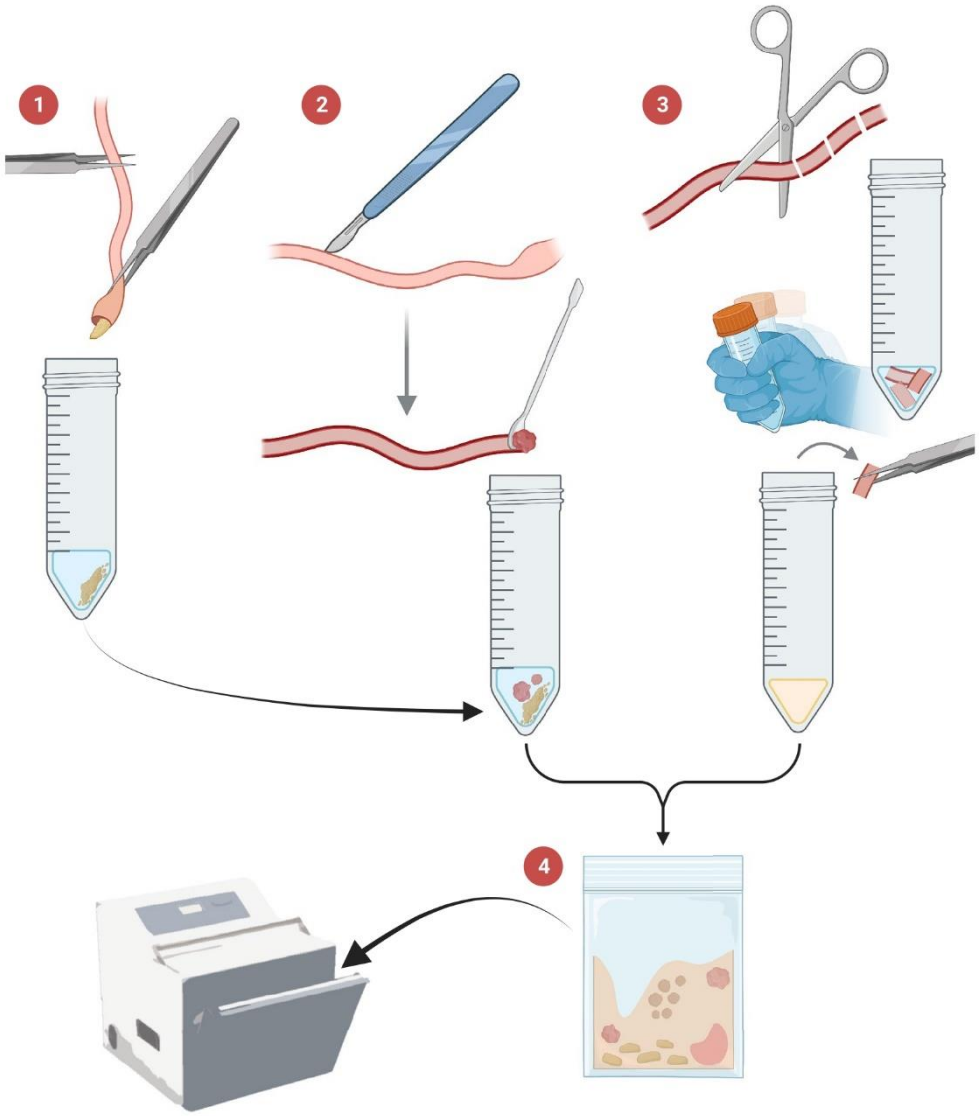

Figure 2. Schematic representation of the steps followed for intestinal sampling of bacteria: 1, stripping for chyme collection; 2, scraping for collection of mucosal content; 3, gathering of bacteria still associated to the tissue by shaking; and 4, pooling and homogenization in a Stomacher. Created with BioRender.com.

Assessment of the diet impact in the establishment of the bacterial communities after the intestinal microbiota transplant

To assess the effect of the diet in the establishment and/or maintenance of bacterial communities after the IMT, gilthead seabream from the first tank continued being fed with the GSB diet, while those from the second tank received the salmon experimental diet. Fish in both tanks were fed by hand three times per day at a feed ratio of 1.5% of the stocked biomass for 36 days. Samples from intestinal content were obtained at different times to study the progression of development over time of the diversity, structure, and composition of the bacterial communities. Sample collection was performed at 2, 7, 16 and 36 days post-IMT (n = 6 per dietary treatment at each time, n = 7 at final time; Fig. 3). At each sampling point after the 2 days, fish BW was measured. Three samples of each diet (salmon and GSB) were also analyzed to compare the microbiota present in them with that from the gut samples.

DNA extraction and 16S rRNA gene amplification for sequencing

Extractions of DNA were performed following the manufacturer instructions with the DNeasy PowerSoil Pro Kit (ref. 47,016, QIAGEN, Germany). The concentrations and purity of extracted DNA were evaluated by means of a Nanodrop-2000® spectrophotometer (Thermo Fisher Scientific, USA). The DNA concentrations were higher than 20 ng/µL and the A_{260}/A_{280} absorbance ratios were higher than 1.80.

The V3-V4 region of the 16S rRNA gene was amplified with the primers 341F (5′-CCTACGGGNGGCWGC AG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) according to the 16S Metagenomic Sequencing Library Preparation guide[25]. First PCR was performed with Q5® High-Fidelity DNA Polymerase (ref. M0491L, New England BioLabs, USA) using the following programme: a step of 30 s at 98 °C for initial denaturation and polymerase activation, followed by 30 cycles of 10 s at 98 °C, 30 s at 55 °C, 30 s at 72 °C, and 2 min at 72 °C of final extension. After that, a second amplification of 8 cycles was run in order to add the specific barcodes to the templates. The amplified region was then sequenced on an Illumina MiSeq Platform (2 × 300 bp paired-end).

Data analyses and statistics

Forward and reverse primers were removed from the *fastq* files by means of the Cutadapt tool in the open-source software QIIME2 (v2022.2)[26]. A workflow based on the R package DADA2 (v1.24.0), which is applied to model and correct Illumina-sequenced amplicon errors[27], was carried out. In brief, reads were subjected to quality filtering, excluding those with a Phred quality score < 28; then, paired-end reads were merged and the sequences with an overlap length < 12 nucleotides, more than 0 mismatches, or identified as chimeras were also removed. During this process, six samples were discarded from the analysis (one from the group gilthead seabream 8 days

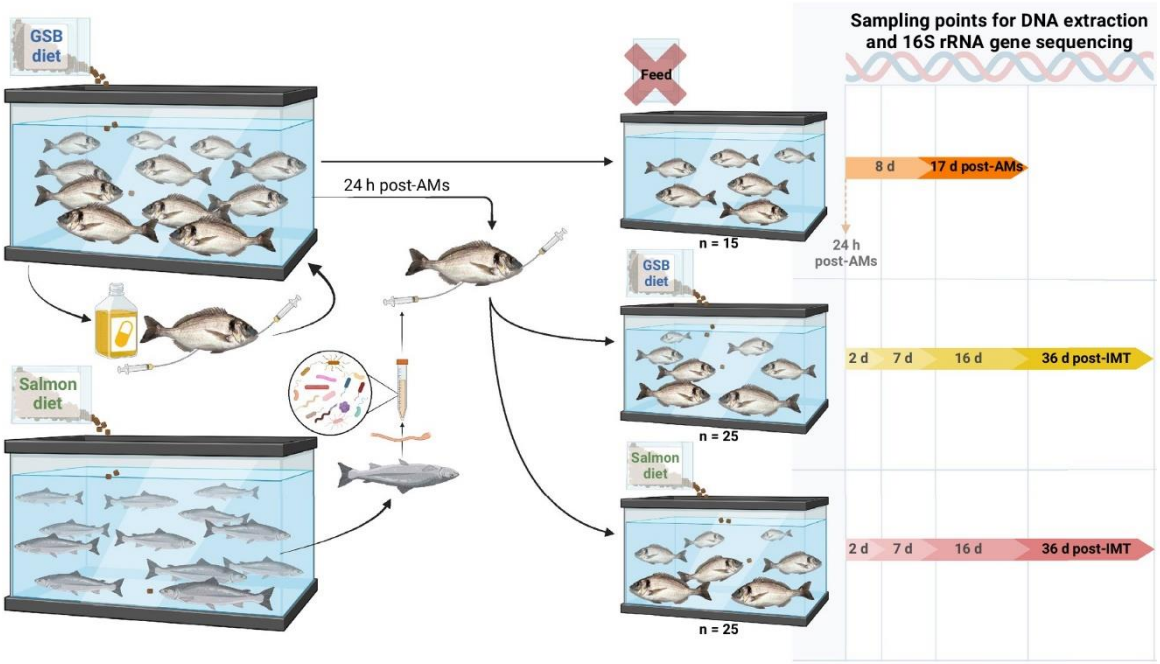

Figure 3. Schematic representation of the intestinal microbiota transplant (IMT) from Atlantic salmon (*Salmo salar*) to gilthead seabream (*Sparus aurata*) and subsequent nutritional assay carried out for assessing the effect of the diet in the gut bacterial communities. After the antimicrobial (AM) treatment, one group of gilthead seabream (n = 15) was fasted for 17 days in order to assess the effect of AMs in the absence of dietary influences. The rest of the gilthead seabream (n = 50) were submitted to the IMT 24 h post-AMs, and in order to assess the effect of the diet, gilthead seabream given the IMT were divided in two tanks (n = 25 per tank): one which continued with the GSB diet, and the other fed with the salmon diet. Created with BioRender.com.

post-AMs, two from the group 17 days post-AMs, one from the group fed the GSB diet 2 days post-IMT, and two from the group fed the salmon diet 2 days post-IMT). DADA2 resolves differences at the single-nucleotide level and the end products are amplicon sequence variant (ASVs).

Bacterial taxonomy was assigned using the SILVA database (v138.1) as a reference library. A bootstrapping confidence of 80% was established as a cut-off to be considered as a reliable assignment[28]; otherwise, ASVs were classified as unassigned. Template rarefaction curves were obtained using the R package vegan (v2.6-4). Then, all samples were rarefied to the lowest sample depth by subsampling 15,993 reads per sample, which was a representative sample size of the different ASVs occurring in the samples (Supplementary Fig. S1), and normalized by total sum scaling[29] to calculate the alpha diversity indices of ACE, Shannon, and Faith's phylogenetic diversity[30,31]. Significant differences in these indices among groups were calculated using the Kruskal–Wallis test followed by the Wilcoxon *post-hoc* test ($P \leq 0.05$). With the values of alpha diversity obtained from gilthead seabream fed their initial diet and the salmon diet, a linear mixed model was first performed taking the variables diet and time as fixed factors and the sampling order as a random factor, for testing the significant effect of these variables and their interaction, by means of the lme4 package[32]. The association between alpha diversity values and time was analysed with Spearman's correlation coefficient (r_s; $P \leq 0.05$). For beta diversity and relative abundance, data was normalized by cumulative sum scaling (CSS) which scales counts by dividing the sum of each sample's counts up to and including a percentile quantile in order to avoid the bias that may be introduced by preferential amplification or sequencing of specific sequences[33]. In this study, we scaled the counts by the 50th percentile of the number of ASVs in each sample for normalization by means of the package metagenomeSeq (v1.38.0)[34]. Beta diversity was approached with the quantitative metrics of Bray–Curtis distance[35] and weighted UniFrac distance, which was used to estimate similarities among samples based on the phylogenetic relationships of their ASVs[36]. The phylogenetic distances among ASVs used to calculate the indices of Faith's phylogenetic diversity and weighted UniFrac were also obtained with QIIME2[26]. Results of beta diversity were represented by means of a Principal Coordinate Analysis (PCoA), and a permutational multivariate analyses of variance (PERMANOVA) was used to check significant differences in beta diversity, performing pairwise PERMANOVAs among groups as a *post-hoc* test ($P \leq 0.05$). The significant effect of the variables diet and time, and their interaction were also tested using a PERMANOVA including both fixed variables. Data on relative abundance among ASVs was analysed by Kruskal–Wallis followed by Wilcoxon test, with P set to 0.1 for determination of significance due to the low number of methodological replicates of the diets and of the baseline samples of Atlantic salmon and gilthead seabream. The above-mentioned statistics were partly obtained with the R package microeco (v0.12.0)[37], which was used to generate figures together with ggplot2 (v3.4.1). Significant differences in fish BW among experimental groups post-IMT were checked by means of a one-way ANOVA with Tukey's range *post-hoc* test for multiple comparison among groups ($P \leq 0.05$), after data verification of normal distribution (Shapiro–Wilk test) and homoscedasticity (Levene's test).

Ethics declaration

All procedures involving animal care, manipulation and sampling were carried out by trained competent personnel, complied with the Spanish (law 32/2007 and Royal Decree 1201/2015) and the European legislation (EU2010/63). The experimental protocol was authorized by the Ethical Committee of the Institute of Agrifood Research and Technology and the Generalitat of Catalunya (CEEA 11264/2021).

ARRIVE guidelines

The study was conducted in compliance with the ARRIVE guidelines and all methods were conducted in accordance with relevant guidelines and regulations.

Results

Culture-bacterial growth after antimicrobial treatment for establishment of baseline criteria

The preliminary trial to evaluate the efficacy of the AM cocktail and the timing of recovery of the basal microbial community indicated that gut microbiota recovery was rapidly advancing after 48 h, which indicated that the actual IMT trial might be initiated 24 h post-AM treatment. After 24 h of incubation in TSA + 0.6% NaCl media, only 6 CFUs were growing from the intestinal microbiota obtained from the AM-treated fish at 24 h, and the number of CFUs increased to 10^2 CFUs at 48 h after AM gavage, while the bacterial growth from control animals remained at 10^3 CFUs in both times. In addition, the absence of clearance zones of growth in the Mueller–Hinton media after 24 h- and 48 h-incubations of the blood of gilthead seabream 24 h post-AM gavage, indicated that there was no risk of retention of antimicrobial residues after 24 h.

Fish body weight

As expected, fasted gilthead seabream showed lower BW values when compared to the fed groups at all sampling times (Table 1), displaying significant differences with respect to GSB fed the salmon diet at 7 days post-IMT, and with respect to gilthead seabream fed both diets (GSB diet and salmon diet) at 16 days post-IMT ($P < 0.05$). There were no differences seen regarding the administered diet after the IMT ($P > 0.05$).

Effect of the antimicrobial treatment on the diversity, structure, and composition of the gut bacterial communities

Regarding microbial diversity, no differences in the ACE index were observed after the AM treatment in fasted gilthead seabream (Kruskal–Wallis, $P = 0.067$; Fig. 4A). However, when performing the Wilcoxon test between the experimental groups, gilthead seabream at 24 h post-AMs showed a tendency towards increasing estimated richness values with respect to gilthead seabream from the pre-AM treatment ($P = 0.1$), whereas at 8 and 17 days

	Time after intestinal microbiota transplant		
	7 days	16 days	36 days
Fasted GSB*	524.2 ± 41.7[a]	536.8 ± 49.0[a]	–
GSB fed GSB diet	608.7 ± 106.8[ab]	668.7 ± 59.9[b]	752.4 ± 72.8
GSB fed salmon diet	688.0 ± 73.9[b]	740.3 ± 84.6[b]	769.4 ± 120.8

Table 1. Body weight (BW, g) of gilthead seabream (*Sparus aurata*) (GSB) under the different experimental conditions. The days in the table header indicate the number of days after the intestinal microbiota transplant (IMT) from donor Atlantic salmon. Values are represented as mean ± SD (n = 6 fish individuals per group; n = 7 at 36 days). Significant differences among the three experimental groups at days 7 and 16 are indicated in the same row by the different superscript letters (one-way ANOVA, with Tukey's range post-hoc test; $P \leq 0.05$). There were no differences regarding diet after the IMT (GSB fed GSB diet vs. GSB fed salmon diet; Student's t-test; $P > 0.05$). *Fasted GSB were not subjected to the IMT and were used as a control to assess the effect of AMs over time and to observe what microbiota profile was recovered in the absence of the influence of feed: in that case, the times 7 and 16 days (post-IMT) correspond to 8 and 17 days post-AMs respectively. The two other groups of fish were submitted to the IMT after which they were fed either the GSB diet or the salmon diet.

post-AMs there was a downward tendency with respect to specimens from the 24 h post-AMs sampling point ($P = 0.036$; $P = 0.057$, respectively). When using both the Shannon and Faith's phylogenetic diversity (PD) indices, a significant decrease was observed at 8 days post-AMs with respect to 24 h post-AMs ($P = 0.036$ in both cases; Fig. 4B,C). Likewise, there was a tendency to an increase in diversity at 24 h post-AMs with respect to gilthead seabream pre-AMs ($P = 0.1$), while at 17 days post-AMs tended to decrease with respect to the 24 h post-AMs sampling point ($P = 0.057$ in both cases). Further, the values for the Shannon index were negatively correlated to the number of days that had passed after AM treatment (Spearman's correlation coefficient $r_s = -0.77$, $P = 0.003$).

In terms of beta diversity, when using the Bray–Curtis metric (Fig. 4D), the PCoA distribution of the samples clearly showed that the structure of the bacterial communities was very different among gilthead seabream from the pre-AMs group, and those collected at 24 h post-AMs, and with respect to the other groups post-AMs ($P < 0.05$). In contrast, the distribution of gilthead seabream at 8 days post-AMs and at 17 days post-AMs was very similar ($P = 0.281$). When also considering the phylogenetic relationships among ASVs within samples (Weighted UniFrac; Fig. 4E), the structures of the bacterial communities in gilthead seabream at 17 days post-AMs and from the pre-AMs group resemble each other ($P = 0.060$) more than the groups at 8 days post-AMs and at 17 days post-AMs ($P = 0.031$). In that latter case, except between gilthead seabream from the pre-AMs group and at 17 days post-AMs, the remaining were different among them ($P < 0.05$).

Regarding composition of gut bacterial communities, the relative abundance of the phylum Proteobacteria was reduced by 50% at 24 h after the AM treatment ($P \leq 0.1$; Fig. 4F; Supplementary Table S1). On the other hand, the relative abundance of the rest of phyla ≥ 0.5% increased ($P \leq 0.1$), except for Spirochaetota ($P > 0.1$). At eight days post-AMs, the relative abundances of Proteobacteria, Planctomycetota, Verrucomicrobiota, Bacteroidota and Dependientae were already re-established with respect to gilthead seabream from the pre-AMs group ($P > 0.1$) and they were maintained in the same range at 17 days post-AMs. The relative abundance of the other phyla (Firmicutes and Actinobacteriota) did not reach values similar to the initial ones until 17 days post-AMs, except for Cyanobacteria, that showed a gradual decrease after the AM treatment that was maintained at 17 days ($P \leq 0.1$). At the level of genus, the AM treatment induced a change in the relative abundance of 70% of the represented genera (those with a relative abundance ≥ 0.5% excluding those classified as unassigned) at 24 h post-AMs; in particular, a decrease in *Vibrio*, while an increase in *Synechococcus* CC9902, *Escherichia-Shigella*, *Cyanobium* PCC-6307, *Staphylococcus*, *Acinetobacter*, *Coxiella*, DS001, and *Streptococcus* was observed ($P \leq 0.1$; Fig. 4G; Supplementary Table S2). *Photobacterium*, *Catenococcus* and *Brevinema* were the only genera to remain unchanged at 24 h post-AMs. At 8 days post-AMs, the relative abundances of 54% of the genera that were originally represented in the baseline samples were already restored, including *Cyanobium* PCC-6307, *Staphylococcus*, *Coxiella*, DS001, and *Streptococcus*. The appearance of *Aliivibrio* at this sampling time was remarkable, as this was not present in gilthead seabream at 24 h post-AMs and pre-AMs ($P \leq 0.1$). At 17 days post-AMs, the relative abundance of *Synechococcus* CC9902 and *Acinetobacter* was also re-established with respect to gilthead seabream from the pre-AMs group, so that 85% of the genera contained a relative abundance similar to the baseline microbiota, and only that of *Vibrio* and *Escherichia-Shigella* was different at the final sampling point of day 17 ($P \leq 0.1$).

Effect of the intestinal microbiota transplant and species-specific diets on the diversity, structure and composition of the gut bacterial communities

There was no significant effect of the time ($F = 0.83$, $P = 0.516$) and the diet ($F = 0.15$, $P = 0.704$) on the ACE index, but there was for their interaction ($F = 11.48$, $P \leq 0.001$). When using the Shannon and Faith's phylogenetic diversity (PD) indices, there was significance on both variables; time (Shannon: $F = 4.40$, $P = 0.005$; PD: $F = 9.85$, $P \leq 0.001$) and diet (Shannon: $F = 5.46$, $P = 0.025$; PD: $F = 18.17$, $P \leq 0.001$), as well as on their interaction (Shannon: $F = 3.21$, $P = 0.023$; PD: $F = 7.36$, $P \leq 0.001$). Thus, the significant differences among experimental groups regarding these two variables were individually evaluated.

For the ACE index, gilthead seabream fed the GSB diet after the IMT and until day 16 post-IMT (included) displayed a similar diversity than that from the pre-IMT samples from Atlantic salmon and gilthead seabream

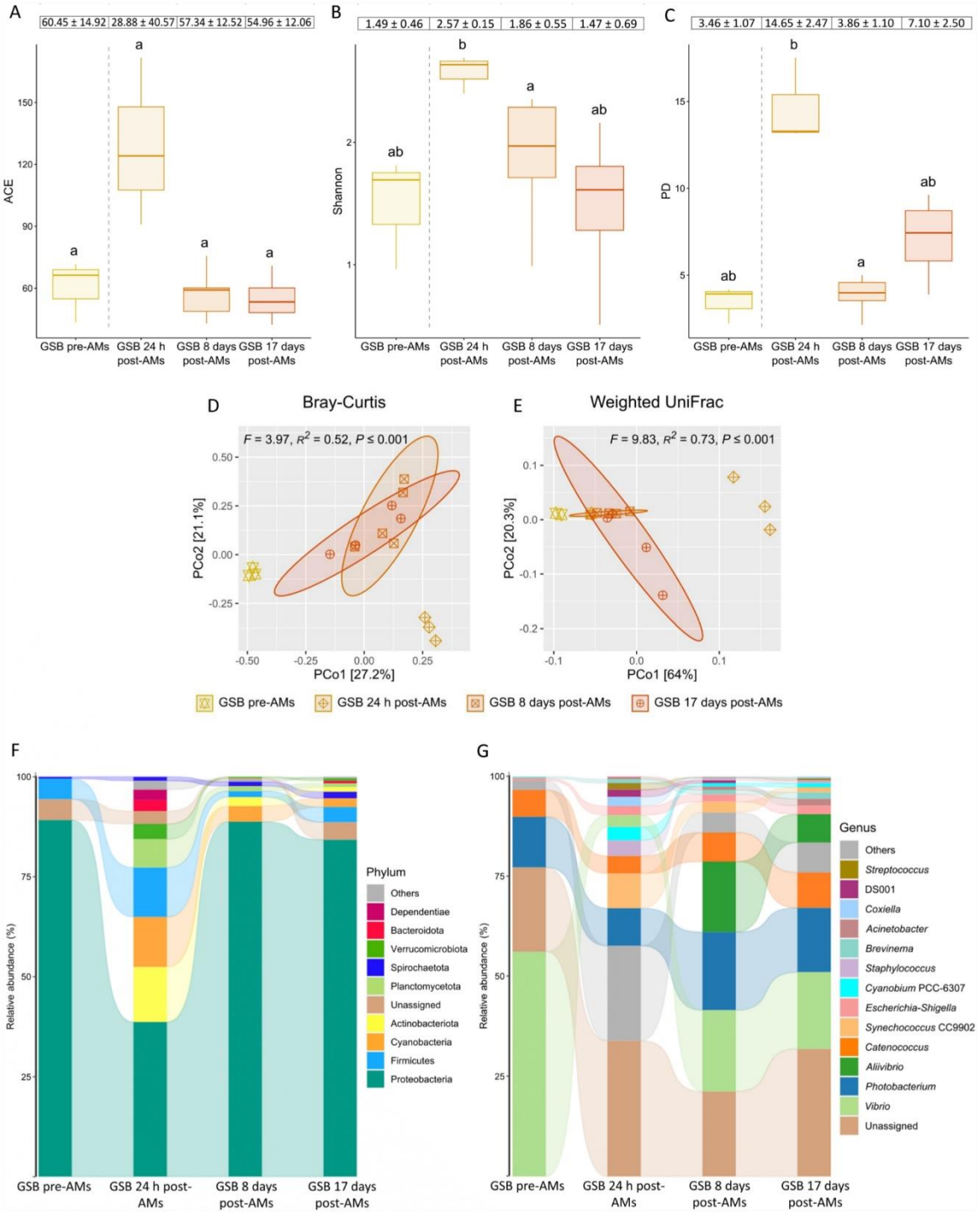

Figure 4. Gut bacterial communities in fasted gilthead seabream (*Sparus aurata*) from the pre- antimicrobial treatment (GSB pre-AMs) during the first 17 days: Alpha diversity measured by (**A**) ACE index, (**B**) Shannon index, and (**C**) Faith's phylogenetic diversity index (PD). The letters show significant differences among experimental groups (Kruskal–Wallis with Wilcoxon *post-hoc* test; $P \leq 0.05$). Microbial structure analyzed with (**D**) Bray–Curtis and (**E**) weighted UniFrac distances. Significant differences among experimental groups are shown in black captions (PERMANOVA; $P \leq 0.05$). Microbial composition at the level of (**F**) phylum and (**G**) genus. Taxa with an abundance < 0.5% are classified as "Others". Mean ± SD values and significant differences among experimental groups (Kruskal–Wallis with Wilcoxon *post-hoc* test; $P \leq 0.1$) at the level of phylum and genus can be found at Supplementary Tables S1 and S2, respectively.

(Fig. 5A). However, at 36 days post-IMT, gilthead seabream fed the GSB diet showed a microbial diversity different to the one of Atlantic salmon ($P<0.05$), although still similar to the pre-IMT group ($P>0.05$). Completely the opposite was seen for gilthead seabream fed the salmon diet, which did not display significant differences over time with respect to the Atlantic salmon microbiota ($P>0.05$); however, at 36 days post-IMT, microbial diversity was lower than in gilthead seabream from the pre-IMT ($P<0.05$). Values of the ACE index in gilthead seabream fed the GSB diet were positively correlated with the time of post-IMT from 7 days onwards (Spearman's correlation coefficient $r_s=0.85$, $P\leq0.001$). Differences regarding the species-specific diet were observed at 16 and 36 days post-IMT ($P<0.05$). Similarly, no differences in the Shannon index for gilthead seabream fed the GSB diet were found at any time post-IMT with respect to the pre-IMT group ($P>0.05$); while at 7 days there was a significant decrease in comparison to the Atlantic salmon microbial diversity ($P<0.05$), but afterwards it increased again reaching similar values to those seen for Atlantic salmon (Fig. 5B). Indeed, these values of Shannon's alpha diversity found in gilthead seabream fed the GSB diet were also positively correlated with the time of post-IMT from 7 days onwards ($r_s=0.69$, $P\leq0.001$). On the other hand, fish fed with the salmon diet after the IMT did not show differences among sampling times nor with the baseline microbial diversities from gilthead seabream or donor Atlantic salmon specimens ($P>0.05$). In this case, significant differences regarding each species-specific diet were only observed at 7 days post-IMT ($P<0.05$). Faith's phylogenetic diversity in gilthead seabream fed the GSB diet did not vary among times and with respect to the values from the baseline fishes (Atlantic salmon microbiota and gilthead seabream from the pre-IMT group) (Fig. 5C). In contrast, gilthead seabream fed the salmon diet showed higher PD values at 7 and 16 days post-IMT than the Atlantic salmon microbiota and gilthead seabream from the pre-IMT group ($P<0.05$). In fact, there was a positive correlation between the samples from days post-IMT and PD from 2 days post-IMT to 16 days post-IMT groups ($r_s=0.79$, $P\leq0.001$); whereas at 36 days the alpha diversity values were restored with respect to the initial ones of Atlantic salmon and gilthead seabream from pre-IMT groups ($P>0.05$). Furthermore, there were significant differences in PD values between gilthead seabream fed the GSB diet and those fed the salmon diet from 7 days onwards ($P<0.05$).

In terms of beta diversity, after the IMT the gut microbial structure of gilthead seabream was significantly affected by both factors: the diet (Bray–Curtis: $F=4.71$, $R^2=0.07$, $P\leq0.001$; weighted UniFrac: $F=17.82$, $R^2=0.17$, $P\leq0.001$) and the time after the IMT (Bray–Curtis: $F=4.81$, $R^2=0.22$, $P\leq0.001$; weighted UniFrac: $F=10.33$, $R^2=0.29$, $P\leq0.001$); as well as by their interaction (Bray–Curtis: $F=2.39$, $R^2=0.11$, $P\leq0.001$; weighted UniFrac: $F=6.28$, $R^2=0.18$, $P\leq0.001$). Therefore, in order to find out the individual effect of the diet on the structure of the gut bacterial communities post-IMT, the different distribution in the PCoA and significant differences among gilthead seabream fed both types of diets, as well as among the diets themselves, were separately analysed at the different sampling points.

Pairwise differences were observed among the four experimental groups at 2 days post-IMT when using the Bray–Curtis metric ($P<0.05$) except for the salmon diet and the GSB diet, with $P=0.1$ in all times, which may be attributed to the low number of replicates (n = 3 for both diets). While the diets appeared separately in the PCoA between them and with respect to gilthead seabream irrespective of the administered diet, fish fed each diet were grouped close to each other (Fig. 6; despite $F=1.75$, $R^2=0.20$, $P=0.035$). Pairwise significant differences among all groups were maintained at the different sampling times ($P<0.05$), whereas the groups fed with the salmon diet and the GSB diet were found further apart as the days passed away. After 36 days from the IMT, the microbial structure of gilthead seabream fed the GSB diet was closer to the GSB diet ($F=3.08$, $R^2=0.28$, $P=0.014$) than to their congeners fed the salmon diet ($F=3.76$, $R^2=0.24$, $P=0.003$). When approaching the beta diversity with the weighted UniFrac metric, all fish displayed a similar microbial structure regardless of the diet administered after the IMT ($F=0.69$, $R^2=0.09$, $P=0.572$; Fig. 6). During the rest of times, all the groups remained separate ($P<0.05$), with the exception of the salmon diet and gilthead seabream fed the salmon diet at 36 days post-IMT, which clustered together ($F=2.33$, $R^2=0.23$, $P=0.083$). Although the distances of weighted UniFrac in the GSB diet and in gilthead seabream fed the GSB diet 36 days post-IMT were significantly different ($F=9.43$, $R^2=0.54$, $P=0.012$), they showed a very close distribution in the PCoA.

Regarding microbial composition, we first compared the different relative abundances of phyla and genera in gilthead seabream from the post-IMT groups, which were fed throughout the trial with the GSB diet, to the gilthead seabream from the pre-IMT group, the Atlantic salmon microbiota, and the administered diet (GSB diet). Thus, at two days post-IMT, all the phyla ≥ 0.5% had a relative abundance like that of Atlantic salmon microbiota and gilthead seabream from the pre-IMT group ($P>0.1$; Fig. 7A; Supplementary Table S3 and Supplementary Fig. S2). However, the diet did show differences with respect to the fish in the relative abundance of the following phyla: for Proteobacteria it was higher in gilthead seabream 2 days post-IMT than in the GSB diet, while for Firmicutes and Fusobacteriota it was lower ($P\leq0.1$), the latter remaining at 0% from this time onwards. These differences were maintained at days 7 and 16, with an increase in Spirochaetota also appearing with respect to the GSB diet and Atlantic salmon microbiota. At 7 days post-IMT, a decrease in the relative abundance of Proteobacteria with respect to the baseline fishes (Atlantic salmon microbiota and gilthead seabream from the pre-IMT group) was also observed ($P\leq0.1$). At the final sampling point (36 days post-IMT), the relative abundance for Proteobacteria was similar to that of the GSB diet, for Spirochaetota it was similar to the GSB diet and to the microbiota of Atlantic salmon (0%), and for Fusobacteriota it resembled that of Atlantic salmon microbiota and gilthead seabream from the pre-IMT groups (0%). In contrast, the relative abundance for Bacteroidota and Cyanobacteria was similar with respect to the baseline samples from fishes (Atlantic salmon microbiota and gilthead seabream from the pre-IMT group) and GSB diet (very close to 0%; $P>0.1$). The relative abundance of Firmicutes at the final sampling point was very close to that of the GSB diet (gilthead seabream 36 days post-IMT: 77.2 ± 4.6%; GSB diet: 68.3 ± 6.9%) despite $P<0.1$ ($P=0.063$).

At the level of genus, the relative abundance of *Acinetobacter*, *Synechococcus* CC9902, *Corynebacterium*, *Peptostreptococcus*, and *Acidibacter* in gilthead seabream fed the GSB diet from 2 days until 16 days post-IMT (included) were in the same range as for the GSB diet, Atlantic salmon microbiota and gilthead seabream from

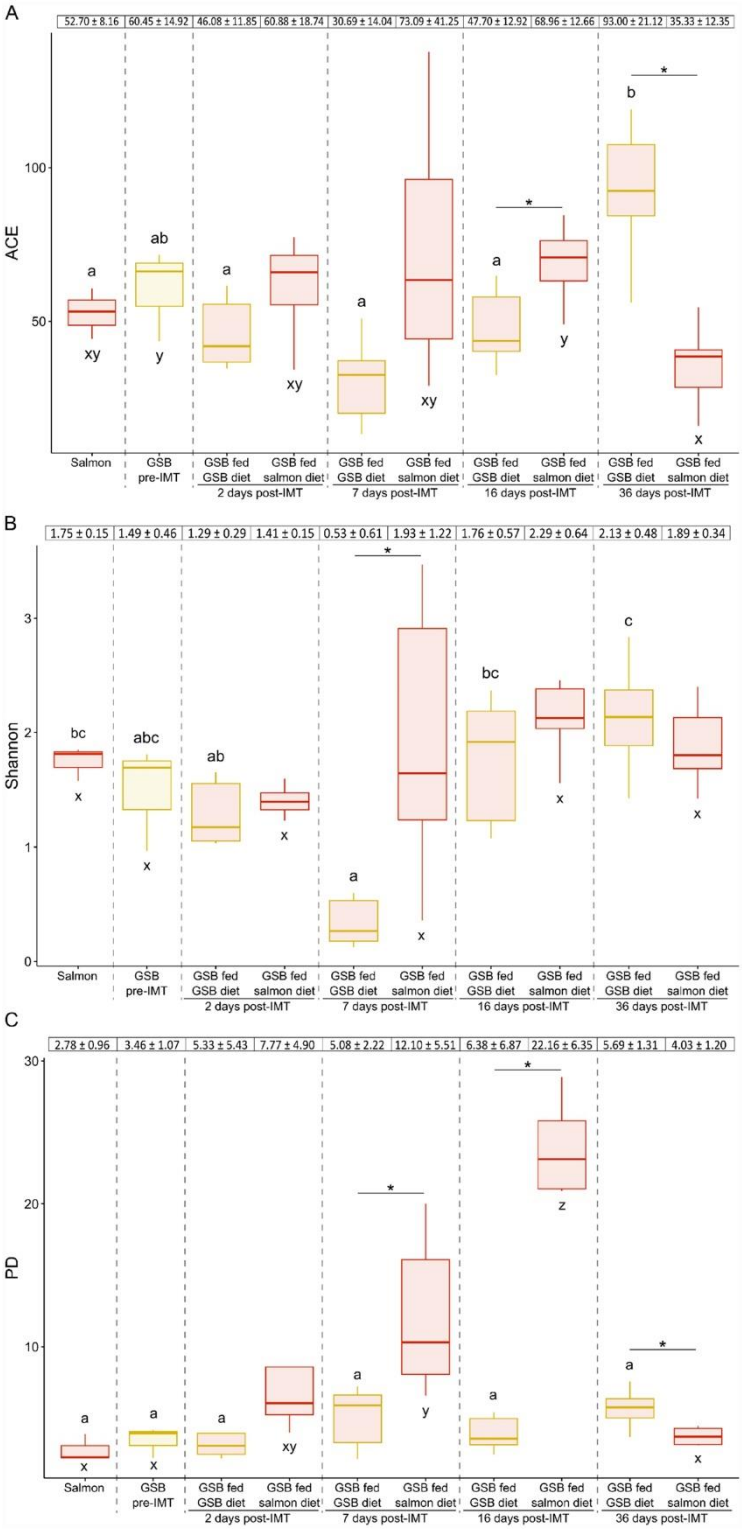

Figure 5. Microbial alpha diversity of the gut bacterial communities in Atlantic salmon (*Salmo salar*), in gilthead seabream (*Sparus aurata*) previous to the intestinal microbiota transplant from donor Atlantic salmon specimens (GSB pre-IMT and after AM treatment) and in gilthead seabream fed either their typical diet (GSB fed GSB diet) or the Atlantic salmon diet (GSB fed salmon diet) at 2, 7, 16 and 36 days post-IMT: (**A**) ACE index, (**B**) Shannon index, and (**C**) Faith's phylogenetic diversity index (PD). The top table represents the index values per group as mean ± SD. Box plots represents the minimum, maximum and the median of the sample values obtained from the diversity indices, and the letters show significant differences among experimental groups (Kruskal–Wallis with Wilcoxon *post-hoc* test; $P \leq 0.05$).

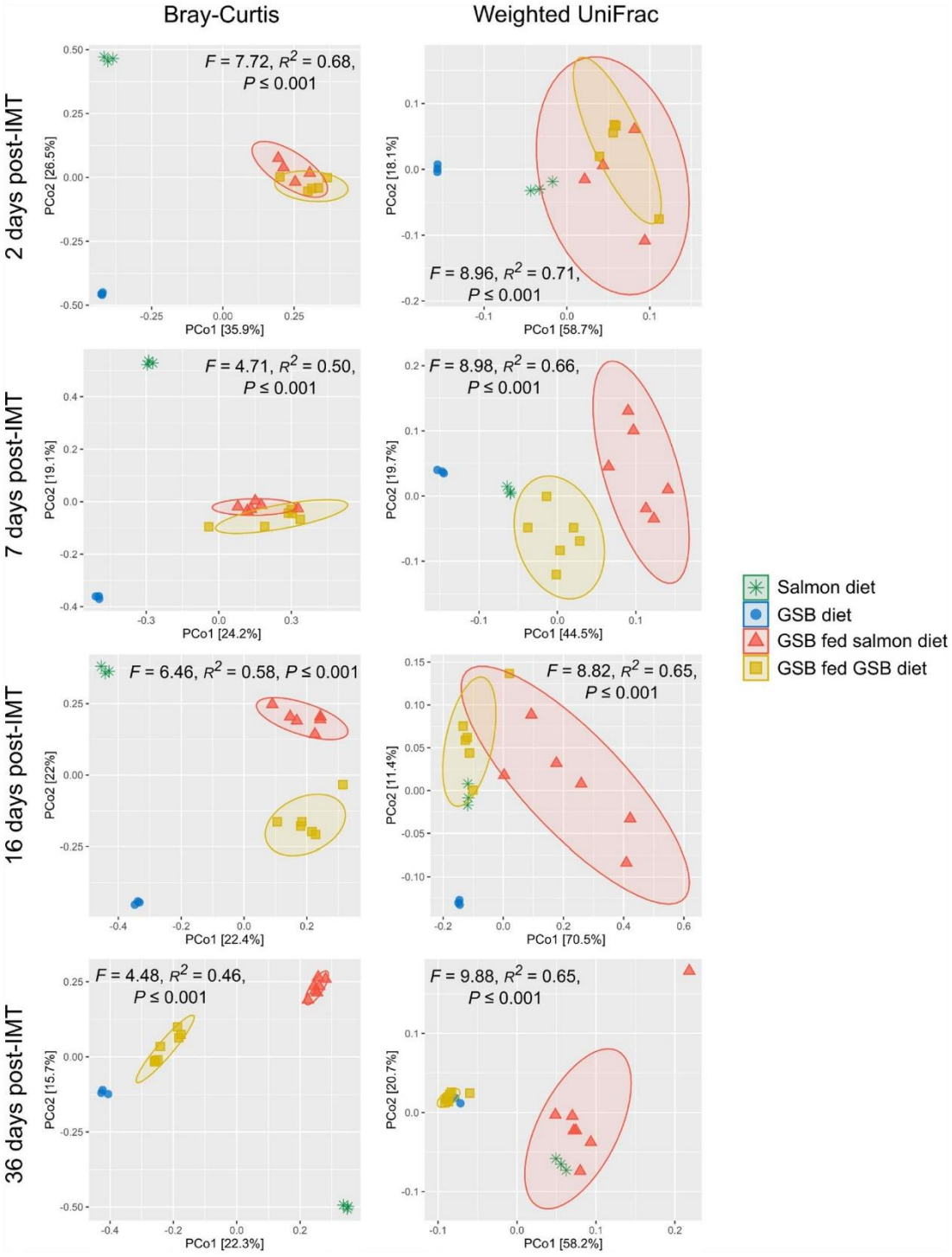

Figure 6. Microbial structure of the gut bacterial communities in gilthead seabream (*Sparus aurata*) fed either their typical diet (GSB fed GSB diet) or the Atlantic salmon diet (GSB fed salmon diet) at 2, 7, 16 and 36 days after the intestinal microbiota transplant from donor Atlantic salmon (post-IMT), as well as of GSB and salmon diets. Beta diversity was analyzed with the metrics of Bray–Curtis and weighted UniFrac distances, and represented by individual distributions in the principal coordinate analyses. Significant differences among experimental groups are shown (PERMANOVA; $P \leq 0.05$).

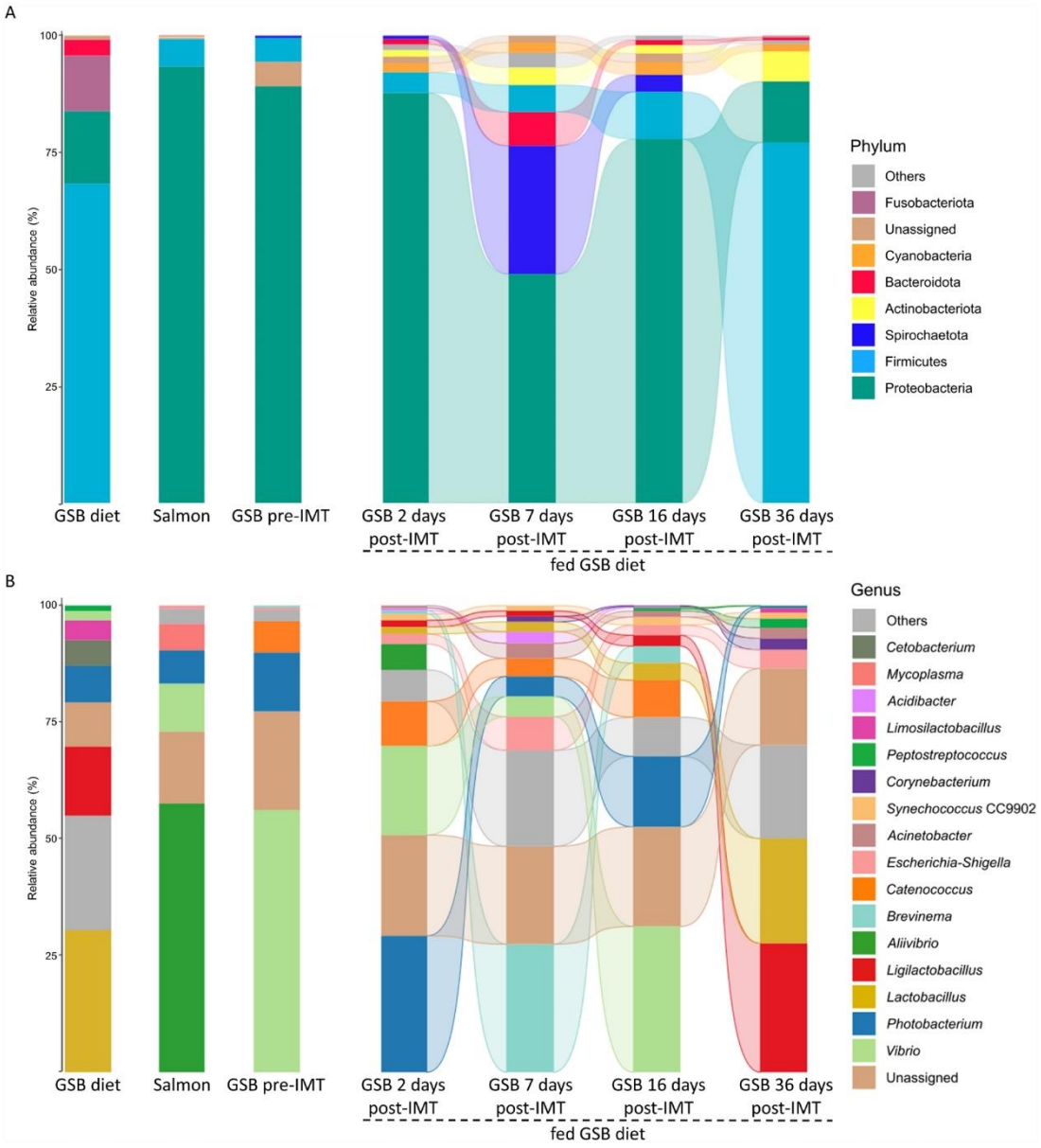

Figure 7. Contrasting control treatments with gilthead seabream (*Sparus aurata*) fed an experimental gilthead seabream diet (GSB diet): Mean relative abundances of the gut bacterial communities in GSB diet, Atlantic salmon (microbiota donor), gilthead seabream previous to the intestinal microbiota transplant (GSB pre-IMT and after AM treatment) and in gilthead seabream fed the GSB diet at 2, 7, 16 and 36 days post-IMT (**A**) at the level of phylum, and (**B**) at the level of genus. Taxa with an abundance < 0.5% are classified as "Others". Mean ± SD values for phyla and genera are compiled in Supplementary Tables S3 and S4, respectively. Significant differences among experimental groups (Kruskal–Wallis with Wilcoxon *post-hoc* test; $P \leq 0.1$) at the level of phylum and genus can be found in Supplementary Figs. S2 and S3, respectively.

the pre-IMT group ($P > 0.1$; Fig. 7B; Supplementary Table S4 and Supplementary Fig. S3). Noteworthy was the increase in relative abundance of *Brevinema* at 7 days post-IMT, a genus common to gilthead seabream from the pre-IMT group, though this was reduced again at 16 days post-IMT ($P \leq 0.1$). Additionally, *Catenococcus*, a genus exclusive from gilthead seabream from the pre-IMT group (which was not present in Atlantic salmon microbiota nor in GSB diet), was maintained at a similar relative abundance to the gilthead seabream from the pre-IMT group during 2, 7 and 16 days post-IMT, but it was not observed at 36 days (0%). The dynamics of *Aliivibrio* and *Mycoplasma* were also remarkable, because neither were found in the gilthead seabream from the pre-IMT group nor in the GSB diet, but they were present in the Atlantic salmon microbiota. In particular, *Aliivibrio* appeared at 2 days-post IMT in gilthead seabream, but its presence was not sustained over time, and *Mycoplasma* was not found at any time. On the other hand, *Cetobacterium* appeared in the GSB diet, while its relative abundance was 0% in the rest of the post-IMT fish, as in the baseline fishes (Atlantic salmon microbiota

and gilthead seabream from the pre-IMT group). At the final sampling time, only 31% of the represented genera had a similar relative abundance to that from the gilthead seabream from the pre-IMT group, and 31% of the genera also displayed similar relative abundances to those of the Atlantic salmon microbiota. Thus, the effect of the donor and host microbial profiles lost significance over time, considering that at 2 days post-IMT 69% of the genera contained a similar relative abundance to that from the gilthead seabream from the pre-IMT group and 50% of the genera showed similar relative abundances to those of the Atlantic salmon microbiota. On the other hand, the administered diet gained significance at 36 days post-IMT, showing more than 56% of genera with a relative abundance similar to the GSB diet group with respect to an initial similarity of 44% at 2 days post-IMT in gilthead seabream fed the GSB diet. It was worth noting the increase in relative abundance of *Lactobacillus* and *Ligilactobacillus* with respect to the previous sampling point (16 days post-IMT), which were common to the GSB diet and they did not appear in the Atlantic salmon microbiota or gilthead seabream from the pre-IMT group ($P \leq 0.1$).

There were distinctly different results when we compared the microbial composition in gilthead seabream from the post-IMT group, which were fed during all the trial with the salmon diet, to the gilthead seabream from the pre-IMT group, the Atlantic salmon microbiota and the administered diet (salmon diet). At 2 days post-IMT, all the phyla displayed a similar relative abundance to that of the donors ($P > 0.1$; Fig. 8A; Supplementary Table S5 and Supplementary Fig. S4). In the same way, the only phylum which showed a differential relative abundance with respect to gilthead seabream from the pre-IMT group was Bacteroidota, which did not appear in any sample of gilthead seabream from the pre-IMT group ($P \leq 0.1$). The abundance of this phylum ranged between 1.4 and 8.9% over time, not showing significant differences with respect to the salmon diet at any time (with a mean relative abundance of $7.9 \pm 1.5\%$; $P > 0.1$). Only the phylum Firmicutes showed significant differences at all sampling points after the IMT with respect to the salmon diet ($20.1 \pm 3.0\%$; these values were always lower in GSB post-IMT: 5.1–12.9%; $P \leq 0.1$). The relative abundance of this phylum over time was maintained in the same range as for gilthead seabream from the pre-IMT group ($5.1 \pm 1.1\%$) and the microbiota of the donors ($5.9 \pm 2.2\%$) ($P > 0.1$). On the other side, the relative abundance of Proteobacteria prominently decreased from 7 days post-IMT onwards with respect to donors' microbiota and gilthead seabream from the pre-IMT group, while that of Actinobacteriota and Bacteroidota increased ($P \leq 0.1$). Although the relative abundance of Cyanobacteria and Spirochaetota was also higher in gilthead seabream at 7 and 16 days post-IMT with respect to both reference microbiota (Atlantic salmon and gilthead seabream before the AM treatment) ($P \leq 0.1$), these significant differences were not sustained at 36 days post-IMT ($P > 0.1$). The relative abundance of Spirochaetota at 7 and 16 days post-IMT also displayed significant differences with respect to that of the salmon diet, which was of $0.2 \pm 0.3\%$ ($P \leq 0.1$).

At the level of genus, no significant differences were observed in the relative abundance of *Corynebacterium*, *Staphylococcus*, *Pseudomonas*, *Alloiococcus*, *Cutibacterium*, *Asinibacterium*, *Sphingomonas*, *Heliimonas*, *Bradyrhizobium*, *Cyanobium* PCC-6307 and *Turicella* in gilthead seabream fed the salmon diet at any of the times post-IMT with respect to the microbiota of donors, gilthead seabream from the pre-IMT group nor the salmon diet ($P > 0.1$; Fig. 8B; Supplementary Table S6 and Supplementary Fig. S5). Although immediately after the microbiota transplant (2 days post-IMT), the relative abundances of *Acinetobacter*, *Synechococcus* CC9902, *Brevinema*, *Vibrionimonas*, and *Bacillus* were also within the same range (very close to 0%) as in the two baseline groups (gilthead seabream from the pre-IMT group, and Atlantic salmon microbiota) and in the salmon diet, their abundances tended to increase and changed over time ($P \leq 0.1$). Conversely, the relative abundance of *Escherichia-Shigella* was significantly higher in all the times post-IMT than in Atlantic salmon microbiota, in gilthead seabream from the pre-IMT group and in the salmon diet, especially at 36 days post-IMT ($19.1 \pm 10.6\%$; $P \leq 0.1$). Additionally, the relative abundances of *Aliivibrio* and *Mycoplasma* tended to decrease after the IMT in comparison to the microbiota of donors ($P \leq 0.1$). Regarding differences in composition maintained over time with respect to the gilthead seabream from the pre-IMT group, the relative abundance of *Vibrio* decreased around 50% after the IMT ($P \leq 0.1$). In comparison to the salmon diet, *Halomonas*, *Chromohalobacter*, *Idiomarina*, and *Pseudoalteromonas* showed a lower relative abundance in all sampled times post-IMT. At the end of the study (36 days post-IMT), 77% of the represented genera (≥0.5% and excluding those classified as unassigned) showed a relative abundance similar to that for the Atlantic salmon microbiota, the same percentage as when compared to gilthead seabream from the pre-IMT group. In terms of the salmon diet, the relative abundances of 65% represented genera matched with those of gilthead seabream fed the salmon diet at 36 days post-IMT.

Comparison of the microbial communities at 7 and 16 days post-IMT with respect to the baseline group not submitted to the intestinal microbiota transplant

Beta diversity values were statistically compared among gilthead seabream fed the GSB and the salmon diets and the baseline group not submitted to the IMT (fish fasted after the AM treatment). Significant differences were found among the three groups at both 7 and 16 days post-IMT when Bray–Curtis distances were used ($P < 0.05$; Fig. 9A,B). Considering phylogenetic relations (weighted UniFrac distances), there were also significant differences among the three groups at 7 days ($P < 0.05$; Fig. 9C). However, at 16 days post-IMT, gilthead seabream fed the GSB diet, and the fasted fish not submitted to the IMT were similar ($F = 1.20$, $R^2 = 0.13$, $P = 0.30$; Fig. 9D), while gilthead seabream fed the salmon diet showed significant differences when compared to both groups ($P < 0.05$).

When representing the Venn diagram with the ASVs found in the three groups, a total of 14 and 21 ASVs where shared among the three groups at 7 and 16 days post-IMT, which constituted a total of 18.4% and 26.9% relative abundance, respectively (Fig. 9E,F).

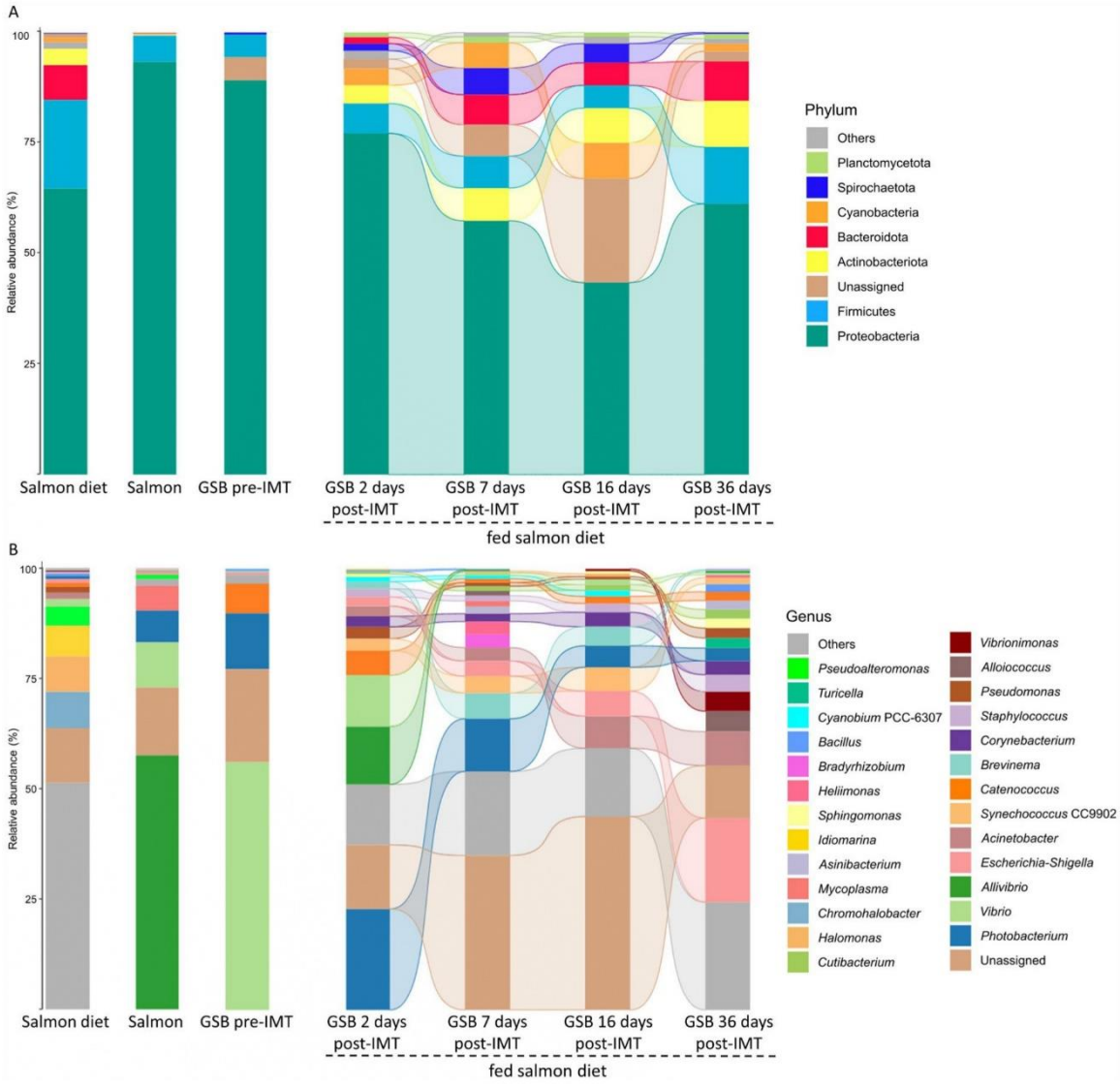

Figure 8. Contrasting control treatments with gilthead seabream (*Sparus aurata*) fed an experimental salmon diet: mean relative abundances of the gut bacterial communities in gilthead seabream fed the salmon diet, Atlantic salmon (microbiota donor), gilthead seabream previous to the intestinal microbiota transplant (GSB pre-IMT and after AM treatment) and in gilthead seabream fed the salmon diet at 2, 7, 16 and 36 days post-IMT (**A**) at the level of phylum, and (**B**) at the level of genus. Taxa with an abundance < 0.5% are classified as "Others". Mean ± SD values for phyla and genera are compiled at Supplementary Tables S5 and S6, respectively. Significant differences among experimental groups (Kruskal–Wallis with Wilcoxon *post-hoc* test; $P \leq 0.1$) at the level of phylum and genus can be found in Supplementary Figs. S4 and S5, respectively.

Discussion

In this study, we have undertaken an experiment to investigate the progression of development of the microbiota when the intestinal microbiota is transplanted into a new host that is then fed with two different diets: the homologous diet given the new host (recipient) and the heterologous diet previously given to the donor Atlantic salmon. To understand what is meant by autochthonous microbiota, we additionally followed the progression of recovery of the native microbiota during 17 days under fasting conditions, after "erasing" most of the microbiota with a cocktail of multiple broad-spectrum antibiotics.

Antimicrobial treatment effects

Under current experimental conditions, the gut bacterial communities of the baseline gilthead seabream (pre-AMs) were dominated by the phylum Proteobacteria, with *Vibrio* and *Photobacterium* as the most dominant genera, results that were in agreement with previous studies in gilthead seabream[38,39]. In this study, the preliminary trial using a limited number of fish to establish the proper timing of IMT administration after the AM treatment

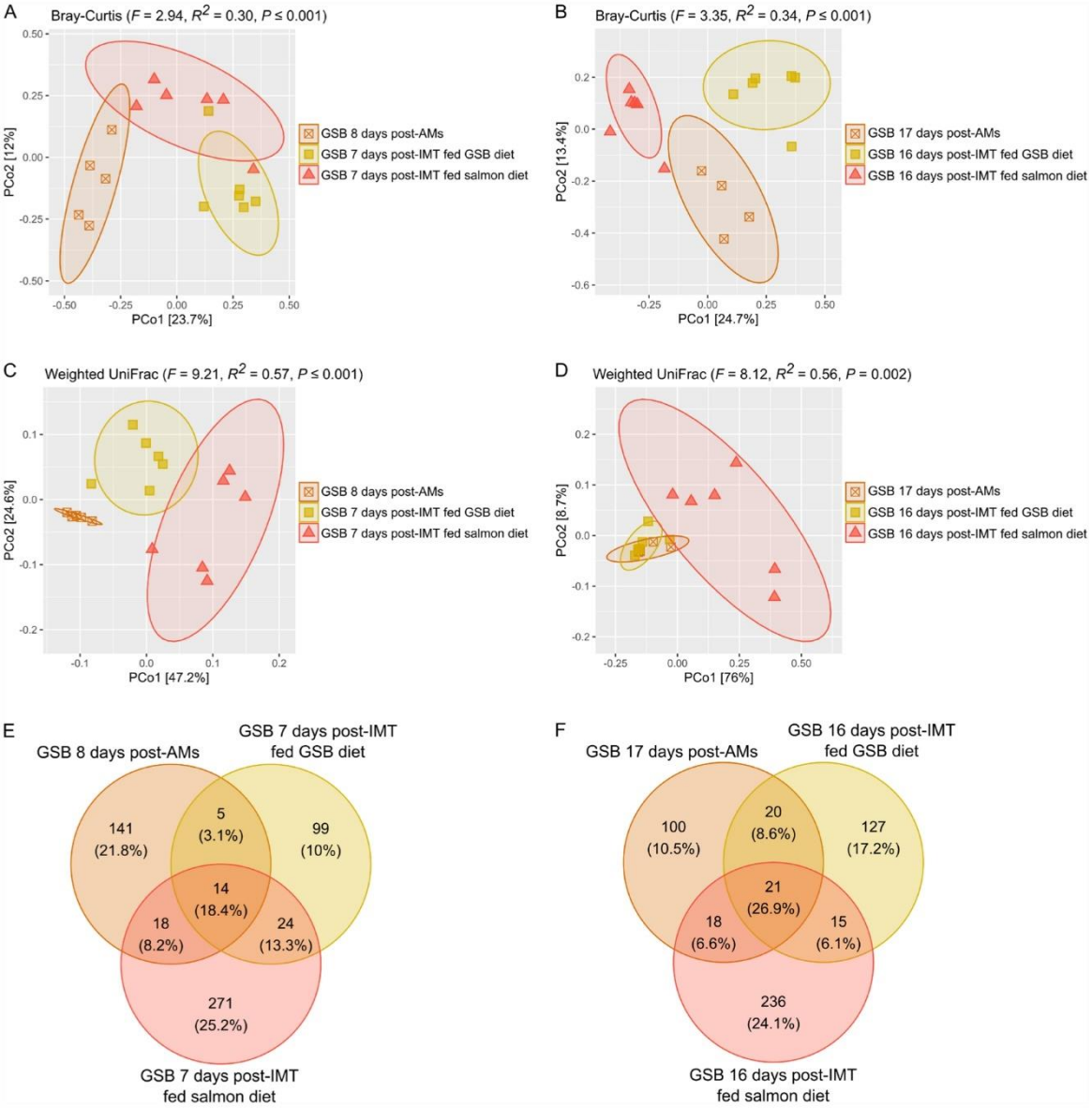

Figure 9. Microbial structure of the gut bacterial communities in gilthead seabream (*Sparus aurata*) from the group which was fasted after the antimicrobial treatment (post-AMs) and the groups which were submitted to the intestinal microbiota transplant and then fed either their typical diet (GSB fed GSB diet) or the Atlantic salmon diet (GSB fed salmon diet). Beta diversity was analyzed using (**A**,**B**) Bray Curtis, and (**C**,**D**) weighted UniFrac distances, at 7 and 16 days post-IMT (8 and 17 days post-AMs), respectively. Venn diagram plotting the number of unique and shared ASVs (and relative abundance %) of the three groups (**E**) at 7 days post-IMT, and (**F**) at 16 days post-IMT.

showed that the relative abundance of the phyla Proteobacteria dramatically decreased at 24 h post-AMs, in line with the significant decrease of the genus *Vibrio* and, to a lesser extent, of *Photobacterium* and *Catenococcus*, which also belong to this phylum. However, based on existing trials in humans and other animal species, when administering the AM combination described herein, there could have been expected an increase in the abundance of Proteobacteria[40–43]. Under current experimental conditions, the decrease of Proteobacteria may be caused by the lower concentration of Vancomycin (0.02 g/L) in comparison to the former studies (0.25–0.5 g/L), which usually leads to a relative boost in the abundance of this phylum when supplied individually[44], likely due to elimination of other competing species, or the possibility of the antibiotic serving as a carbon/energy source for resistant strains/species[45]. The other antibiotics present in the AM cocktail used in this study (Metronidazole, Neomycin, Ampicillin) have been reported to have a more significant effect on the increase of the abundance of other phyla such as Bacteroidota or Actinobacteriota depending on the host species and on the different experimental conditions of each assay[40–43]. On the other hand, and in agreement with the above-mentioned studies,

despite the impact of the AM cocktail on the relative abundance of *Vibrio*, *Photobacterium* and *Catenococcus*, other genera belonging to Proteobacteria did show a significant increase, such as *Coxiella*, *Escherichia-Shigella*, and *Acinetobacter*. The changes in abundance of Proteobacteria members were coupled to an increase in the relative abundance of strains belonging to Firmicutes (i.e., bacteria from the genera *Staphylococcus* and *Streptococcus*), and the appearance of new ones that were not present before the AM treatment, such as Cyanobacteria (*Synechococcus* CC9902, *Cyanobium* PCC-6307) and Actinobacteriota (DS001), among others. The increase in the relative abundance of Firmicutes may be caused by the reduced competition for colonizing space and available resources in the intestine because of Proteobacteria death or growth inhibition by the AMs, or just be a bias inherent to the complexity of analyzing microbiota datasets, as they are compositional (that is, each sample consists of proportions of various organisms with a sum constrained to a constant). In this sense, when approaching a 16S rRNA gene experiment with relative abundances, if a taxon goes up while another goes down, it is not possible to know if that occurred just because one taxon has increased, or because the other has decreased, or both[46]. On the other hand, the emergence of taxa which were not present before the AM treatment (members of Actinobacteriota, Cyanobacteria, Planctomycetota, Verrucomicrobiota, Bacteroidota and Dependientae) was reflected in the tendency towards increased richness (ACE), diversity (Shannon) and phylogenetic diversity (PD) values. However, the culture-dependent bacterial growth test showed that most of the gut content lacked viable bacteria after the first 24 h, using TSA + 0.6% NaCl media, which suggested that most of the bacteria sequenced from this sampling time were not viable on the chosen media, while the surviving taxa from the microbiota seemed to readily re-colonize the gut within 48 h after the AM treatment, as evinced by growth on culture media. Nonetheless, it is also important to consider that a large portion of the microbiota is not culturable, or they need more selective media to grow[47], meaning that the above-mentioned approach was only a proxy of the proportion of viable bacteria culturable on TSA + 0.6% NaCl medium, but we could not determine the total viability of the microbiota. Consequently, we decided to select the time 24 h post-AMs as the best time to perform the IMT from donor Atlantic salmon. Furthermore, among the fasted group, sequencing results showed a gradual trend at 8- and 17-days post-AMs towards re-establishing the microbiota profile that existed before the AM-treatment. At 17 days post-AMs, the only represented genera showing significant differences with respect to the baseline gilthead seabream were *Vibrio* and *Escherichia-Shigella*. The reestablishment of the initial gut microbiota might be facilitated by many biotic and abiotic factors, such as the environmental pressures during the trial, including water temperature and salinity[48,49], the environmental microbiota composition[50], the fish origin[51], species, sex, age[52], and host's genetic background[53]. Meanwhile, the few changes observed with respect to the gilthead seabream pre-AM treatment (baseline state) may be caused by possible residual effects of the antibiotics and/or by the effect of the fasting conditions. Regarding the fasting state, for instance, many *Vibrio* species acting as symbionts produce hydrolytic enzymes (i.e., amylase, lipase, cellulase, and chitinase), which contribute to the breakdown of dietary components[54], so the pronounced decrease in *Vibrio* relative abundance may be a response to the absence of feed in the digestive tract of fish. Whatever the reason, the restoration of the intestinal microbiota composition was in line with the recovery of initial values of alpha diversity over time, which was particularly evident for the Shannon diversity index, which showed a negative correlation with the number of days post-AMs until reaching values like those of the baseline samples of gilthead seabream pre-AMs after 17 days ($r_s = -0.77$, $P = 0.003$). When comparing inter-individual variation (beta diversity), both of the metrics used (Bray–Curtis and weighted UniFrac) clearly reflected the different structure among gilthead seabream from the pre-AMs group and at 24 h post-AMs; while samples from 8 days post-AMs clustered in a different group than the previous ones. In addition, when using Bray–Curtis dissimilarity, which considers the abundance of each species in the community, the gut bacterial communities from gilthead seabream at 8 days post-AMs resembled those of 17 days post-AMs, which were still different to those of fish from the pre-AMs group. Inversely, when also considering the inter-individual phylogenetic relationships among species by the weighted UniFrac distance, the structure of the gut communities from gilthead seabream at 8- and 17-days post-AMs remained different, while the latter was similar to that of the pre-AMs. Thus, the above-mentioned results seemed to indicate that after 17 days the gut microbial diversity, structure and composition was recovered from the AM treatment and were fairly stable. Considering these results, we are confident that by measuring the microbial dynamics during 36 days after the IMT, at the final sampling point we were assessing only the effect of the transplantation in the gut microbiota rather than the long-term effect of the AMs applied to the recipient fish before the transplant.

Alpha and beta diversity for IMT

The fact that at 36 days post-IMT, gilthead seabream groups fed with the GSB and the salmon diets displayed ACE values that were only similar to those of gilthead seabream from the pre-IMT group and to the Atlantic salmon microbiota, respectively, suggested that the diet was the main driver of the gut bacterial richness. These results are in agreement with previous studies assessing the influence of the diet in humans and fish[55,56]. The impact of the diet was also supported by our beta diversity results. Indeed, at the end of the trial, the gut microbial structure of gilthead seabream was similar to the diet which each group was fed, as shown in weighted UniFrac comparison. On the other hand, Shannon diversity results suggested that the microbial diversity of the fish fed the GSB diet tended to a differentiation from the donor in the medium-term after the transplantation (at 7 days post-IMT)[30]. However, the diversity reached an intermediate state between the donor and recipient diversity in a longer-term, in terms of evenness, already at 16 days post-IMT, and phylogeny, at 36 days post-IMT, as demonstrated by both Shannon and Faith's phylogenetic diversity indices. In addition, similar to our results, when performing a FMT in yellowtail kingfish (both via gavage or through the water), Legrand et al.[18] found a modulation at 8–15 days post-FMT towards a reestablishment in the diversity from 0 days post-FMT and from that of their congeners that were not submitted to a FMT. Altogether, our results suggested that the microbiota of gilthead seabream after 36 days from the IMT is the sum of the synergy between the host, the donor, and the administered diet.

Bacterial community composition of gilthead seabream fed with their homologous (GSB) diet post-IMT

When comparing the bacterial composition of the animals that received the transplanted microbiota and fed with their homologous diet to their initial microbiota from pre-IMT and at 2 days post-IMT, it was notable the emergence of the genera *Lactobacillus* and *Ligilactobacillus*, which showed a very pronounced increase in their relative abundance at the end of the trial with respect to the pre-IMT group. Many species of these lactic acid bacteria (LAB) have been proposed as promising probiotics for humans and in animal production, that can serve as efficient alternatives to antibiotics for improving animal performance and preventing diseases[57,58]. As mentioned above, these genera were not present in the bacterial populations of gilthead seabream from the pre-IMT group nor in the Atlantic salmon microbiota, so it was clear that their origin in the intestine was through their abundant presence in the experimental GSB diet. It is difficult to categorically conclude whether these bacteria colonized the intestinal mucosa of gilthead seabream or were present just in the transient microbiota that circulates through the intestinal tract, or both, since we collected both the autochthonous and allochthonous microbiota in a single analytical sample from each fish. However, their increasing abundance over time seemed to indicate that these probiotic species introduced through the diet were colonizing the gut as an adaptation to a healthy gut condition once the effects of the AM treatment had subsided following the introduction of the IMT. Two other remarkable genera showing significant differences at 2 days post-IMT with respect to gilthead seabream from the pre-IMT group were *Vibrio*, which decreased over time, and *Escherichia-Shigella*, which increased over time. In this case, it was difficult to determine to what extent these differences could be attributed to the influence of the microbial profile from donor Atlantic salmon, to the GSB diet, as it also showed differences for the abundance of these genera with respect to the fish post-IMT at all the times, or to the effect of the AMs, which generated a similar trend in both genera: decrease of *Vibrio* and increase of *Escherichia-Shigella*. These potential pleiotropic differences may also be due to many other factors, such as the thermal impact from the use of Atlantic salmon donors grown at 12 °C rather than the 20 °C at which the seabream were kept, or due to the interaction among gut bacterial communities. As an example of such potential interactions among different bacterial species, LAB and in particular, *Lactobacillus*, are well-known to produce antimicrobial substances that have activity towards many *Vibrio* species[59], which has been applied to the commercial farming of aquatic species to prevent and control vibriosis[60,61]. Therefore, the reduction of *Vibrio* over time under current conditions, may be partly attributed to the mentioned exponential increase in the abundance of *Lactobacillus* and *Ligilactobacillus*. In the case of *Aliivibrio*, it was clear that its emergence in gilthead seabream (2 days) after the IMT was due to its presence in the donor Atlantic salmon microbiota, as it was not found in the recipient animals before the transplant and in the diet. Some species belonging to this genus are found seasonally in the hindgut of Atlantic salmon[62]. Under current conditions, *Aliivibrio* was not maintained over time, showing relative abundances equal or very close to 0% from 7 days post-IMT onwards, probably due to the water temperature differences between both hosts' rearing conditions (12 °C in the Atlantic salmon and 20 °C in gilthead seabream). This hypothesis is supported by the fact that many *Aliivibrio* species have an optimal growth rate within the range of 12–18 °C and lose growth capacity at temperatures of approximately 20 °C[63–65]. On the other hand, Legrand et al.[18] showed a maintenance of various ASVs belonging to the genus *Aliivibrio* when performing a FMT among individuals of yellowtail kingfish reared at 12.7–14 °C at 2 days post-FMT, but these were lost at 8 days post-FMT. This may indicate that this genus, not only to thermal changes, but is also very sensitive to shifts in the environmental conditions. In addition, it is not uncommon to observe that part of the donor bacterial taxa which initially colonized the recipient gut after a FMT are not maintained over time[18,20]. In this sense, a higher efficiency in the persistence of the bacterial composition may be achieved by repeating IMT/FMT over time, as already shown in mice[66], and considering the higher efficiency of multiple dose-FMTs in humans for therapeutic applications with respect to single dose-FMTs[67–69]. It is also important to note that the current trial was a first conceptual approach in an attempt to understand the microbial dynamics when performing an IMT between two fish spices reared under quite opposite conditions, being in this case the temperature the determining factor, but for greater efficiency in interspecific IMT/FMT, two species cultured at similar conditions must be sought.

At 36 days post-IMT, the results from gilthead seabream fed the GSB diet showed the importance of the homologous diet for defining the fish microbiota in the long-term after an IMT, with 56% of the genera showing a relative abundance similar to the diet. This is consistent with the already demonstrated high proficiency of the diet for determining the bacterial composition of the fish gut during non-starvation periods[56,70]. The microbiota from the Atlantic salmon and baseline gilthead seabream from the pre-IMT group also played an important, but slighter, role in shaping the gut bacterial communities (31% of the genera had a relative abundance similar to gilthead seabream from the pre-IMT group and Atlantic salmon microbiota). These results were partly in agreement with the previous studies of microbial transplants that have been performed in fish. In particular, while Smith et al.[15] and Zhang et al.[17] showed successful results for African turquoise killifish and large yellow croaker respectively, their microbial composition in terms of phyla and genera and their abundances varied with respect to both the donor and recipient animals. On the other hand, in the work of Legrand et al.[18] in yellowtail kingfish microbiota, the FMT only worked in some individuals, and in any case, the transplanted animals displayed a composition profile much more similar to the recipient fish than to the donors. Regarding microbial transplants among different species, when Rawls et al.[21] transplanted the gut microbiota of mouse into germ-free zebrafish, the vast majority of phyla from the donor mice were maintained, adapting their abundances to the recipient animals. However, few genera from the donors were maintained after the transference, similar to what was observed in the study of Valenzuela et al.[20] after performing a FMT from human to zebrafish. In line with this, Toh et al.[19] performed an inoculation of bacteria isolated from cultures derived from the human gastrointestinal tract into zebrafish, but only two out of 22 species were established in the zebrafish gut microbiota. Nonetheless, our study is the first attempt to perform an IMT between two different fish species and none of the

above-mentioned studies considered to evaluate the impact of the diet on the microbiota of the transplanted fish. In this sense, in the present work, at the final sampling point the effect of the bacterial composition from the diet was clear for the relative abundances of *Peptostreptococcus* and *Limosilactobacillus*, as these genera found in the GSB diet were not present in the Atlantic salmon microbiota nor in the gilthead seabream from the pre-IMT group. Another genus which was probably influenced by the diet over time was *Photobacterium*, since the presence of *Lactobacillus,* either present in the diet as a probiotic or in the fish intestine, has been suggested to negatively regulate the abundance of *Photobacterium* in shrimp[71] and fish species such as gilthead seabream[72], in the same manner as described above for *Vibrio*. Related to this, some species belonging to the Vibrionaceae family (*Photobacterium* and *Vibrio* genera) may act as potential pathogens[54], so it may be hypothesized that the microbial transplant executed within the present assay, and the homologous experimental diet provided to the recipient fish thereafter, may have a beneficial effect on the host's health by the exclusion of potential pathogenic bacteria. However, the genera *Photobacterium* and *Vibrio* also contain many species that are typical symbionts in fish intestines that can produce hydrolytic enzymes that help with digestion[39,54], so for demonstrating their effect on the host intestinal health further studies focused on characterizing these species and their metabolic potentials would be needed.

Apart from the impact of the diet during the trial, these results showed that the initial gilthead seabream microbiota (pre-AMs and pre-IMT) also had an important influence in the short-term establishment of the microbiota after the IMT. This was reflected in the presence of *Catenococcus* and *Brevinema* up to 16 days post-IMT, as both genera were found only in gilthead seabream from the pre-IMT group, but not in the Atlantic salmon microbiota, nor in the GSB diet. Therefore, their presence after the IMT may be associated to either environmental factors or to factors intrinsic to the fish species such as their physiology, or genetic background, as both bacterial genera have been previously reported as part of the autochthonous fish microbiota[72–74]. Neither of these genera were maintained in the long-term (36 days post-IMT), though it may be due to the higher impact of the GSB diet, as the abundance of *Brevinema* in the fish gut mucosa has been shown to be easily modulated by the ingredient composition of the diet[75,76], or due to competition with other bacteria after the IMT. Indeed, the competition among bacterial populations and their subsequent decrease in abundance of some may free up gut niches and resources needed for the emergence and growth of novel bacterial species, such as those belonging to the genera *Acinetobacter* and *Corynebacterium*, which were not present in the gut microbiota of Atlantic salmon or gilthead seabream from the pre-IMT group, nor in the GSB diet. Curiously, a previous assay in Atlantic salmon has shown a correlation between the increase of rearing water temperatures up to 21 °C and the disappearance of *Acinetobacter* and LAB, and increase of *Vibrio*[77]. Thus, under current experimental conditions the above-mentioned differences in the abundance of these genera before and after the IMT may also be related to microbial communities coming from fish normally restricted to lower water temperatures (12 °C) being introduced into an environment with higher temperatures (20 °C). However, interestingly, in the present study, the observed dynamics were opposite to those that would be expected based on the work of the former authors, which indicated that some bacteria have the capacity to adapt to new conditions, which supports the relevance and viability of performing interspecific IMTs[21,78]. In this sense, it is well-known that the fish microbiota is a constantly changing element, which has the capacity to coevolve with the host and create very unique adaptations that are not found in other vertebrate groups[79,80]. There are many ways the holobiont may be influenced, and the microbial communities within can adapt, thereby altering the holobiont. Such complexity of the holobiont is clearly reflected in the difficulty to elucidate what are the primary drivers altering the composition of the microbiota, whether by the composition of the donors, recipients, or by the diet, as exemplified in the current study. In addition, there are few assays performing inter-species microbiota transfers in fish[19–21] and none of them had considered the diet as a key factor, until now. More studies of this type will be needed to gain better knowledge about the modulation of the fish gut microbiota through host-, donor-, and diet-microbiota interactions within the holobiont.

Bacterial community composition of gilthead seabream fed with their heterologous (salmon) diet post-IMT

Similar to the gilthead seabream subjected to the IMT and then fed with their homologous diet, at 2 days post-IMT in the fish fed the heterologous diet (for salmon) the abundances of all represented phyla were similar to those observed in the Atlantic salmon microbiota. On the other hand, although most phyla also presented a relative abundance like that of gilthead seabream from the pre-IMT group, Bacteroidota increased significantly after the IMT, possibly due to the effect of AMs that boosted their abundance, or due to their significant abundance in the salmon diet, as compared to the lower value in the GSB diet. At the genus level, at 2 days post-IMT the decreased abundance in *Vibrio* and increase in *Escherichia-Shigella*, with respect to gilthead seabream from the pre-IMT group, evinced that the effects of the AMs were mainly responsible for such changes in abundance in the above mentioned genera, as these same dynamics were also observed in the other two groups (gilthead seabream fed with the GSB diet and for those that were fasted) shortly after the AM treatment. These results were partly expected since some *Vibrio* strains have been shown to be sensitive to both ampicillin and neomycin (both included in the AM cocktail)[81–83]. In addition, *Escherichia-Shigella* also showed an increase in the gut of mice treated with an antibiotic cocktail containing the same antibiotics that we used[41]. Furthermore, the decrease in *Vibrio* and increase in *Escherichia-Shigella* were maintained in all sampling times post-IMT, with the latter showing an increasing colonization of gilthead seabream gut over time. *Escherichia-Shigella* is habitually found in the intestine of farmed gilthead seabream[75,84], and have been reported as part of the core gut microbiota in some fish species[85–87]; thus, its constant increasing abundance may be attributed to its survival and persistent growth over time as part of the gilthead seabream core microbiota. The persistency of the core microbiota may be consistent with the high abundance of the ASVs which were shared among gilthead seabream fed the GSB and the salmon

diets and the group not submitted to the IMT (post-AMs) at both 7 and 16 days post-IMT, as shown in the Venn diagram (Fig. 9E,F). In addition, the fact that there were no significant differences in beta diversity among fasted gilthead seabream at 17 days post-AMs and the group fed the GSB diet at 16 days post-IMT suggested a progression towards a common status in both groups once the microbial communities are recovered from the AM treatment and adapted to the changes caused by the transplant. However, this was not observed in the group fed the salmon diet, which showed significant differences with respect to fasted gilthead seabream at 17 days post-AMs, underlining again the essential role of the diet in the definition of the microbiota, as discussed above.

On the other hand, the up-regulation of *Photobacterium* at 2 days after the IMT seemed to be mainly a direct consequence of the abundant presence of this genus in the gut microbiota of donor fish, which may be also increased by its presence in the gut of the recipient gilthead seabream. At this point, the effect of the salmon diet in the modulation of the abundance of *Photobacterium* was probably irrelevant as only 2 days had passed since the IMT and its presence in the diet was very low. However, over a longer-term scope, the initial high density of this genus in donor and recipient fishes apparently moved to the background, and it might be plausible that other factors such as the diet took priority. In this sense, as happened with fish fed the GSB diet, the decrease of *Vibrio* and *Photobacterium* over time might have been a response to the presence of LAB in the feed, albeit at a lower percentage compared to the GSB diet. However, for fish fed the salmon diet, the LAB did not ultimately colonize the intestinal mucosa (absence of both genera at 36 days post), but these bacteria were included in the diet being fed, and bacterial colonization is well-known to not be necessary to induce host microbiota modification[88–90]. On the other hand, the increased abundance of *Aliivibrio* at 2 days after the IMT was clearly a consequence of its abundance in the Atlantic salmon microbiota, as it was present neither in the recipient fish microbiota nor in the salmon diet, and again its disappearance over time may be related to the change of environmental temperatures. A remarkable appearance of *Mycoplasma* at 7 days post-IMT was noted, which was only present in the Atlantic salmon microbiota and is commonly found in the intestinal mucosa of salmonids[91], thus indicating that the *Mycoplasma* likely originated from the donor microbiota. Interestingly, when the relative abundance of *Mycoplasma* increased at 7 days post-IMT, that of *Aliivibrio* decreased to 0%, in accordance with the model proposed by Scheuring et al.[92], which suggested resource competition between these two genera and toxicity of some *Aliivibrio* strains towards *Mycoplasma*. However, *Aliivibrio* did not remain over time, as its abundance was very low and probably there were not optimal growing conditions for species of this genus, or the competition with other bacteria prevented their growth.

The influence of the three tested factors (feed, donor microbiota and recipient microbiota) on the establishment of the gut bacterial communities after an IMT was much higher in the case of fish fed the salmon diet than in their congeners fed the GSB diet. In particular, 77% of the genera were similar to gilthead seabream from the pre-IMT group and Atlantic salmon microbiota, while 65% of genera presented a relative abundance similar to the microbial profile of the salmon diet. Apart from *Photobacterium*, *Vibrio* and *Escherichia-Shigella*, the only genera that showed significant differences at the final sampling point, with respect to gilthead seabream from the pre-IMT group, were: *Acinetobacter*, *Catenococcus* and *Vibrionimonas*. Regarding *Acinetobacter*, probably as happened with *Pseudomonas*, there was a colonization of the gut mucosa by these genera from dietary origin. Both genera from the order Pseudomonadales tend to inhabit the intestinal mucosa of fish[93] and have been reported to possess enzymatic activities that favour a higher nutrient digestion[94]. The Pseudomonadales are also noted for their ability to metabolize a wide variety of substrates, including various antibiotics[45], which may explain their higher plasticity to grow out in fish gut after the AM treatment. This metabolic activity in the current context, would give those bacterial species an added benefit towards survival during the initial stages of this trial, as seen in the fasted gilthead seabream with the increased relative abundance of *Acinetobacter* and *Pseudomonas* at 24 h post-AMs. In gilthead seabream fed the salmon diet after the IMT, some genera which were present at lower abundances in the salmon diet (≤0.5%), and were not present in the donor's microbiota, nor in gilthead seabream from the pre-IMT group showed a similar trend to that shown for the Pseudomonodales. However, such genera showed greater abundances at the final sampling time: *Vibrionimonas*, *Corynebacterium*, *Sphingomonas*, and *Cutibacterium*. As *Acinetobacter* and *Corynebacterium* also appeared at 36 days post-IMT (though at lower %) in fish fed the GSB diet (which were not part of the core microbiota of the recipient fish), the abundances of the mentioned genera in the fish gut may not only be attributed to their dietary origin. However, other hypotheses may not be excluded such as the emergence of some strains, possibly induced by specific compounds provided in the diet or by other factors, like the competition among bacteria for resources after the IMT. Finally, unlike fish fed the GSB diet, the feeding of the salmon diet led to the incorporation of *Catenococcus* through the diet and this genus was maintained until 36 days post-IMT, reaffirming that the microbial composition of the diet is a fundamental element in defining the microbiota after a dysbiosis caused by such an invasive strategy as both anal and oral antibiotic gavage followed by an IMT.

Conclusions

The methodological approach for performing an intestinal microbiota transplant between fish as outlined in the current study sheds light on the mechanisms underlying the modulation of the host microbiota after a gut microbial transplant between different fish species, with the added value that it not only considers the microbiota and the different environmental conditions of the donor and the recipient fishes, but also the bacteria present in the diets of donor and recipient. Among our findings, the effect of the diet type was notable for defining the gut estimated richness (ACE index) in the long-term and the bacterial diversity (Shannon) and phylogenetic diversity (Faith index) in the short-term, that reached an equilibrium inherent to the host environment or to the animal over the longer term. The diet displayed a high level of influence in defining the gut microbial structure, as reflected by the weighted UniFrac values. Regarding composition, it was difficult to assess to what extent the similarities or differences in abundances found over time after the IMT were due to the donor and recipient fish

microbiota, to the bacteria present in the feed, or to the summation of all of them, since there are many factors which indirectly may affect bacterial abundance, such as the change of environmental conditions (i.e., inhibition of growth at certain environmental conditions for *Aliivibrio*), competition among bacteria (i.e., inhibition of *Photobacterium* and *Vibrio* by LAB, or competition between *Aliivibrio* and *Mycoplasma*), release of metabolites to the gut environment (i.e., toxins by *Aliivibrio*), etc. What can undoubtedly be drawn from this study is that: (i) the "core" microbiota of the animal is capable of being reestablished over time in the absence of outside influence such as diet, despite subjecting the host to different dysbiosis causing strategies such as a treatment of AM or an IMT; (ii) the diet plays a very important role in defining the gut microbiota of the host animal, as seen in those gilthead seabream fed the GSB diet, where the bacterial composition more closely resembled that of the feed; and (iii) in fish fed the salmon diet, while there was a large part of the bacterial taxa composition from the Atlantic salmon gut microbiota that was also maintained or reestablished by the end of the assay, the overall microbiota was more unique arising from a combination of extrinsic and intrinsic drivers. From a future perspective, there exists some potential for application of IMT for improvement of digestive capabilities and enhancement of fish health that is worthy of future exploration. In this sense, microbial transplants have been shown to be a good strategy to improve growth and feed performance, not only in ruminants[13,95], but also in swine[96] and, slightly, in poultry[97]. Overall, the application of IMT in fish seems to be a promising strategy with multitude of potential applications to be developed, but for that, in parallel with this, it is important to continue evaluating the impact of the different factors affecting the gut microbial composition and functionality when applying this strategy. However, it is also important to consider that a limiting factor of IMTs in fish is the number of individuals that are managed in aquaculture, which makes their massive application on an industrial scale difficult.

Data availability

Raw sequencing data and metadata for all samples included in this study have been uploaded to the Sequence Read Archive (SRA) database of NCBI (https://www.ncbi.nlm.nih.gov/sra) and can be accessed with the Bioproject accession number PRJNA1029014, while the processed data outcomes have been included within the manuscript or additional files.

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References

1. Brugman, S. *et al.* A comparative review on microbiota manipulation: lessons from fish, plants, livestock, and human research. *Front Nutr.* **5**, 80. https://doi.org/10.3389/fnut.2018.00080 (2018).
2. Mills, S., Stanton, C., Lane, J. A., Smith, G. J. & Ross, R. P. Precision nutrition and the microbiome, part I: current state of the science. *Nutrients* **11**, 923. https://doi.org/10.3390/nu11040923 (2019).
3. Sommer, F., Anderson, J. M., Bharti, R., Raes, J. & Rosenstiel, P. The resilience of the intestinal microbiota influences health and disease. *Nat. Rev. Microbiol.* **15**, 630–638. https://doi.org/10.1038/nrmicro.2017.58 (2017).
4. Brugman, S. *et al.* T lymphocytes control microbial composition by regulating the abundance of *Vibrio* in the zebrafish gut. *Gut Microbes* **5**, 737–747. https://doi.org/10.4161/19490976.2014.972228 (2014).
5. Astudillo-García, C. *et al.* Evaluating the core microbiota in complex communities: A systematic investigation. *Environ. Microbiol.* **19**, 1450–1462. https://doi.org/10.1111/1462-2920.13647 (2017).
6. Hasan, N. & Yang, H. Factors affecting the composition of the gut microbiota, and its modulation. *PeerJ* **7**, e7502. https://doi.org/10.7717/peerj.7502 (2019).
7. Kuziel, G. A. & Rakoff-Nahoum, S. The gut microbiome. *Curr. Biol.* **32**, R257–R264. https://doi.org/10.1016/j.cub.2022.02.023 (2022).
8. Wegierska, A. E. *et al.* The connection between physical exercise and gut microbiota: Implications for competitive sports athletes. *Sports Med.* **52**, 2355–2369. https://doi.org/10.1007/s40279-022-01696-x (2022).
9. Cheng, G. *et al.* Antibiotic alternatives: The substitution of antibiotics in animal husbandry?. *Front. Microbiol.* **5**, 217. https://doi.org/10.3389/fmicb.2014.00217 (2014).
10. Bakken, J. S. *et al.* Treating *Clostridium difficile* infection with fecal microbiota transplantation. *Clin. Gastroenterol. Hepatol.* **9**, 1044–1049. https://doi.org/10.1016/j.cgh.2011.08.014 (2011).
11. Choi, H. H. & Cho, Y. S. Fecal microbiota transplantation: current applications, effectiveness, and future perspectives. *Clin. Endosc.* **49**, 257–265. https://doi.org/10.5946/ce.2015.117 (2016).
12. DePeters, E. J. & George, L. W. Rumen transfaunation. *Immunol. Lett.* **162**, 69–76. https://doi.org/10.1016/j.imlet.2014.05.009 (2014).
13. Ribeiro, G. O. *et al.* Repeated inoculation of cattle rumen with bison rumen contents alters the rumen microbiome and improves nitrogen digestibility in cattle. *Sci. Rep.* **7**, 1276. https://doi.org/10.1038/s41598-017-01269-3 (2017).
14. Li, N. *et al.* Spatial heterogeneity of bacterial colonization across different gut segments following inter-species microbiota transplantation. *Microbiome* **8**, 161. https://doi.org/10.1186/s40168-020-00917-7 (2020).
15. Smith, P. *et al.* Regulation of life span by the gut microbiota in the short-lived African turquoise killifish. *Elife* **6**, e27014. https://doi.org/10.7554/eLife.27014 (2017).
16. Hu, C. *et al.* Young fecal transplantation mitigates the toxicity of perfluorobutanesulfonate and potently refreshes the reproductive endocrine system in aged recipients. *Environ. Int.* **167**, 107418. https://doi.org/10.1016/j.envint.2022.107418 (2022).
17. Zhang, J. *et al.* Effects of fecal bacteria on growth, digestive capacity, antioxidant capacity, intestinal health of large yellow croaker (*Larimichthys crocea*) larvae. *Aquaculture* **562**, 738796. https://doi.org/10.1016/j.aquaculture.2022.738796 (2023).
18. Legrand, T. P. *et al.* Antibiotic-induced alterations and repopulation dynamics of yellowtail kingfish microbiota. *Anim. Microbiome* **2**, 26. https://doi.org/10.1186/s42523-020-00046-4 (2020).
19. Toh, M. C., Goodyear, M., Daigneault, M., Allen-Vercoe, E. & Van Raay, T. J. Colonizing the embryonic zebrafish gut with anaerobic bacteria derived from the human gastrointestinal tract. *Zebrafish* **10**, 194–198. https://doi.org/10.1089/zeb.2012.0814 (2013).
20. Valenzuela, M. J. *et al.* Evaluating the capacity of human gut microorganisms to colonize the zebrafish larvae (*Danio rerio*). *Front. Microbiol.* **9**, 1032. https://doi.org/10.3389/fmicb.2018.01032 (2018).
21. Rawls, J. F., Mahowald, M. A., Ley, R. E. & Gordon, J. I. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell* **127**, 423–433. https://doi.org/10.1016/j.cell.2006.08.043 (2006).
22. FAO. The State of World Fisheries and Aquaculture 2022. Towards Blue Transformation. Rome (2022).

23. Salomón, R. *et al.* A bioactive extract rich in triterpenic acid and polyphenols from *Olea europaea* promotes systemic immunity and protects Atlantic salmon smolts against furunculosis. *Front. Immunol.* **12**, 737601. https://doi.org/10.3389/fimmu.2021.737601 (2021).
24. Quaranta, G. *et al.* Laboratory handling practice for faecal microbiota transplantation. *J. Appl. Microbiol.* **128**, 893–898. https://doi.org/10.1111/jam.14522 (2020).
25. Illumina. 16S Metagenomic Sequencing Library Preparation: Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System. https://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf (2013).
26. Hall, M. & Beiko, R. G. 16S rRNA gene analysis with QIIME2: Methods and protocols. *Methods Mol. Biol.* **1849**, 113–129. https://doi.org/10.1007/978-1-4939-8728-3_8 (2018).
27. Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **13**, 581–583. https://doi.org/10.1038/nmeth.3869 (2016).
28. Smith, P. E. *et al.* Synthetic sequencing standards: a guide to database choice for rumen microbiota amplicon sequencing analysis. *Front. Microbiol.* **11**, 606825. https://doi.org/10.3389/fmicb.2020.606825 (2020).
29. McKnight, D. T. *et al.* Methods for normalizing microbiome data: An ecological perspective. *Methods Ecol. Evol.* https://doi.org/10.1111/2041-210X.13115 (2019).
30. Kim, B. R. *et al.* Deciphering diversity indices for a better understanding of microbial communities. *J. Microbiol. Biotechnol.* **27**, 2089–2093. https://doi.org/10.4014/jmb.1709.09027 (2017).
31. Faith, D. P. Conservation evaluation and phylogenetic diversity. *Biol. Conserv.* **61**, 1–10. https://doi.org/10.1016/0006-3207(92)91201-3 (1992).
32. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1–48. https://doi.org/10.18637/jss.v067.i01 (2015).
33. Paulson, J.N., Pop, M. & Bravo, H. C. metagenomeSeq: Statistical analysis for sparse high-throughput sequencing. https://www.bioconductor.org/packages/devel/bioc/vignettes/metagenomeSeq/inst/doc/metagenomeSeq.pdf (2023).
34. Paulson, J. N., Stine, O. C., Bravo, H. C. & Pop, M. Differential abundance analysis for microbial marker-gene surveys. *Nat. Methods* **10**, 1200–1202. https://doi.org/10.1038/nmeth.2658 (2013).
35. Bray, J. R. & Curtis, J. T. An ordination of the upland forest communities of southern Wisconsin. *Ecol. Monogr.* **27**, 326–349. https://doi.org/10.2307/1942268 (1957).
36. Chang, Q., Luan, Y. & Sun, F. Variance adjusted weighted UniFrac: A powerful beta diversity measure for comparing communities based on phylogeny. *BMC Bioinform.* **12**, 1–14. https://doi.org/10.1186/1471-2105-12-118 (2011).
37. Liu, C., Cui, Y., Li, X. & Yao, M. *microeco*: an R package for data mining in microbial community ecology. *FEMS Microbiol. Ecol.* **97**, fiaa255. https://doi.org/10.1093/femsec/fiaa255 (2021).
38. Kormas, K. A., Meziti, A., Mente, E. & Frentzos, A. Dietary differences are reflected on the gut prokaryotic community structure of wild and commercially reared sea bream (*Sparus aurata*). *Microbiologyopen* **3**, 718–728. https://doi.org/10.1002/mbo3.202 (2014).
39. Piazzon, M. C. *et al.* Under control: How a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. *Microbiome* **5**, 164. https://doi.org/10.1186/s40168-017-0390-3 (2017).
40. Gong, Y. *et al.* Antibiotic changes host susceptibility to *Eimeria falciformis* infection associated with alteration of gut microbiota. *Infect. Immun.* **90**, e00229-e322. https://doi.org/10.1128/iai.00229-22 (2022).
41. Ray, P., Chakraborty, S., Ghosh, A. & Aich, P. Effects of treatment with three antibiotics, vancomycin, neomycin, and AVNM on gut microbiome in C57BL/6 mice. *BioRxiv.* https://doi.org/10.1101/2021.02.08.430372 (2021).
42. Rodrigues, R. R. *et al.* Antibiotic-induced alterations in gut microbiota are associated with changes in glucose metabolism in healthy mice. *Front. Microbiol.* **8**, 2306. https://doi.org/10.3389/fmicb.2017.02306 (2017).
43. Lee, J. G. *et al.* Role of the global gut microbial community in the development of colitis-associated cancer in a murine model. *Biomed. Pharmacother.* **135**, 111206. https://doi.org/10.1016/j.biopha.2020.111206 (2021).
44. Rosa, C. P., Brancaglion, G. A., Miyauchi-Tavares, T. M., Corsetti, P. P. & de Almeida, L. A. Antibiotic-induced dysbiosis effects on the murine gastrointestinal tract and their systemic repercussions. *Life Sci.* **207**, 480–491. https://doi.org/10.1016/j.lfs.2018.06.030 (2018).
45. Dantas, G., Sommer, M. O. A., Oluwasegun, R. D. & Church, G. M. Bacteria subsisting on antibiotics. *Science* **320**, 100–103. https://doi.org/10.1126/science.1155157 (2008).
46. Tsilimigras, M. C. & Fodor, A. A. Compositional data analysis of the microbiome: Fundamentals, tools, and challenges. *Ann. Epidemiol.* **26**, 330–335. https://doi.org/10.1016/j.annepidem.2016.03.002 (2016).
47. Spanggaard, B. *et al.* The microflora of rainbow trout intestine: A comparison of traditional and molecular identification. *Aquaculture* **182**, 1–15. https://doi.org/10.1016/S0044-8486(99)00250-1 (2000).
48. Kim, P. S. *et al.* Host habitat is the major determinant of the gut microbiome of fish. *Microbiome* **9**, 166. https://doi.org/10.1186/s40168-021-01113-x (2021).
49. Rudi, K. *et al.* Stable core gut microbiota across the freshwater-to-saltwater transition for farmed Atlantic salmon. *Appl. Environ. Microbiol.* **84**, e01974-e2017. https://doi.org/10.1128/AEM.01974-17 (2018).
50. Roeselers, G. *et al.* Evidence for a core gut microbiota in the zebrafish. *ISME J* **5**, 1595–1608. https://doi.org/10.1038/ismej.2011.38 (2011).
51. Dhanasiri, A. K., Brunvold, L., Brinchmann, M.F., Korsnes, K., Bergh, Ø. & Kiron, V. Changes in the intestinal microbiota of wild Atlantic cod *Gadus morhua* L. upon captive rearing. *Microb. Ecol.* **61**, 20–30 (2011).
52. Piazzon, M. C. *et al.* Sex, age, and bacteria: How the intestinal microbiota is modulated in a protandrous hermaphrodite fish. *Front. Microbiol.* **10**, 2512. https://doi.org/10.3389/fmicb.2019.02512 (2019).
53. Navarrete, P. *et al.* PCR-TTGE analysis of 16S rRNA from rainbow trout (*Oncorhynchus mykiss*) gut microbiota reveals host-specific communities of active bacteria. *PloS One* **7**, e31335. https://doi.org/10.1371/journal.pone.0031335 (2012).
54. Egerton, S., Culloty, S., Whooley, J., Stanton, C. & Ross, R. P. The gut microbiota of marine fish. *Front. Microbiol.* **9**, 873. https://doi.org/10.3389/fmicb.2018.00873 (2018).
55. Leeming, E. R., Johnson, A. J., Spector, T. D. & Le Roy, C. I. Effect of diet on the gut microbiota: Rethinking intervention duration. *Nutrients* **11**, 2862. https://doi.org/10.3390/nu11122862 (2019).
56. Viver, T. *et al.* Food determines ephemerous and non-stable gut microbiome communities in juvenile wild and farmed Mediterranean fish. *Sci. Total Environ.* **889**, 164080. https://doi.org/10.1016/j.scitotenv.2023.164080 (2023).
57. Guerrero Sanchez, M., Passot, S., Campoy, S., Olivares, M. & Fonseca, F. *Ligilactobacillus salivarius* functionalities, applications, and manufacturing challenges. *Appl. Microbiol. Biotechnol.* **106**, 57–80. https://doi.org/10.1007/s00253-021-11694-0 (2022).
58. Van Doan, H. *et al.* Host-associated probiotics: A key factor in sustainable aquaculture. *Rev. Fish Sci. Aquac.* **28**, 16–42. https://doi.org/10.1080/23308249.2019.1643288 (2020).
59. Koga, T., Mizobel, T. & Takumi, K. Antibacterial activity of *Lactobacillus* species against *Vibrio* species. *Microbiol. Res.* **153**, 271–275. https://doi.org/10.1016/s0944-5013(98)80011-6 (1998).
60. Naiel, M. A. *et al.* Using lactic acid bacteria as an immunostimulants in cultured shrimp with special reference to *Lactobacillus* spp. *Aquac. Int.* **29**, 219–231. https://doi.org/10.1007/s10499-020-00620-2 (2021).

61. Dhanasekaran, D., Saha, S., Thajuddin, N., Rajalakshmi, M. & Panneerselvam, A. Probiotic effect of *Lactobacillus* isolates against bacterial pathogens in fresh water fish. *J. Coast Dev.* **13**, 103–112 (2010).
62. Klemetsen, T., Karlsen, C. R. & Willassen, N. P. Phylogenetic revision of the genus *Aliivibrio*: intra-and inter-species variance among clusters suggest a wider diversity of species. *Front. Microbiol.* **12**, 626759. https://doi.org/10.3389/fmicb.2021.626759 (2021).
63. Khrulnova, S. A., Manukhov, I. V. & Zavil'gel'skii, G. B. "Quorum sensing" regulation and the structure of *lux* the operon in marine bacteria *Aliivibrio logei*. *Russ. J. Genet.* **47**, 1415–1421. https://doi.org/10.1134/S1022795411120052 (2011).
64. Söderberg, J. J., Grgic, M., Hjerde, E. & Haugen, P. *Aliivibrio wodanis* as a production host: development of genetic tools for expression of cold-active enzymes. *Microb. Cell Fact.* **18**, 197. https://doi.org/10.1186/s12934-019-1247-1 (2019).
65. Colquhoun, D. J., Alvheim, K., Dommarsnes, K., Syvertsen, C. & Sørum, H. Relevance of incubation temperature for *Vibrio salmonicida* vaccine production. *J. Appl. Microbiol.* **92**, 1087–1096. https://doi.org/10.1046/j.1365-2672.2002.01643.x (2002).
66. Amorim, N. *et al.* Refining a protocol for faecal microbiota engraftment in animal models after successful antibiotic-induced gut decontamination. *Front. Med.* **9**, 770017. https://doi.org/10.3389/fmed.2022.770017 (2022).
67. Shimizu, H. *et al.* Repeated fecal microbiota transplantation in a child with ulcerative colitis. *Pediatr. Int.* **58**, 781–785. https://doi.org/10.1111/ped.12967 (2016).
68. Fehily, S. R., Basnayake, C., Wright, E. K. & Kamm, M. A. Fecal microbiota transplantation therapy in Crohn's disease: Systematic review. *J. Gastroenterol. Hepatol.* **36**, 2672–2686. https://doi.org/10.1111/jgh.15598 (2021).
69. Rossen, N. G. *et al.* Fecal microbiota transplantation as novel therapy in gastroenterology: A systematic review. *World J. Gastroenterol.* **21**, 5359–5371. https://doi.org/10.3748/wjg.v21.i17.5359 (2015).
70. Silva, F. C. D. P., Nicoli, J. R., Zambonino-Infante, J. L., Kaushik, S. & Gatesoupe, F. J. Influence of the diet on the microbial diversity of faecal and gastrointestinal contents in gilthead sea bream (*Sparus aurata*) and intestinal contents in goldfish (*Carassius auratus*). *FEMS Microbiol. Ecol.* **78**, 285–296. https://doi.org/10.1111/j.1574-6941.2011.01155.x (2011).
71. Huynh, T. G., Hu, S. Y., Chiu, C. S., Truong, Q. P. & Liu, C. H. Bacterial population in intestines of white shrimp, *Litopenaeus vannamei* fed a synbiotic containing *Lactobacillus plantarum* and galactooligosaccharide. *Aquac. Res.* **50**, 807–817. https://doi.org/10.1111/are.13951 (2019).
72. Rosado, D., Canada, P., Marques Silva, S., Ribeiro, N., Diniz, P. & Xavier, R. Disruption of the skin, gill, and gut mucosae microbiome of gilthead seabream fingerlings after bacterial infection and antibiotic treatment. *FEMS Microbes* **4**, xtad011. https://doi.org/10.1093/femsmc/xtad011 (2023).
73. Quero, G. M. *et al.* Host-associated and environmental microbiomes in an open-sea Mediterranean gilthead sea bream fish farm. *Microb. Ecol.* **86**, 1319–1330. https://doi.org/10.1007/s00248-022-02120-7 (2022).
74. Ruiz, A. *et al.* Modulation of gut microbiota and intestinal immune response in gilthead seabream (*Sparus aurata*) by dietary bile salt supplementation. *Front. Microbiol.* **14**, 1123716. https://doi.org/10.3389/fmicb.2023.1123716 (2023).
75. Naya-Català, F. *et al.* Genetics and nutrition drive the gut microbiota succession and host-transcriptome interactions through the gilthead sea bream (*Sparus aurata*) production cycle. *Biology* **11**, 1744. https://doi.org/10.3390/biology11121744 (2022).
76. Huyben, D. *et al.* Effect of dietary oil from *Camelina sativa* on the growth performance, fillet fatty acid profile and gut microbiome of gilthead Sea bream (*Sparus aurata*). *PeerJ* **8**, e10430. https://doi.org/10.7717/peerj.10430 (2020).
77. Neuman, C., Hatje, E., Zarkasi, K. Z., Smullen, R., Bowman, J. P. & Katouli, M. The effect of diet and environmental temperature on the faecal microbiota of farmed Tasmanian Atlantic Salmon (*Salmo salar* L.). *Aquac. Res.* **47**, 660–672. https://doi.org/10.1111/are.12522 (2016).
78. Park, J. C. & Im, S. H. Of men in mice: The development and application of a humanized gnotobiotic mouse model for microbiome therapeutics. *Exp. Mol. Med.* **52**, 1383–1396. https://doi.org/10.1038/s12276-020-0473-2 (2020).
79. Kelly, C. & Salinas, I. Under pressure: interactions between commensal microbiota and the teleost immune system. *Front. Immunol.* **8**, 559. https://doi.org/10.3389/fimmu.2017.00559 (2017).
80. Ikeda-Ohtsubo, W. *et al.* How can we define "optimal microbiota?" A comparative review of structure and functions of microbiota of animals, fish, and plants in agriculture. *Front. Nutr.* **5**, 90. https://doi.org/10.3389/fnut.2018.00090 (2018).
81. Węgrzyn, G. & Czyż, A. Detection of mutagenic pollution of natural environment using microbiological assays. *J. Appl. Microbiol.* **95**, 1175–1181. https://doi.org/10.1046/j.1365-2672.2003.02121.x (2003).
82. Heng, S. P. *et al. Vibrio vulnificus*: An environmental and clinical burden. *Front. Microbiol.* **8**, 997. https://doi.org/10.3389/fmicb.2017.00997 (2017).
83. Baig, M. Z., Abdullah, U. H., Shafquat, Y., Humayun, K. N. & Zafar, A. Non O1, non O139 *Vibrio cholerae* bacteraemia in an infant; case report and literature review. *Springerplus* **4**, 1–9. https://doi.org/10.1186/s40064-015-1346-3 (2018).
84. Marchi, A. *et al.* Feeding gilthead sea bream with increasing dietary bacterial single cell protein level: Implication on growth, plasma biochemistry, gut histology, and gut microbiota. *Aquaculture* **565**, 739132. https://doi.org/10.1016/j.aquaculture.2022.739132 (2023).
85. Gatesoupe, F. J. *et al.* The highly variable microbiota associated to intestinal mucosa correlates with growth and hypoxia resistance of sea bass, *Dicentrarchus labrax*, submitted to different nutritional histories. *BMC Microbiol.* **16**, 1–13. https://doi.org/10.1186/s12866-016-0885-2 (2016).
86. Gao, Y. M. *et al.* Deep insights into gut microbiota in four carnivorous coral reef fishes from the South China Sea. *Microorganisms* **8**, 426. https://doi.org/10.3390/microorganisms8030426 (2020).
87. Deng, Y., Kokou, F., Eding, E. H. & Verdegem MC. Impact of early-life rearing history on gut microbiome succession and performance of Nile tilapia. *Anim. Microbiome* **3**, 1–17. https://doi.org/10.1186/s42523-021-00145-w (2021).
88. Marco, M. L. Is Probiotic Colonization Essential? International Scientific Association for Probiotics and Prebiotics (ISAPP). https://isappscience.org/is-probiotic-colonization-essential/ (2019).
89. Schoultz, I. & Keita, Å. V. Cellular and molecular therapeutic targets in inflammatory bowel disease-focusing on intestinal barrier function. *Cells* **8**, 193. https://doi.org/10.3390/cells8020193 (2019).
90. Moroni, F. *et al.* The effects of nisin-producing *Lactococcus lactis* strain used as probiotic on gilthead sea bream (*Sparus aurata*) growth, gut microbiota, and transcriptional response. *Front. Mar. Sci.* **8**, 659519. https://doi.org/10.3389/fmars.2021.659519 (2021).
91. Llewellyn, M. S. *et al.* The biogeography of the Atlantic salmon (*Salmo salar*) gut microbiome. *ISME J.* **10**, 1280–1284. https://doi.org/10.1038/ismej.2015.189 (2016).
92. Scheuring, I., Rasmussen, J. A., Bozzi, D. & Limborg, M. T. A strategic model of a host–microbe–microbe system reveals the importance of a joint host–microbe immune response to combat stress-induced gut dysbiosis. *Front. Microbiol.* **13**, 912806. https://doi.org/10.3389/fmicb.2022.912806 (2022).
93. Nayak, S. K. Role of gastrointestinal microbiota in fish. *Aquac. Res.* **41**, 1553–1573. https://doi.org/10.1111/j.1365-2109.2010.02546.x (2010).
94. Ray, A. K., Ghosh. K. & Ringø, E. J. A. N. Enzyme-producing bacteria isolated from fish gut: A review. *Aquac. Nutr.* **18**, 465–492. https://doi.org/10.1111/j.1365-2095.2012.00943.x (2012).
95. Pounden, W. D. & Hibbs, J. W. Rumen inoculations in young calves. *J. Am. Vet. Med. Assoc.* **114**, 33–35 (1949).
96. Hu, L. *et al.* Exogenous fecal microbiota transplantation from local adult pigs to crossbred newborn piglets. *Front. Microbiol.* **8**, 2663. https://doi.org/10.3389/fmicb.2017.02663 (2018).
97. Siegerstetter, S. C. *et al.* Fecal microbiota transplant from highly feed-efficient donors shows little effect on age-related changes in feed-efficiency-associated fecal microbiota from chickens. *Appl. Environ. Microbiol.* **84**, e02330-e2417. https://doi.org/10.1128/AEM.02330-17 (2018).

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Author contributions
E.G. and K.B.A. financed the research, conceived and designed the study, and collected the microbiota samples. A.R. processed the microbial samples, analyzed the data, and wrote the original draft. E.G. and K.B.A. contributed to the review and editing of the final draft. All authors have read and approved the final version of the manuscript.

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DISCUSSION

Discussion

The present doctoral thesis aims to propose different strategies to reduce fat accumulation in fish and subsequently improve the animal health and condition, using gilthead seabream (*Sparus aurata*) as a biological model. Two different kinds of strategies were proposed: i) the supplementation of the fish diets with feed additives that possess digestive and hypolipidemic properties; and ii) the regulation of the gut microbial communities through an intestinal microbiota transplant. Under this context, the efficiency of a blend of bile salts on improving fish performance, lipid digestion, promoting lipid catabolism, and reducing the levels of fat accumulation was evaluated (Chapter I; Ruiz et al., 2023a), as well as its modulatory effects on the gut microbial communities and the host's immune response (Chapter II; Ruiz et al., 2023b). Furthermore, the efficiency of a combination of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde (denoted as "SPICY feed additive"; Chapter III; Ruiz et al., 2023c) and of a combination of turmeric, capsicum, black pepper, and ginger oleoresins (denoted as "SO feed additive"; Chapter IV; Ruiz et al., 2024a) was also assessed in gilthead seabream considering the above-mentioned parameters. To test the above-mentioned additives, a basal diet was manufactured with low levels of fish oil (3% in the diet) and poultry fat as the main lipid source (8% in the diet) in order to promote body adiposity. Regarding the intestinal microbiota transplant, although its efficiency in reducing fat accumulation was not tested in this thesis since the use of different feed additives did not allow us to identify a particular bacterial profile associated to lower body adiposity, we proposed a first methodological approach and studied its applicability in terms of success in the intended microbial modulation, as well as the role that the diet plays in shaping the gut microbial communities after the transplant (Chapter V; Ruiz et al., 2024b). In this section, the main results of these five studies will be evaluated together and comparing them to relevant works of fish nutrition literature to facilitate an overview of the importance of the advances made in this thesis.

1. Effect of the blend of bile salts and combinations of spices on fish performance

Animal growth is generally associated with a good health status and indicates a proper fulfilment of nutritional requirements (Lupatsch et al., 1998; Breck, 2014). In addition, animal growth is a key indicator of productivity and economic viability to be considered when evaluating feed formulations in the blue food sector (Tsikliras and Polymeros, 2014). In the present thesis, three different types of dose-response effects in gilthead seabream growth were observed regarding the specific additives tested.

In the case of the blend of bile salts (equal parts of sodium cholate and sodium deoxycholate, and sodium taurocholate hydrate in a proportion of 30/70), we observed a dose-dependent quadratic response, with the highest final body weight (BW_f) values observed when supplementing the diet with a dose of 0.06% of total dietary levels ($BS_{0.06\%}$), and intermediate values with a dose of 0.12% ($BS_{0.12\%}$). The same dose-dependent response was observed for the final standard length (SL_f) and specific growth rate (SGR) (Chapter I; Ruiz et al., 2023a). In consistency with our results, previous studies have also shown a similar quadratic dose-response effect when supplementing bile salts in fish diets, with the highest growth rate observed at the intermediate inclusion levels tested (Appendix 1). For instance, Ding et al. (2020) reported an increased growth of large yellow croaker (*Larimichthys crocea*) when supplementing its diets with bovine bile salts at an inclusion level of 0.03%, but not at 0.015 nor 0.045%. Similarly, Yu et al. (2019) reported an increased fish growth when including a blend of bile acids at 0.03% in largemouth bass (*Micropterus salmoides*), but not at lower (0.008, 0.016, 0.024%) nor higher (0.06%) inclusion levels. In hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂), no effects in growth performance were observed when using a dietary supplement of sodium taurocholic acid at 0.03, 0.06, 0.12, and 0.15%, but this bile salt had a positive growth effect at 0.09% (Xu et al., 2022b). In leopard coral grouper (*Plectropomus leopardus*), a similar significant increase in growth, in terms of BW_f, SGR, and weight gain rate (WGR), was observed when supplementing its diet with a blend of porcine bile acids at 0.3% (Gao et al., 2023). However, the former authors reported that only at an inclusion level of 0.45% was a significant increase in SGR observed, though without an effect on BW_f and WGR with respect to the control diet, while at 0.15 and 0.6% the porcine bile acid blend did not have any positive effect on fish growth (Gao et al., 2023). Several of these studies concluded that the optimal inclusion levels of bile salts in fish diets depends on the fish species and on the type of bile acids (Yu et al., 2019; Xu et al. 2022b). A deficiency or an overdose of bile acids with respect to the species-specific optimum range can even compromise the growth of the fish (Jiang et al., 2018; Adam et al., 2023; Gao et al., 2023). In this sense, Jiang et al. (2018) reported that the dietary supplementation of genetically improved farmed tilapia (GIFT; *Oreochromis niloticus*) diets with bile acids at an inclusion level of 0.005 and 0.015% improved the fish growth. On the other hand, the former authors observed that at higher supplementation levels, the bile acid blend did not promote (at 0.045%), and even decreased (at 0.135%) the fish growth in terms of WGR (Jiang et al., 2018).

Apart from the above mentioned studies in which a dose-dependent quadratic response is observed, the positive effects of bile salts and bile acids on fish growth performance have been well-demonstrated in a wide range of fish species. Some of such species are rainbow trout (*Oncorhynchus mykiss*; Yamamoto et al., 2007; Iwashita et al., 2008), turbot (*Scophthalmus maximus*; Gu et al., 2017), tongue sole (*Cynoglossus semiliaevis*; Li et al., 2021b; Wang et al., 2022), grass carp (*Ctenopharyngodon idella*; Zhou et al., 2018a), Chinese perch (*Siniperca chuatsi*; Zhang et al., 2022a), yellow catfish (*Pelteobagrus fulvidraco*; Yao et al., 2022), and striped catfish (*Pangasianodon hypophthalmus*; Adam et al., 2023). However, controversial results on the effects of bile salt supplementation on fish growth have been found. For instance, some works have shown an absence of improvement when dietary bile acids/salts have been supplemented in the diets of rainbow trout (Iwashita et al., 2009), largemouth bass (Yin et al., 2021), large yellow croaker (Li et al., 2023), and black seabream (*Acanthopagrus schlegelii*; Jin et al., 2019).

Apart from the composition of the blend of bile salts, and dietary inclusion levels, many factors may influence the effect of bile salts on fish performance, including the feeding period, and the diet composition (Gu et al., 2017; Zhang et al., 2022a). Among these factors, the diet composition and bile salt inclusion levels seem to be main drivers, as demonstrated by Bhusare et al. (2023). In brief, the former authors reported that in fish fed a diet containing 38% crude protein and 8% crude lipids, cholic acid improved growth at inclusion levels of 0.05 and 0.1%, while when feeding the fish with a diet with 35% crude protein and 11% crude lipids, only the inclusion level of 0.05% showed a positive effect on growth. On the other hand, the former authors also observed that neither of the tested inclusion levels (0.05 nor 0.1%) had any effect on tilapia growth when feeding with 32% crude protein and 14% crude lipids. In addition, Kortner et al. (2016) observed a growth dependency with respect to the dietary protein source and to the type of bile salts used in Atlantic salmon (*Salmo salar*). In this study, when a diet with partial fishmeal replacement by soy and pea protein concentrates (41% crude protein and 30% crude lipids) was supplemented with sodium taurocholate at 1.8% inclusion, no effects on growth were observed, while when supplementing the same diet with bovine bile salts (not specified) at 1.8% a decrease in growth was observed. On the other hand, when feeding Atlantic salmon with a diet with the same proximate composition but with higher fishmeal levels and partial fishmeal replacement by soy protein, the inclusion of bovine bile salts at 1.8% had no effects on the fish growth (Kortner et al., 2016).

Overall, many reasons have been suggested for the improvement in fish growth observed upon dietary supplementation with bile salts, such as an increased feed intake and feed utilization efficiency, an optimized lipid digestion, and an enhancement in fish lipid metabolism and antioxidant status (Yu et al., 2019; Ding et al., 2020; Gao et al., 2023). Similarly, deficiencies in bile acid content have been associated to low lipid digestion and a consequent decrease in growth performance (Gao et al., 2023). On the other hand, considering that bile acids are absorbed in the intestine and metabolized in the liver and intestinal tract (Romano et al., 2020), their high levels may be toxic for the hepatocytes, leading to impaired liver function, formation of gallstones, and depressed growth (Adam et al., 2023; Jiang et al., 2018), as well as for the enterocytes, damaging the intestinal tissue, impairing its function (Yao et al., 2022).

Concerning the first combination of spices tested in this thesis, known as SPICY, which contained a mixture of capsicum, black pepper, ginger, and cinnamaldehyde, a dose-

dependent plateau response was observed in terms of growth performance indicators. In particular, at an inclusion level of 0.1% ($SPICY_{0.1\%}$) an improvement in BW_f and SGR was observed, which was maintained at 0.15% ($SPICY_{0.15\%}$; Chapter III; Ruiz et al., 2023c). To date, this combination of spices has already been tested in 1-day-old male chicks at a dietary inclusion level of 0.025%, showing an improved growth performance in terms of BW at 7 days of age (Herrero-Encinas et al., 2023). However, no differences in growth performance were observed from 14 days of age on with respect to chicks fed the basal diet without the SPICY supplementation (Herrero-Encinas et al., 2023). In a recent study, we have tested the same combination of spices at inclusion levels of 0.05, 0.1 and 0.15%, in gilthead seabream fed with a diet that included partial fish oil replacement by mammalian-rendered fat (45% substitution; Ruiz et al., 2024c). In that study, we observed no changes in growth with respect to the group of fish fed the basal diet over time (from 29 to 112 days). This indicates that the effect of spices on fish growth may depend on the dietary lipid source and/or on the age of the fish, since in that study gilthead seabreams had a BW_i of 85 ± 4 g (Chapter IV; Ruiz et al., 2024a), and in the trial included in the present thesis (Chapter III; Ruiz et al., 2023c) their BW_i was of 44 ± 4 g. On the other hand, the second tested combination of spices, denoted as the SO additive, which contains turmeric, capsicum, black pepper, and ginger and was only tested at a dietary inclusion level of 0.2%, caused no changes in any of the growth performance indicators measured (Chapter IV; Ruiz et al., 2024a).

Apart from the above-mentioned study supplementing the SPICY feed additive in a gilthead seabream diet with partial fish oil replacement by mammalian-rendered fat (Ruiz et al., 2024c), there are no other studies evaluating the combined effects of the spices evaluated herein in fish. However, the effects of capsicum, black pepper, ginger, turmeric (or their active principles), and cinnamaldehyde on growth performance, have been individually evaluated (Appendix 2). Regarding the three spices common to both tested combinations (capsicum, black pepper, and ginger), different results have been found since several factors, such as the fish species, feeding period, additive composition (the spices or their active principles), and the dietary inclusion level of the additive have not been applied universally to enable comparison (Appendix 2).

For capsicum, many studies have shown no effects on growth in perciform fish, including blue streak hap (*Labidochromis caeruleus*; Yılmaz and Ergün, 2011), jewel cichlid (*Hemichromis guttatus*; Yigit et al., 2021), gilthead seabream (Wassef et al., 2010), and Mozambique tilapia (*Oreochromis mossambicus*; Yılmaz et al., 2013a). Nonetheless, Ibrahim et al. (2024) reported an improved growth in Nile tilapia (*Oreochromis niloticus*) when diets were supplemented with capsicum at inclusion levels of 0.04, 0.08, and 0.16%. It is important to note that the above-mentioned studies in perciform fish diets used much higher dietary inclusion levels (0.3-15%; Wassef et al., 2010; Yılmaz and Ergün, 2011; Yılmaz et al., 2013a; Yigit et al., 2021) than Ibrahim et al. (2024), which may be the cause of the absence of effects on fish growth. Similar to perciform fish, capsicum dietary dosage also seems to be a paramount factor influencing the growth of salmoniform fish, as demonstrated by Yılmaz et al. (2024). These authors observed that the BW_f, WGR and SGR of rainbow trout increased when including a capsicum oleoresin at 0.7 and 1.4% in the diet, but not at 2.1 and 2.8% inclusion levels. In addition, Yanar et al. (2016) showed no improvement on rainbow trout growth over time (20, 40, 60, and 80 days) at inclusion levels of 0.5, 2, and 4.4%. On the other hand, Talebi et al. (2013) found an amelioration

in rainbow trout BW and total length (TL) when capsicum was incorporated to the diet at lower inclusion levels (0.003, 0.004, and 0.006%), which was maintained in all the tested feeding times (20, 40, and 60 days). The former authors suggested that the absence of effect reported in some studies at high capsicum doses, may be due to the high levels of cellulose in some extracts (21-24% in Yanar et al., 2016), which is non digestible by the host and can negatively affect fish growth (Talebi et al., 2013). On the other hand, Yılmaz and Ergün (2011) stated that the absence of effect of capsicum at high doses in fish may be likely due to the higher concentrations of antinutritional factors, such as excessive pungency, tannin, and saponin contents.

Similar to capsicum, the effect of black pepper on the growth of many fish species depends on its dietary inclusion levels, having an optimum range which varies among species. Indeed, according to the existing literature, the optimum range of black pepper is of 1-2% in rohu (*Labeo rohita*; Ullah et al., 2021), while for piperine (the main active principle of black pepper) the optimum range is of 0.1-0.4% in common carp (*Cyprinus carpio*; Giri et al., 2023), and 0.05-0.075% in olive flounder (*Paralichthys olivaceus*; Malintha et al., 2023). No effects on growth in such fish species were observed at higher nor lower inclusion levels (Wojno et al., 2021; Giri et al., 2023; Malintha et al., 2023; Ullah et al., 2021), even compromising weight gain (WG) in common carp when black pepper was supplemented at 0.02% (Wojno et al., 2021). On the other hand, El-Houseiny et al. (2019) and Stoev and Zhelyazkov (2021) did not find differences in growth performance when using a dietary supplement of black pepper at 0.1% in African catfish (*Clarias gariepinus*) and rainbow trout, respectively.

Regarding ginger, most studies have shown positive results on the growth of different fish species, such as Asian sea bass (*Lates calcarifer*; Talpur et al., 2013), rohu (Sukumaran et al., 2016), common carp (Fazelan et al., 2020; Mohammadi et al., 2020), striped catfish (Ashry et al., 2023), and rainbow trout (Aqmasjed et al., 2023). For instance, the study of Sukumaran et al. (2016) demonstrated that the effect of ginger on fish growth may be influenced by the duration of feeding and dietary inclusion levels. Indeed, while no amelioration on rohu growth was reported when supplementing ginger at 0.6% for 30 days, an improved WG, WGR and SGR were observed at 60 days. In addition, the former authors reported that while at higher inclusion levels (0.8 and 1%) the positive effect of ginger on growth was maintained, no effects were observed at lower inclusion levels (0.2 and 0.4%) (Sukumaran et al., 2016). Conversely, no beneficial effects in growth were observed under supplementation of Nile tilapia diets with ginger at 0.5 and 1%, while a diminishment in growth was observed at an inclusion of 1.5% (Brum et al., 2017).

The fourth component of the SPICY feed additive, cinnamaldehyde, is the main active principle of cinnamon, and has shown growth-promoting effects on many fish species, including tongue sole (Wang et al., 2021a), fat greenling (*Hexagrammos otakii*; Gu et al., 2022), grass carp (Zhou et al., 2020), and Nile tilapia (Abd El-Hamid et al., 2021). On the other hand, Amer et al. (2018) found no differences in Nile tilapia growth when supplementing its diets with cinnamaldehyde. The differential effect reported in the works of Amer et al. (2018) and Abd El-Hamid et al. (2021) may be due to the distinct feeding period (75 days *vs*. 12 weeks, respectively), additive format (essential oil *vs*. nanoemulsion, respectively), and/or dietary inclusion level (0.105, 0.210% *vs*. 0.01, 0.02, 0.03%, respectively).

Growth promoting effects of turmeric and curcumin (the main active principle of turmeric) have also been observed in different fish species, such as common carp (Abdel-Tawwab and Abbass, 2017), grass carp (Ming et al., 2020), rainbow trout (Yonar et al., 2019), and Nile tilapia (Diab et al., 2014). On the other hand, Wojno et al. (2021) observed no effect on the fish growth when supplementing the diets of common carp with 0.02% turmeric. The different effect with respect to the work of Abdel-Tawwab and Abbass (2017) may be attributed to the different feeding period (10 weeks in Abdel-Tawwab and Abbass, 2017 *vs*. 40 days in Wojno et al., 2021), diet composition (9% crude protein + 4% crude lipids *vs*. 53% crude protein + 7% crude lipids), turmeric origin and format (powder, from a local market in Egypt *vs*. unknow format, from the company Verdure Sciences in Indiana, U.S.A.), and inclusion levels (0.1-0.5% *vs*. 0.02%, respectively). The results of many fish studies suggest that, as with many spices, one of the determinants of the efficiency of turmeric on growth performance is the dosage used. For instance, for gilthead seabream, curcumin was shown to be effective at a dietary inclusion higher than 2% (2-3%; Ashry et al., 2021); for largemouth bass, at 1%, but not at 0.5% (Wang et al., 2023); and for crucian carp, at 0.5%, but not at 0.1% (Jiang et al., 2016). In large yellow croaker, turmeric improved the growth at a dietary inclusion level of 0.04%, but not at lower (0.02%) and higher (0.06%) inclusion levels (Ji et al., 2021).

Overall, many studies have attributed the growth-promoting effects of capsicum, black pepper, ginger, turmeric, and cinnamaldehyde to an enhanced feed utilization, increased nutrient digestion and absorption, and a positive modulation of the gut microbiota that leads to an improved health status (El-Houseiny et al., 2019; Zhou et al., 2020; Ashry et al., 2021; Aqmasjed et al., 2023; Ashry et al., 2023; Ibrahim et al., 2024; Malintha et al., 2023), which is in line with the results observed in the present thesis. In addition, the growth-supporting effect of turmeric may be associated to the enhancement of the immune and antioxidant systems, and anti-stress properties of curcumin (Ming et al., 2020; Aqmasjed et al., 2023). Considering that the SPICY feed additive was able to improve the growth in gilthead seabream (Chapter III; Ruiz et al., 2023c), the reasons why the SO feed additive did not have an effect on growth performance (Chapter IV; Ruiz et al., 2024a) might be associated to the absence of cinnamaldehyde. Furthermore, although turmeric, capsicum, black pepper, and ginger have growth promoting effects when administered individually, their combined impact is unpredictable. Chowdhury et al. (2021) reported that the supplementation of turmeric, ginger, and garlic in rohu worsened the metabolic enzyme activities, feed utilization, and growth performance of rohu; meanwhile, the pairwise combinations of turmeric and ginger, turmeric and garlic, and ginger and garlic had a positive effect in lipase and metabolic activities, and feed and growth performance. In this sense, the combination of certain spices can lead to a reduced growth performance due to the antagonistic effect of their active principles (Parasuraman et al., 2014). In the case of the SO feed additive, it did not produce an adverse effect on the growth of gilthead seabream, but a null effect, which may be associated to the absence of changes in feed utilization, while the SO additive positively affected the fish lipid metabolism, fat deposition, immune status, and composition of the gut microbiota (Chapter IV; Ruiz et al., 2024a). In summary, the bioactive compounds present in phytogenic additives, their proportions, interactions, origin and type of processing influence the effect of the feed additive on fish growth and health (Chowdhury et al., 2021; Firmino et al., 2021a).

Concerning feed performance, none of the tested additives influenced the feed intake of gilthead seabream, even though an increased feed consumption could have been expected based on the attractant properties of the additives used. In this sense, bile salts have a high olfactory sensitivity in fish (Buchinger et al., 2014), and can act as a taste stimulus in some species, such as channel catfish (*Ictalurus punctatus*; Rolen and Caprio, 2008) and Mexican cavefish (*Astyanax fasciatus*; Kasumyan and Vinogradskaya, 2019). However, in other fish species, like Nile tilapia, silver dollar (*Metynnis argenteus*), green swordtail (*Xiphophorus helleri*), and roach (*Rutilus rutilus*), bile salts have neutral or aversive effect on fish taste response (Kasumyan and Vinogradskaya, 2019). Moreover, ginger contains gingerol, which increases the feed palatability for the fish (Ashry et al., 2023). Some studies have also suggested that curcumin and cinnamaldehyde may also be used as feed attractants in fish to increase their feed intake due to their attractive flavours (Alagawany et al., 2021; Gu et al., 2022). Furthermore, it has been demonstrated that capsicum is a very good feed attractant for whiteleg shrimp (*Litopenaeus vannamei*; Kawamura et al., 2019), but this feature has not been reported for fishes so far. Thus, the absence of effect of the tested additives on the feed intake may be due to their low dietary inclusion levels, or the presence of other ingredients in diets (*i.e.*, protein hydrolysates) that had a more prevalent effect on diet palatability (Hattori et al., 2021).

Regarding feed utilization, it has been demonstrated that bile salts, at certain dietary concentrations, are able to reduce feed conversion rate (FCR) values in different fish species like the hybrid grouper (at 0.09%; Xu et al., 2022b), Chinese perch (at 0.09%; Zhang et al., 2022a), striped catfish (0.025-0.150%; Adam et al., 2023), and yellow catfish (at 0.06%; Yao et al., 2022), and/or to increase feed efficiency ratio (FER) values, as in turbot (at 0.5%; Gu et al., 2017), and rainbow trout (1.0-1.5%; Yamamoto et al., 2007; Iwashita et al., 2008) (Appendix 1). However, when the dietary inclusion levels of bile salts exceed or do not reach an optimum range, they may have null or even negative effects on feed utilization parameters, as exemplified by Jiang et al. (2018). The former authors reported that while feed efficiency (FE) values were improved at a 0.015% inclusion of a blend of bile salts in GIFT tilapia diets, FE values did not change at relatively lower (0.005%) or higher levels (0.045%) with respect to the basal feed. Meanwhile, at much higher levels (0.135%), FE was compromised. In addition, the proximate composition of the diet also influences the potential effect of bile salts on feed utilization. This was shown in the study by Bhusare et al. (2023), where it was reported that in GIFT tilapia diets supplemented with 0.05 and 0.1% cholic acid, there was an improved FCR when decreasing the dietary crude protein to 32% and increasing crude lipids to 14%. Furthermore, in some studies with the same species, controversial results have been found. In this sense, while Ding et al. (2020) reported lower FCR values in large yellow croaker when diets were supplemented with bile salts at 0.015, 0.03, and 0.045%, Li et al. (2023) found no changes at 0.03 nor at 0.12% dietary inclusion levels, and improved FCR values were only observed at 0.06% inclusion. This may suggest that the optimum inclusion range of bile salts for a species may vary regarding the differential diet composition (45% crude protein + 18% crude lipids *vs*. 42% crude protein + 12% crude lipids) and/or the different composition and origin of bile salts due to the wide range of bile salts used in different studies (unknow composition, bovine bile salts *vs*. hyodeoxycholic acid, hyocholic acid, chenodeoxycholic acid, unknow origin, respectively).

The above-mentioned factors (diet composition, bile salt composition, origin, and inclusion levels) may be the reasons for the absence of effect of bile salts reported in some works in leopard coral grouper (Gao et al., 2023), black seabream (Jin et al., 2019), largemouth bass (Yu et al., 2019; Yin et al., 2021), and grass carp (Zhou et al., 2018a). In line with these studies, in our case, we also did not observe significant changes in the fish FCR values, even though there was a numerical trend towards a decrease with the increasing inclusion levels of bile salts (0% bile salts: 1.21 ± 0.05, 0.6%: 1.19 ± 0.05; 0.12%: 1.16 ± 0.03; Chapter I; Ruiz et al., 2023a). However, this downward trend in FCR was probably not the cause of the growth-promoting effect of bile salts observed in our study, rather than the effect of bile salts on fish lipid metabolism and health status (Chapter I; Ruiz et al., 2023a; Chapter II; Ruiz et al., 2023b).

From the two tested combinations of spices, only the SPICY feed additive was able to improve FCR in gilthead seabream at an inclusion level of 0.1%, while intermediate FCR values were observed at 0.15% inclusion (Chapter III; Ruiz et al., 2023c; Chapter IV; Ruiz et al., 2024a). The success of the SPICY feed additive on reducing FCR values was probably mainly due to its content of ginger and cinnamaldehyde. In this sense, positive results on FE have been observed in several fish species under dietary supplementation with ginger, including Asian sea bass (Talpur et al., 2013), rohu (Sukumaran et al., 2016), common carp (Fazelan et al., 2020; Mohammadi et al., 2020), striped catfish (Ashry et al., 2023), and rainbow trout (Aqmasjed et al., 2023) (Appendix 2). Similarly, cinnamaldehyde has shown positive effects on FE on most of the fish species in which it has been tested, such as tongue sole (Wang et al., 2021a), fat greenling (Gu et al., 2022), grass carp (Zhou et al., 2020), and Nile tilapia (Abd El-Hamid et al., 2021). On the other hand, the effect of capsicum on most fish species was rather neutral, as was the case in blue streak hap (Yılmaz and Ergün, 2011), jewel cichlid (Yigit et al., 2021), gilthead seabream (Wassef et al., 2010), Mozambique tilapia (Yılmaz et al., 2013a), and rainbow trout (Talebi et al., 2013; Yanar et al., 2016). Positive effects of capsicum on FE have only been reported in a few studies like those of Ibrahim et al. (2024) in Nile tilapia, and Yılmaz et al. (2024) in rainbow trout. Controversial results among studies have been found for black pepper, showing potential to ameliorate FCR values in common carp (Giri et al., 2023), and rainbow trout (Stoev and Zhelyazkov, 2021). However, other studies have shown that dietary supplementation of black pepper or piperine did not always result in FCR changes (Malintha et al., 2023), and could even compromise FE values under certain experimental conditions. That was the case of Ullah et al. (2021), which observed an increase in FCR values when supplementing rohu diets with high concentrations (1, 2, and 3%) of black pepper, and of Wojno et al. (2021), who also reported higher FCR values when supplementing common carp diets with 0.02% piperine compared to the basal diet, while no changes were observed when the diet was supplemented with the same concentration of black pepper.

The absence of effects on feed utilization of the SO feed additive in gilthead seabream (Chapter IV; Ruiz et al., 2024a) was probably a combined consequence of: 1) the presence of capsicum and black pepper as the main components of the SO feed additive, which had little or no effects on fish FE, as discussed above; 2) the possible antagonistic effect of different bioactive compounds from the spices included in the additive (Parasuraman et al., 2014); and 3) the relatively low inclusion levels of turmeric in the feed in comparison to the inclusion levels indicated in available literature. Regarding this last point, Ashry et al. (2023) observed that the optimum inclusion range of curcumin to improve FCR in gilthead seabream was between 2.5

and 3.0%; while at lower inclusion levels, FCR values did not vary (2%) or even increase (1.5%). Although in other fish species lower levels of inclusion of turmeric have been shown to enhance feed utilization, as was the case with crucian carp (0.1-0.5%; Jiang et al., 2016) and grass carp (0.02-0.08%; Ming et al., 2020), positive results have also been reported at relatively high inclusion levels in rainbow trout (1-4%; Yonar et al., 2019), and Nile tilapia (1-2%; Aqmasjed et al., 2023). Otherwise, some works have found no differences in feed utilization regardless the difference in inclusion levels tested of turmeric or curcumin, as reported for largemouth bass (Wang et al., 2023) and common carp (Abdel-Tawwab and Abbass, 2017; Wojno et al., 2021). Overall, this indicates that if higher inclusion levels of turmeric had been tested, we might have observed an improved feed performance in gilthead seabream. However, the composition of the SO feed additive and inclusion level tested within this thesis were selected based on the close composition and inclusion levels used for the SPICY feed additive in our previous trial (Chapter III; Ruiz et al., 2023c), and probably, a higher turmeric inclusion would have influenced the beneficial effects of the SO additive observed for lipid metabolism, immune status, and gut microbial modulation, which will be discussed in the next sections of this thesis.

2. Dietary modulation of the blend of bile salts and combinations of spices on lipid digestion, metabolism, and accumulation

As reviewed in the Introduction section, the replacement of fish oil by alternative ingredients often results in disorders related to lipid metabolism, increasing fat accumulation in digestive organs, including the liver (Fountoulaki et al., 2009; Wassef et al., 2015; Monteiro et al., 2018) and the intestine (Caballero et al., 2002; Torrecillas et al., 2017), which subsequently can result in physiological disorders. For instance, fat accumulation in digestive organs can deregulate nutrient metabolism, digestion, and absorption (Serna-Duque and Esteban, 2020), and compromise the immune status of the fish (Weisman and Miller, 2006). In addition, accumulation of perivisceral fat can negatively affect the flesh quality, by going rancid and producing an unpleasant smell, causing consumer rejection (Hsieh and Kinsella, 1989; Grigorakis, 2007). Thus, many efforts have been made in the aquaculture industry to keep fish lipid levels within an ideal range to fulfill consumer nutritional needs and quality standards (Salmerón, 2018). One of the main objectives of the present thesis was to reduce fat accumulation through the supplementation of the tested additives on gilthead seabream diets.

In view of the histomorphological results from the liver and intestine, the three tested additives were able to reduce the levels of fat deposits in the liver and intestine, as well as the perivisceral fat index (PVFI) values (Chapter I; Ruiz et al., 2023a; Chapter III; Ruiz et al., 2023c; Chapter IV; Ruiz et al., 2024a). One of the most striking observations in the present thesis was the differential dose-response found among distinct tissues regarding fat accumulation. In this sense, in the liver, the effect of the blend of bile salts and of the SPICY feed additive on reducing hepatic fat accumulation was much more marked at the lowest concentration tested (0.06% and 0.1%, respectively); while at higher concentrations (0.12% and 0.15%, respectively), the number of individuals with large hepatic lipid accumulation were only slightly decreased with respect to the control group. Similarly, the values of the PVFI were significantly reduced under supplementation of gilthead seabream diets with the blend of bile salts and the SPICY additive at inclusion levels of 0.06 and 0.1% respectively, while at higher inclusion levels, PVFI values were intermediate. On the other hand, in the intestine, such additives were highly effective in reducing fat deposit accumulation at both inclusion levels tested, but especially at the highest levels (0.12% and 0.15%, respectively). The distinct dose-response observed in the distinct tissues may be attributed to the variations in the enzymatic activities involved in lipid digestion and metabolism among tissues, as well as to the molecular mechanisms underlying lipid metabolism inherent to each tissue. In this sense, the activity of the bile salt-activated lipase was much higher in the anterior intestine than in the pyloric caeca (Chapter I; Ruiz et al., 2023a; Chapter III; Ruiz et al., 2023c; Chapter IV; Ruiz et al., 2024a). In addition, previous studies have reported that the enzyme lipoprotein lipase (LPL) has a high activity in the liver and mesenteric adipose tissue of gilthead seabream (Saera-Vila et al., 2005), while LPL activity is almost non-existent in the fish intestine (Feng et al., 2014). Further, while a higher effect of bile acids could be expected in the enterocytes due to the well-known role of the fish intestine in bile acid absorption (Romano et al., 2020), the gene expression results of fish liver suggested some counter-regulatory mechanism regarding bile salt and lipid metabolism to balance their

levels, as will be further discussed below. The SO feed additive, which was only assessed at an inclusion level of 0.2%, usefully reduced the levels of perivisceral fat and the accumulation of fat deposits in the liver and intestine as well, being especially effective in the intestine. In addition, only the supplementation of gilthead seabream diet with the SO feed additive was able to reduce the levels of lipids in the liver with respect to the fish fed the control diet (Chapter IV; Ruiz et al., 2024a).

In agreement with the results of the present thesis, supplementation of bile salts in aquafeeds usually leads to a decrease in hepatic lipid accumulation, which is evinced by the reduction in the number and size of hepatic vacuoles and lipid droplets in the liver, as demonstrated in black seabream (Jin et al., 2019), largemouth bass (Yin et al., 2021), hybrid grouper (Xu et al., 2022b), yellow catfish (Yao et al., 2022), and Chinese perch (Zhang et al., 2022a). On the other hand, similar to what was observed for key performance indicators associated with growth and feed performance in some fish species, at high inclusion levels bile salts may not be as effective (or even harmful) in reducing hepatic fat accumulation. This was the case of Jiang et al. (2018), which found an increase in hepatic vacuolization and hepatocyte nuclear migration under dietary supplementation of a blend of bile salts at an inclusion level of 0.14% in GIFT tilapia. Regarding the intestine, some fish studies have also shown reduced levels of fat accumulation in the enterocytes under dietary supplementation of bile salts, marked by a reduced vacuolization, central nuclear position, enlargement of the mucosal folds, and decreased leukocyte infiltration. This was the case of rainbow trout (Yamamoto et al., 2007; Iwashita et al., 2008; Iwashita et al., 2009) and largemouth bass (Yin et al., 2021).

Despite the diminishment in fat deposits in the liver and intestine of gilthead seabream when diets were supplemented with bile salts, no significant differences in the proximate composition of the liver and fillet were found in our study (Chapter I; Ruiz et al., 2023a). On the other hand in some fish diets, supplementation with bile salts at different concentrations reduced levels of lipids in the liver of GIFT tilapia (Jiang et al., 2018), grass carp (Zhou et al., 2018a), largemouth bass (Yu et al., 2019), large yellow croaker (Ding et al., 2020), hybrid grouper (Xu et al., 2022b), and Chinese perch (Zhang et al., 2022a), and in the muscle of GIFT tilapia (Jiang et al., 2018), large yellow croaker (Ding et al., 2020), and hybrid grouper (Xu et al., 2022b). Reduced lipid contents in whole-body proximate composition have also been found under bile salt supplementation in GIFT tilapia (Jiang et al., 2018; Bhusare et al., 2023) and leopard coral grouper (Gao et al., 2023), while the whole-body lipids levels have been shown to increase in turbot (Gu et al., 2017) and largemouth bass (Yu et al., 2019). Nonetheless, in the present thesis we did not evaluate the fish whole-body proximate composition.

As explained above, in the fish nutrition literature regarding the inclusion of spices in aquafeeds, the majority of studies have been devoted to evaluating the effect of spices on the fish growth and feed performance. Only a few studies have focused on the modulation of the levels of fat accumulation, though in these controversial results were obtained. For instance, in gilthead seabream, capsicum dietary supplementation did not influence the muscle lipid content (Wassef et al., 2010). Conversely, dietary inclusion of capsicum increased the whole-body content of lipids in Mozambique tilapia (Yılmaz et al., 2013a), and the levels of hepatic fat accumulation in jewel cichlid (Yigit et al., 2021). However, it is important to remember the high dietary inclusion levels of capsicum in these assays (3-15% in Yigit et al., 2021; 0.7-2.8%

in Yılmaz et al., 2013a), which can be detrimental, but at lower concentrations may have health-promoting effects (Guldiken et al., 2018). Contradictory results among species have also been obtained for black pepper supplementation, which has been shown to decrease the whole-body content of lipids in African catfish (El-Houseiny et al., 2019), while no differences were found in common carp (Wojno et al., 2021). On the other hand, the former authors reported that dietary supplementation of piperine rather than black pepper, increased common carp whole-body content of lipids (Wojno et al., 2021). In rohu, an increase in the levels of fat in the fillet was also reported under black pepper dietary supplementation (Ullah et al., 2021). Concerning ginger dietary supplementation, Mohammadi et al. (2020) reported decreased whole-body lipid levels in common carp, while in rainbow trout no differences with respect to fish fed the basal diet were found (Aqmasjed et al., 2023). On the other hand, Ashry et al. (2023) found a higher carcass lipid content in striped catfish when diets were supplemented with 1.5% ginger, but not at 0.5 and 1% (Fazelan et al., 2020).

Regarding turmeric and curcumin, some studies have reported a reduction in the lipid content in the liver, as shown in large yellow croaker (Ji et al., 2021), or in the whole-body in common carp (Wojno et al., 2021). Other studies have reported no effect from turmeric nor curcumin supplementation on the lipid content in carcass in gilthead seabream (Ashry et al., 2023), nor in the whole-body in crucian carp (Jiang et al., 2016), common carp (Abdel-Tawwab and Abbass, 2017), rainbow trout (Aqmasjed et al., 2023), and largemouth bass (Wang et al., 2023). Cinnamaldehyde dietary supplementation usually does not influence the fish lipid content in muscle and whole-body, as demonstrated in fat greenling (Gu et al., 2022), and Nile tilapia (Amer et al., 2018), respectively.

The combination of spices may enhance their potential to reduce lipid accumulation in the fish body, as happened in rohu when diets were supplemented with ginger and turmeric (Chowdhury et al., 2021). Similar findings were reported in African catfish under dietary supplementation of black pepper and turmeric (El-Houseiny et al., 2019). Nonetheless, other studies have shown no changes in lipid levels when combining some of such spices, as it was the case for ginger and curcumin in rainbow trout (Aqmasjed et al., 2023). In line with the results observed in the present thesis, when in our previous assay we supplemented the SPICY feed additive at 0.1 and 0.15% in a diet with partial fish oil replacement by mammalian-rendered fat, we observed a decrease in fat deposit accumulation in the liver, in addition to a reduction in fillet lipid levels (Ruiz et al., 2024c).

None of the additives tested in this thesis altered the proximate macronutrient composition nor the fatty acid profile of the fish fillets. However, there was a significant increase in the docosahexaenoic acid (C22:6 n-3; DHA) / eicosapentaenoic acid (C20:5 n-3; EPA) ratio when the diet was supplemented with the SPICY additive at an inclusion level of 0.1%, which was linked to a numerical non-significant increase in DHA and decrease in EPA levels in gilthead seabream fillet (Table 1). This was in line with the increase in DHA, and consequently n-3 LC-PUFA, contents observed in the liver under the dietary SPICY supplementation, even though such an increase in fatty acids was only significant at an inclusion level of 0.15%. In this sense, animal fats (such as poultry fat in this case) are high in SFAs and MUFAS, which are preferentially oxidized to produce energy in fish, sparing LC-PUFA from catabolism and increasing their availability and tissue deposition (Henderson, 1996; Fonseca-Madrigal et al.,

2005; Trushenski and Lochmann, 2009). Regarding the well-demonstrated stimulatory role of spices on fatty acid oxidation in mammals (Westerterp-Plantenga et al., 2006), which may also be extrapolated to fish as discussed below, the SPICY additive may have potentiated the preferential oxidation of SFAs and MUFAs over LC-PUFAs. Among LC-PUFAs, many studies have pointed to a preferential deposition of DHA over EPA and most n-3 LC-PUFAs, which may be the reason of the increase in DHA levels in the liver, and DHA/EPA ratio in fillets. In addition, part of the content of EPA and other n-3 PUFAs may be metabolized into DHA to help meet the fish requirements (Coccia et al., 2014; Emery et al., 2016; Morais et al., 2020). The DHA/EPA ratio values of the fillets of fish fed the $SPICY_{0.1\%}$ diet (1.37 ± 0.08) were closer to the values of farmed gilthead seabream juveniles fed a fish oil-based diet (1.45-2.04) reported in previous works (Lenas et al., 2011; Ruiz et al., 2024c) than in fish fed the control diet (1.23 ± 0.03) that was rich in SFAs and MUFAs. However, other studies have reported much lower DHA/EPA ratios for the same fish species fed with fish oil-based diets (*i.e.*, 0.56-081; Izquierdo et al., 2005; Fountoulaki et al., 2009; Benedito-Palos et al., 2010).

Considering the wide range of DHA/EPA values reported in different studies, it is difficult to interpret the results of DHA/EPA ratio in terms of improvement in nutritional quality. In any case, apart from the increase in the DHA/EPA values observed in fish fed the $SPICY_{0.1\%}$ diet, no other differences were observed in the fatty acid profile of the fillet, nor in the n-6/n-3 ratio. In addition, there were no differences in the indices of atherogenicity (IA), thrombogenicity (IT), and hypocholesterolemic/ hypercholesterolemic fatty acids' ratio (h/H) in the fillets among different dietary treatments (Table 1). These indices evaluate the nutritional quality of the lipid fraction from the edible flesh for the consumer (Chen and Liu, 2020). The IA indicates the relationship between the sum of the main SFAs, which are considered pro-atherogenic, which favour the adhesion of lipids to cells of the circulatory system, and the sum of unsaturated fatty acids, which are considered anti-atherogenic. The IT is denoted as the relationship between the pro-thrombogenic and anti-thrombogenic fatty acids, and this index indicates the amenability to clot formation in blood vessels. The h/H characterizes the relationship between hypocholesterolemic and hypercholesterolemic fatty acids (Chen and Liu, 2020). However, one critical point of the IA and IT indices is the consideration of n-6 PUFAs as anti-atherogenic and anti-thrombogenic agents, since recent studies have demonstrated that n-6 PUFAs produce eicosanoids with pro-inflammatory, vasoconstrictory, and pro-aggregatory properties (Saini and Keum, 2018). Nonetheless, the values obtained for the IA and IT, which were similar among all dietary treatments, were far below the limits considered harmful for the consumer's health (1.0; Marques et al., 2022), and within the value range considered as beneficial for human health (below 0.5; Bazarsadueva et al., 2021). Regarding the h/H ratio, the values of all gilthead seabream dietary groups were maintained within the ordinary value range of fish, ranging from 1.54 to 4.83 (Chen and Liu, 2020; Bazarsadueva et al., 2021). Overall, these results suggest that the tested additives were able to reduce the fat accumulation in digestive organs (liver and intestine) and in the visceral cavity without compromising the proximate composition nor the nutritional quality indices of the fish fillets, which is an advantage considering that their organoleptic properties, including general taste and flavour, are largely attributed to the content of fat in the edible part (Grigorakis et al., 2003).

Table 1. Nutritional quality markers of the fillet fatty acid profile in gilthead seabream (*Sparus aurata*) fed the control diet and the diets supplemented with bile salts at an inclusion level of 0.06% ($BS_{0.06\%}$) and 0.12% ($BS_{0.12\%}$), a combination of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde at 0.1% ($SPICY_{0.1\%}$) and 0.15% ($SPICY_{0.15\%}$), and a combination of turmeric, capsicum, black pepper, and ginger oleoresins at 0.2% (SO).

	Control	$BS_{0.06\%}$	$BS_{0.12\%}$	$SPICY_{0.1\%}$	$SPICY_{0.15\%}$	SO
DHA / EPA	1.23 ± 0.03^{a}	1.21 ± 0.04^{a}	1.24 ± 0.04^{a}	1.37 ± 0.08^{b}	1.28 ± 0.06^{ab}	1.28 ± 0.05^{ab}
EPA + DHA	53.24 ± 5.08	51.33 ± 2.25	56.14 ± 4.13	53.41 ± 1.62	54.07 ± 8.50	54.10 ± 4.55
n-6 / n-3	1.97 ± 0.18	2.09 ± 0.08	1.94 ± 0.14	1.99 ± 0.06	2.01 ± 0.29	1.97 ± 0.14
IA	0.32 ± 0.02	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.02	0.32 ± 0.01	0.31 ± 0.01
IT	0.38 ± 0.03	0.38 ± 0.01	0.37 ± 0.01	0.38 ± 0.01	0.38 ± 0.03	0.37 ± 0.01
h / H	2.28 ± 0.07	2.31 ± 0.06	2.31 ± 0.06	2.29 ± 0.12	2.31 ± 0.07	2.35 ± 0.03

Values are represented as mean ± SD (n = 4 tanks per dietary group) and differences among groups (one-way ANOVA; $P \leq 0.05$) are indicated by the different superscript letters. Abbreviations: DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; n6/n3: omega 6 to omega 3 polyunsaturated fatty acids' ratio; IA: index of atherogenicity; IT: index of thrombogenicity; h/H: hypocholesterolemic/ hypercholesterolemic fatty acids' ratio.

The mechanisms underlying the reduction of fat accumulation in gilthead seabream observed in this thesis, and in previous studies, may vary depending on the nutritional strategy and type of additive tested. Regarding bile salts, when they are secreted from the gallbladder into the proximal part of the intestine, they can bind to the interface of lipid aggregates through their hydrophobic regions (steroid rings and angular methyl groups), emulsifying them into small lipid droplets (Maldonado-Valderrama et al., 2011). Lipid emulsification also allows for the formation of mixed micelles, which are structures composed of bile salts, cholesterol, phospholipids, and lipid digestion products, such as fatty acids and monoglycerides, facilitating their transport in the aqueous medium of the digestive tract, and the posterior absorption of lipids and cholesterol through the intestinal epithelium (Carey and Small, 1970). Additionally, the binding of bile salts to the interface of lipid aggregates allows a higher activity of lipases produced by the exocrine pancreas, particularly bile salt-activated lipases, since it causes an orogenic displacement of other compounds adhered to the interface (*i.e.*, surfactants, proteins, free fatty acids). Additionally, the emulsification of lipid aggregates into smaller lipid droplets increases the surface area on which lipases can act (Romano et al., 2020). Bile salts also contribute to the activation of the bile salt-activated lipase, which is the most dominant lipase of marine fish and can hydrolyze a wide range of substrates (wax esters, mono-, di- and triacylglycerides, phospholipids, ceramides, fat-soluble vitamin esters and cholesteryl esters) (Tocher, 2003; Romano et al., 2020).

Taking into account the above-mentioned ideas, the inclusion of bile salts in aquafeeds may have hypolipidemic effects through an increased concentration of bile salts in the intestine, which promote higher lipid digestion and absorption rates, and through an increased activity of the bile salt-activated lipase. In this sense, many studies in fish have shown that dietary bile salt supplementation leads to an increase bile acid content in the fish intestine and/or

gallbladder, suggesting an efficient absorption of supplemented bile salts into the enterohepatic circulation, as demonstrated in rainbow trout (Yamamoto et al., 2007; Iwashita et al., 2008; Iwashita et al., 2009), Atlantic salmon (Kortner et al., 2016), and turbot (Gu et al., 2017). On the other hand, Yao et al. (2022) found a decreased content of whole-body bile acids in yellow catfish. However, in the former assay, the diet was supplemented with glycocholic acid (G-CA), which is not a typical endogenous bile salt in fish, since bile salts are normally conjugated with taurine in fish (Kim et al., 2015; Kortner et al., 2016). Thus, Yao et al. (2022) suggested that, even though G-CA was able to enter the enterohepatic circulation as indicated by the bile salt profile changes, such bile salt promoted a higher excretion of bile acids.

In our results, we did not observe significant alterations in the concentration of total bile salts following their supplementation in gilthead seabream diet due to the high dispersion among samples, but the numerical mean value of total bile salts in the anterior intestine showed a tendency to increase with increasing doses of supplemented bile salts in the diet (Chapter I; Ruiz et al., 2023a). Nonetheless, we reported a dose-dependent modulation of the bile salt profile, with a significant increase in the levels of taurodeoxycholic acid (T-DCA) in the anterior intestine and gallbladder in fish fed the $BS_{0.12\%}$ diet, with respect to the control group. The tested blend of bile salts was composed of a mixture of equal parts of sodium cholate (CA) and sodium deoxycholate (DCA), and sodium taurocholate (T-CA) hydrate in a proportion of 30/70. Thus, the increase in T-DCA may be attributed to the metabolization of CA (dehydroxylation) and T-CA (deconjugation and dehydroxylation) into DCA by the intestinal microbiota, and to the conjugation of reabsorbed and supplemented CA with taurine in the liver (Schubert et al., 2017; Romano et al., 2020). On the other hand, while the two primary bile salts T-CA and taurochenodeoxycholic acid (T-CDCA) were maintained at basal levels in the anterior intestine, we observed a linear decrease of T-CDCA levels in the gallbladder. Such decrease may be a response to a differential absorption rate of bile acids caused by the higher content of T-DCA in the intestine, and/or to a lower synthesis of T-CDCA in the liver. In this sense, Pandak et al. (2002) showed in an *in vitro* study that addition of T-DCA and T-CA into rat hepatocytes inhibited the activity of oxysterol 7α-hydroxylase (CYP7B1), which is the rate-limiting enzyme of the alternative pathway of bile acid synthesis. In addition, taurine is the amino acid with which bile acids are typically conjugated in fish, so its reduced availability can lead to a limited synthesis of conjugated bile salts (Kim et al., 2015). Supporting these hypotheses, and in line with the results of the present thesis, many works have shown a decrease in the content of T-CDCA in the gallbladder when supplementing T-CA in fish diets, as demonstrated in rainbow trout (Iwashita et al., 2008; Iwashita et al., 2009) and Atlantic salmon (Kortner et al., 2016). Similar results have also been found with a mixture of bile salts of bovine origin, as shown in rainbow trout (Yamamoto et al., 2007; Iwashita et al., 2008) and Atlantic salmon (Kortner et al., 2016).

In terms of digestibility, supplementation of bile salts in fish diets usually improves the activity of lipolytic digestive enzymes. In particular, improved lipoprotein lipase, hepatic lipase, and total lipase activities were observed in the livers of GIFT tilapia (Jiang et al., 2018) and large yellow croaker (Ding et al., 2020) fed diets supplemented with bile salts. Similarly, a higher lipase activity has been observed in the livers of tongue sole (Wang et al., 2022) and hybrid grouper (Xu et al., 2022b) under dietary bile salt supplementation. The induction of a higher lipase activity by dietary bile salt supplementation is also evident in the fish intestine, as was

the case in turbot (Gu et al., 2017), GIFT tilapia (Jiang et al., 2018; Bhusare et al., 2023), tongue sole (Li et al., 2021b), and leopard coral grouper (Gao et al., 2023). A higher lipoprotein lipase activity was also measured in the intestine of GIFT tilapia fed with a diet supplemented with bile salts (Jiang et al., 2018). In addition, dietary bile salt supplementation has also been reported to stimulate the activity of other digestive enzymes, such as amylase and protease in tongue sole (Li et al., 2021b; Wang et al., 2022), and trypsin in Atlantic salmon (Kortner et al., 2016) and leopard coral grouper (Gao et al., 2023).

In our study, we did not observe a significant improvement of total alkaline protease and α-amylase activities by the addition of bile salts, at least in the pyloric caeca, nor in the anterior intestine of gilthead seabream (Chapter I; Ruiz et al., 2023a). However, the activity of the bile salt-activated lipase in the anterior intestine increased with escalating doses of supplemented bile salts, being significantly higher at 0.12% inclusion. In this sense, since the anterior intestine is the main site of lipid digestion and absorption, as in higher vertebrates, in fish the highest lipase activity along the gastrointestinal tract is found in the proximal part (González-Félix et al., 2018; Chapter I; Ruiz et al., 2023a), so the enzymatic activity might be more susceptible to be stimulated in this region. Kurtovic et al. (2010) observed that when testing the effect of different types of bile acids (DCA, CA, T-CA) on the lipase activity in Chinook salmon (*Macruronus novaezelandiae*), the highest activity was induced by T-CA, which is the main component (70%) of the blend of bile salts that we tested in gilthead seabream. These results were partly in line with those of Iijima et al. (1998), which evaluated the effect of the same types of bile acids on the activity of purified bile salt-activated lipase from red seabream (*Pagrus major*) using different substrates (p-nitrophenyl myristate and triolein). Iijima et al. (1998) reported the highest lipase activity in response to T-CA when the substrate was p-nitrophenyl myristate, which is the most commonly used substrate to measure lipase activity (Nolasco-Soria et a., 2023) and the one used in our assay. On the other hand, these authors observed a higher lipase activity for CA with triolein as a substrate, which was also present in our blend of bile salts, but it is less commonly used as a substrate (Nolasco-Soria et al., 2023).

Considering the above-mentioned results, the 50% numerical increase in T-CA content (although not statistically significant) in the anterior intestine of gilthead seabream fed the $BS_{0.12\%}$ diet, with respect to the control group, may be the cause of the higher activity reported for bile salt-activated lipase. The extent to which the increase in T-DCA levels also contributed to the increased activity of bile salt-activated lipase cannot be determined, since the presence of this type of bile salt was not tested in the mentioned studies. The increased lipase activity was in line with the improved values of apparent digestibility coefficient (ADC) of lipids observed in gilthead seabream fed the $BS_{0.12\%}$ diet, in concordance with the enhancement in lipid ADC values also observed in previous studies with rainbow trout (Yamamoto et al., 2007; Iwashita et al., 2008) and turbot (Gu et al., 2017) when supplementing their diets with T-CA or bovine bile salts.

Concerning the use of spices, their effect on modulation of body adiposity in mammals has been mainly attributed to an improved lipid digestion, induced by an increased bile acid synthesis and stimulation of digestive enzymatic activity (Platel and Srinivasan, 2004; Srinivasan, 2005). Spices not only increase bile acid synthesis, but they are also able to increase the rate of their secretion, gastrointestinal flow, and intestinal reabsorption (Platel and

Srinivasan, 2004). To our knowledge, apart from the studies included in this thesis (Chapter III; Ruiz et al., 2023c; Chapter IV; Ruiz et al., 2024a), the fish bile salt profile has not been previously evaluated in extant studies of fish diets supplemented with spices. Nonetheless, Yılmaz et al., (2013b) reported an increase in bile somatic index values when European sea bass (*Dicentrarchus labrax*) diets were supplemented with the spice thyme (*Thymus vulgaris*) or the herbs rosemary (*Rosmarinus officinalis*) and fenugreek (*Trigonella foenum graecum*), indicating an increased bile flow rate and bile acid quantity. On the other hand, the combinations of spices tested in this thesis did not produce any significant change in the bile salt profile of the gallbladder and the anterior intestine in gilthead seabream (Chapter III; Ruiz et al., 2023c; Chapter IV; Ruiz et al., 2024a). However, this was partly due to the high deviation found among samples and to the low number of samples (n = 4), since sample pools from each fish tank were made to provide assurance that enough content was obtained to perform the bile acid profile analyses. In any case, a non-significant numerical increase in the mean values of total bile acids of 37% and 54% was observed in the anterior intestine of gilthead seabream fed the $SPICY_{0.1\%}$ and SO diets, respectively, with respect to their congeners fed the control diet. A noteworthy finding was that in fish fed the diets supplemented with the two combinations of spices, as well as in the control group, the only bile salts detected were T-CA and T-CDCA. Therefore, considering that CA and CDCA are the predominant and often the only bile acids found in the bile salt profile of Perciform fish (Hagey et al., 2010), the observed numerical increase in the gallbladder and anterior intestine suggests that the synthesis and conjugation of these two primary bile acids were increased under the SPICY and SO dietary supplementation.

Supporting the hypothesis that in fish, as in mammals, fat accumulation can be reduced through a higher digestive ability, some studies have demonstrated an improved enzymatic activity in fish fed with diets supplemented with the spices tested in this thesis. Indeed, higher activities of protease and amylase have been reported in the anterior intestine of Nile tilapia under dietary capsicum supplementation (Ibrahim et al., 2024). Regarding black pepper, higher activities of intestinal lipase, protease, and amylase have been found when supplementing piperine in the diet of common carp (Giri et al., 2023). In relation to this, even though Malintha et al. (2023) did not measure digestive enzyme activities, they reported higher dry matter and protein ADC values when including piperine at low inclusion levels (up to 0.075% and up to 0.05%, respectively) in olive flounder. Ginger dietary supplementation has also been proven to increase lipase, protease, and amylase activities in the intestine of striped catfish (Ashry et al., 2023). Additionally, curcumin has been shown to be capable of modulating the fish digestive enzyme activities. Jiang et al. (2016) reported an increase in the activities of lipase and trypsin in the hepatopancreas and intestine, as well as in amylase activity in the hepatopancreas of crucian carp. Undoubtedly, among the spices and active principles tested, cinnamaldehyde is the one that has most often been shown to individually be able to improve the digestive capacity of fish. In this sense, increased lipase and pepsin activities were reported in the digestive tract of fat greenling when diets were supplemented with cinnamaldehyde, even though no differences in amylase activity were found (Gu et al., 2022). The former authors also found higher values of lipid, protein, and dry matter ADCs. In grass carp under dietary supplementation of cinnamaldehyde, an enhancement of the activities of lipase, amylase, chymotrypsin, and trypsin was observed in the hepatopancreas

and intestine (Zhou et al., 2020). Similarly, Abd El-Hamid et al. (2021) showed that supplementing Nile tilapia diets with a cinnamaldehyde nanoemulsion increased the activities of lipase, protease, and amylase in the intestine. On the other hand, Wang et al. (2021a) studied the effect of cinnamaldehyde on the activities of digestive enzymes along different intestinal regions of tongue sole. The latter authors observed a higher protease activity only in the anterior intestine, while a higher amylase activity was found in both anterior and mid intestine. On the other hand, a higher lipase activity was observed along the entire intestine of tongue sole when the diet was supplemented with cinnamaldehyde. Some of such spices or their active principles may have synergistic and complementary effects, which may enhance the overall effectiveness of spices in combination (Parasuraman et al., 2014). For instance, higher activities of lipase, protease, and amylase have been observed in the intestine of rohu under dietary supplementation with ginger and turmeric (Chowdhury et al., 2021).

Similarly, despite not registering changes in lipid ADC values, we observed a significantly higher activity of bile salt-activated lipase in the anterior intestine when supplementing gilthead seabream diets with the SPICY and SO feed additives (Chapter III; Ruiz et al., 2023c; Chapter IV; Ruiz et al., 2024a). These results suggest that the hypolipidemic effect of spices in gilthead seabream may be largely caused by the stimulation of bile salt-activated lipase by the active principles of the spices and/or by potentially increased secretion of bile salts. However, modulation of fat levels in fish tissues is a complex mechanism (Salmerón, 2018), and thus, the reduction in fat accumulation cannot solely be attributed to an enhanced intestinal digestion, but also to the mechanisms underlying the regulation of lipid metabolism. Therefore, this thesis also aimed to investigate gene expression in the liver, which is the primary organ involved in lipid metabolism (Bruslé and Gonzàlez i Anadon, 1996), focusing on genes associated with lipid metabolism and oxidative status.

As explained in the Introduction section, there are three main mechanisms underlying the modulation of fat accumulation in fish tissues, which are the conversion of fatty acids into triacylglycerides (lipogenesis) and cell storage; the metabolization of non-lipid substrates into fatty acids and then triacylglycerides for storage (known as *de novo* lipogenesis); and the catabolism of triacylglycerides into fatty acids and glycerol (lipolysis), which are then oxidized to generate energy or released into the blood stream (Salmerón, 2018). Attending to our results of hepatic gene expression in gilthead seabream (Chapter I; Ruiz et al., 2023a; Chapter III; Ruiz et al., 2023c; Chapter IV; Ruiz et al., 2024a), only two out of the 44 genes included in the PCR-array were similarly regulated by the three tested additives. Such genes were *lpl*, which was down-regulated, and fatty acid synthase (*fasn*), which was up-regulated under supplementation of the three additives with respect to the control group.

In context, fatty acids can be incorporated into the cells as free fatty acids, which are transported by albumin, or can be obtained by the enzymatic hydrolysis of triacylglycerides from chylomicrons, very low-density lipoproteins, and to a lesser extent, low-density lipoproteins circulating in the plasma (Proença et al., 2014). The hydrolysis of triacylglycerides from such lipoproteins is carried out by LPL, releasing glycerol and free fatty acids, which are incorporated into the cells, where they are reconverted to triacylglycerides and stored as an energy reservoir (Salmerón, 2018). Thus, the down-regulation of *lpl* may favour a reduced fat deposition in digestive organs, and may be the cause of the reduced PVFI values in gilthead

seabream when the diet was supplemented with the three tested additives. On the other hand, FASN metabolizes acetyl-CoA and malonyl-CoA into palmitic acid (C16:0). The up-regulation of *fasn* may be a counter-regulatory mechanism to maintain stable levels of fatty acids in the liver through *de novo* fatty acid synthesis, in the context of the presumably lower incorporation of fatty acid synthesis associated to the *lpl* down-regulation. In this sense, inverse dynamics in the expressions of *lpl* and *fasn* have already been observed in previous studies like those conducted in Nile tilapia (Tian et al., 2013), silver pomfret (*Pampus argenteus*; Peng et al., 2017), and spotted seabass (*Lateolabrax maculatus*; Huang et al., 2018).

No other differentially regulated genes were shared among fish fed the blend of bile salts and any of the tested combination of spices. Indeed, aside from these two genes, hydroxyacyl-CoA dehydrogenase (*hadh*) and farnesoid X receptor (*fxr*) were the only differentially regulated genes in the liver of gilthead seabream under dietary supplementation of bile salts (Chapter I; Ruiz et al., 2023a). The down-regulation of *hadh*, which encodes for an enzyme involved in β-oxidation, may be in line with the presumably lower entrance of fatty acids into cells associated to the lower *lpl* expression. Regarding FXR, this is a nuclear receptor belonging to the superfamily of ligand-activated transcription factors predominantly expressed in tissues involved in bile acid homeostasis, such as the liver, and intestine (Kuipers et al., 2004). This type of nuclear pleiotropic receptor is involved in several biological processes, such as the metabolism of bile acids, lipids, proteins, and carbohydrates, energy homeostasis, nutrient uptake, immunity, bone formation and remodeling, and, indirectly, in shaping the gut microbial communities (Schubert et al., 2017; Zheng et al., 2017; Chiang and Ferrell, 2022). The main agonist of FXR is the primary bile acid CDCA, so the decreased levels of T-CDCA that we observed in the gallbladder of gilthead seabream when supplementing the diet with the blend of bile salts may be the reason for such reduced *fxr* expression. However, the expression of cholesterol 7α-hydroxylase (*cyp7a1*), which is negatively regulated by activated FXR (Romano et al., 2020), did not exhibit significant differences, indicating that the differential expression of *fxr* was not correlated with an alteration in the synthesis rate of bile acids via the classic pathway (Introduction, Figure 3).

Regarding the two combinations of spices tested, fish fed the diets supplemented with the SPICY and SO additives also shared two other up-regulated genes, the elongation of very long chain fatty acids 6 (*elovl6*) and the stearoyl-CoA desaturase 1b (*scd1b*). The enzymes encoded by these genes are involved in *de novo* fatty acid synthesis, and can use as a substrate palmitic acid, the end product of FASN, so the up-regulation of *elovl6* and *scd1b*, may be a response to a presumably higher FASN activity, accelerating the transformation of palmitic acid into other fatty acids and avoiding their accumulation. In this sense, ELOVL6 elongates saturated (SFAs) and monounsaturated (MUFAs) fatty acids. Among others, this enzyme catalyzes the conversion of palmitic acid into stearic acid (C18:0) (Sampath and Ntambi, 2005; Matsuzaka and Shimano, 2009). Therefore, the increased levels of stearic acid found in the liver of gilthead seabream fed the SO diet with respect to fish fed the control diet may be attributed to the up-regulation of *elovl6* (Chapter IV; Ruiz et al., 2024a). On the other hand, SCD1B transforms palmitic and stearic acids into palmitoleic (C16:1 n-7) and oleic (C18:1 n-9) acids, respectively (Ntambi and Miyazaki, 2004). Although palmitoleic acid can act as a lipokine reducing lipogenic activity and fat accumulation (Bermúdez et al., 2022), and oleic acid has also been shown to regulate lipid metabolism in mammals (García-Escobar et al., 2008), there were no

significant differences in the levels of such fatty acids in the liver of gilthead seabream when supplementing the diet with the SPICY and SO additives (Chapter III; Ruiz et al., 2023c; Chapter IV; Ruiz et al., 2024a).

The SPICY feed additive also led to a change in the expression of peroxisome proliferator-activated receptor β (*pparβ*) and cholesterol 7α-hydroxylase (*cyp7a1*). Although the role of PPARβ is not completely unraveled in fish, nor in mammals, it has been suggested that it may induce the transactivation of genes involved in fatty acid oxidation (Wang et al., 2003). In addition, their ligands are likely fatty acids (Wang et al., 2003; Kidani and Bensinger, 2012), so the down-regulation of *pparβ* may indicate a reduced fatty acid oxidation in relation to the putative lower entrance of fatty acids in cells as indicated by *lpl* down-regulation. In relation to CYP7A1, it is the first and rate-limiting enzyme of the classic bile acid synthesis pathway (Introduction, Figure 3), which is the pathway that generates the major content of primary bile acids (Zhou and Hylemon, 2014; Chiang and Ferrel, 2020). Thus, the up-regulation of *cyp7a1* may indicate a higher bile acid synthesis and subsequent secretion into the intestine, in line with the numerical increase reported in the anterior intestine in fish fed the $SPICY_{0.1\%}$ diet. Considering the above-mentioned important role of bile salts in lipid emulsification and stimulation of digestive enzyme activities (Maldonado-Valderrama et al., 2011; Romano et al., 2020), the expected higher bile acid synthesis and secretion may be the reasons for the reduced fat accumulation found in the liver, intestine, and visceral cavity in gilthead seabream when the diet was supplemented with the SPICY additive (Chapter III; Ruiz et al., 2023c).

Regarding the SO additive, apart from the shared regulation of *lpl*, *fasn*, *elovl6*, and *scd1b* with the SPICY additive, the supplementation of gilthead seabream diet with turmeric, capsicum, black pepper, and ginger oleoresins, also modulated the expression of the hepatocyte nuclear factor 4 alpha (*hnf4a*), sterol regulatory element-binding protein 2 (*srebp2*), citrate synthase (*cs*), and peroxiredoxin 5 (*prdx5*). To give context, the enzyme HNF4α is involved in the transcriptional regulation of multiple biological pathways, including bile acid synthesis and conjugation, lipid homeostasis, gluconeogenesis, ureagenesis, cell adhesion, proliferation, and apoptosis, among other functions (Yeh et al., 2019). In mammals, HNF4α is involved in the transactivation of CYP7A1 (Romano et al., 2020). However, regardless of the up-regulation of *hnf4a*, the expression of *cyp7a1* remained stable in fish fed the SO diet with respect to those fed the control diet (Chapter IV; Ruiz et al., 2024a). This may indicate that in this case, this enzyme would be involved in the regulation of other metabolic processes different to bile acid metabolism due to its pleiotropic profile or, otherwise, it may reflect that gene expression and enzyme activity are not always correlated, such as in cases where activities are controlled post-transcriptionally or post-translationally. On the other hand, *srebp2* up-regulation may be in line with a higher bile acid synthesis. Many studies in mammals have pointed out that hepatic SREBP enzymes, especially SREBP2, are activated in response to a reduction in cholesterol content (Sato, 2010). Regarding fish, previous studies in rainbow trout (Zhu et al., 2018) and Atlantic salmon (Leaver et al., 2008; Kortner et al., 2013; Gu et al., 2014) have also shown an up-regulation of *srebp2* when feeding with a plant-based diet, which has been attributed to the lower dietary cholesterol levels. In addition, Zhu et al. (2020) suggested that SREBP2 is involved in the homeostasis of cholesterol in rainbow trout, by promoting its hepatic synthesis. Thus, the up-regulation of *srebp2* that we observed in gilthead seabream liver when the diet was supplemented with the SO feed additive may be a mechanism to maintain stable levels of

hepatic cholesterol in response to a higher metabolization rate of cholesterol into primary bile acids.

Concerning the regulation of lipid metabolism, CS is a key mitochondrial enzyme involved in the Krebs cycle which converts the products of fatty acid oxidation, oxalacetate and acetyl-CoA, into citrate (Akram, 2014). Consequently, *cs* up-regulation may be attributed to a higher β-oxidation of fatty acids to produce energy, since many spices, such as capsicum (used in the present thesis), have been shown to promote lipid oxidation in mammals (Westerterp-Plantenga et al., 2006). In spite of *cs* up-regulation, none of the genes which encode enzymes from the electron transport chain [NADH-ubiquinone oxidoreductase chain 2 (*nd2*), NADH-ubiquinone oxidoreductase chain 5 (*nd5*) nor cytochrome c oxidase subunit I (*coxi*)] showed a differential regulation among fish fed these diets. On the other hand, some of the active principles present in the SO additive, such as curcumin, as well as capsaicin, have antioxidant properties in mammals (Srinivasan, 2005). Since PRDX5 is an antioxidant enzyme which scavenges reactive oxygen species (ROS) (Kim et al., 2018), it may be speculated that the down-regulation of *prdx5* that we found in gilthead seabream fed the SO diet was a response to a low generation of ROS in fish hepatocytes, but further research would be needed to confirm this hypothesis. Overall, these findings illustrate the complexity of the molecular mechanisms orchestrating the regulation of lipid metabolism under spice supplementation, indicating that deciphering the molecular and physiological mechanisms underlying lower fat accumulation found under current experimental conditions deserves further attention by means of *in vitro* studies, and holistic transcriptomic and proteomic approaches.

Additionally, to determine whether the regulation of lipid and bile salt metabolisms induced by the tested additives persisted over time, the expression of the same array of genes was evaluated after a 48 hour fasting period. At this time, the regulation induced by the $BS_{0.06\%}$ and $SPICY_{0.1\%}$ diets were attenuated, and a transient effect associated to their dietary administration was observed. In particular, only *prdx5* was differentially expressed when supplementing the diet with the blend of bile salts at 0.06% (Chapter I; Ruiz et al., 2023a), which also showed a similar up-regulation in fish fed the $SPICY_{0.1\%}$ (Chapter III; Ruiz et al., 2023c) and SO diets (Chapter IV; Ruiz et al., 2024a). Among the many roles of PRDX5 defined in mammals, it is remarkable that it can also prevent and alleviate adipogenesis and hepatic fat accumulation by promoting fatty acid oxidation and reducing lipogenesis through the regulation of ROS levels (Kim et al., 2018; Kim et al., 2020). In gilthead seabream the supplementation of the diet with the SPICY feed additive at 0.1% also induced an up-regulation of sterol regulatory element-binding protein 1 (*srebp1*) (Chapter III; Ruiz et al., 2023c), which has been shown to promote *de novo* fatty acid synthesis in many fish species, including zebrafish (*Danio rerio*), large yellow croaker, Japanese seabass (*Lateolabrax japonicus*), and gilthead seabream (Xie et al., 2021). Thus, in Ruiz et al. (2023c; Chapter III) the up-regulation of *srebp1* in 48 h fasted-gilthead seabreams fed the SO diet with respect to their control congeners, may be attributed to an attempt to restore hepatic lipid levels after food deprivation.

On the other hand, the 48-h fasting period enhanced the modulation of genes involved in bile salt and lipid metabolism induced by the SO feed additive (Chapter IV; Ruiz et al., 2024a). In this sense, apart from *prdx5*, there was a differential expression of fifteen genes in fish fed the

SO diet with respect to those fed the control diet. Among these genes, it was interesting to observe an up-regulation of *fxr* and liver X receptor α (*lxra*), which regulate bile acid synthesis through the expression of *cyp7a1*. As mentioned in the Introduction section of this thesis, FXR is activated by high levels of bile acids and triggers the transcription of a small heterodimer partner (*shp*), which interacts with the transcription factors α-fetoprotein transcription factor (FTF) and HNF4α, avoiding *cyp7a1* expression. On the other hand, *cyp7a1* expression can be suppressed through the heterodimer complex formed by retinoid X receptor (RXR) and LXR. The activity of LXR is promoted by high levels of oxysterols, which are products of cholesterol metabolism (Frisch and Alstrup, 2018; Romano et al., 2020). In line with the expression results, considering the antagonistic role of FXR and LXR, the up-regulation of both genes may result in an equilibrium that did not modify *cyp7a1* expression with respect to the control diet. Controversial results were also observed for two genes involved in *de novo* fatty acid synthesis: elongation of very long chain fatty acids 1 (*elovl1*), which was down-regulated, and elongation of very long chain fatty acids 4 (*elovl4*), which was up-regulated. In teleost fish, ELOVL4 is involved in the elongation of C18 to C20 PUFAs, resulting in PUFAs with a main chain of up to 36 carbons. Among other functions, the role of ELOVL4 in the biosynthesis of n-3 LC-PUFAs, such as DHA from EPA and docosapentaenoic acid (C22:5 n-3) (Xie et al., 2021), is of utmost importance considering the growth- and health-promoting effects of n-3 LC-PUFAs in fish (Ibeas et al., 1994; Peng et al., 2014). On the other hand, ELOVL1 is believed to be involved in the elongation of C14 to C20 SFAs, MUFAs, and PUFAs in fish (Xie et al., 2021). The inverse regulation of *elovl1* and *elovl4* may be a long-term mechanism of the spices to maintain or increase the levels of n-3 PUFAs in the liver of fasted animals, but further studies are needed to confirm this hypothesis.

The effect of the SO additive on the regulation of fatty acid oxidation was very obvious. In this sense, in 48 h fasted-fish which were fed the SO diet, there was an up-regulation of *pparβ* in comparison to those fed with the control diet. As already explained in mammals, PPARβ promotes the expression of genes involved in fatty acid oxidation (Wang et al., 2003). In the study of Wang et al. (2003), one of the genes up-regulated in the brown adipose tissue of mouse under activation of PPARβ was carnitine palmitoyltransferase 1B (*cpt1β*). This is in line with the up-regulation of carnitine palmitoyltransferase 1A (*cpt1a*) that we found in 48 h fasted-gilthead seabream fed with the SO diet. In this sense, CPT1α and CPT1β are localized in the outer membrane of the mitochondria and catalyze the transport of acyl-CoA from the cytosol into the intermembrane space, transforming acyl-CoA into acyl-carnitine. Then, acyl-carnitine is transported to the mitochondrial matrix and converted back to acyl-CoA, which undergoes β-oxidation (Weil et al., 2013; Wang et al., 2021b). Despite the absence of differences in hydroxyacyl-CoA dehydrogenase (*hadh*), which is involved in the oxidation of fatty acids, the up-regulation of *cs* may indicate an increased flux of acetyl-CoA to the Krebs cycle, leading to a higher generation of $FADH_2$ and NADH + H^+ (Akram, 2014). In line with that, there was also an up-regulation of NADH-ubiquinone oxidoreductase chain 2 (*nd2*) and NADH-ubiquinone oxidoreductase chain 5 (*nd5*), which are part of the mitochondrial electron transport chain, where $FADH_2$ and NADH + H^+ are used to generate energy in the form of ATP (Nolfi-Donegan et al., 2020). Overall, these results indicated that the SO feed additive may promote fatty acid oxidation as an energy source in 48 h fasted-fish to maintain the proper functioning of physiological processes despite the absence of feed.

In summary, these results evinced that the tested SO feed additive, which contains turmeric, capsicum, black pepper, and ginger, reduces fat accumulation in 48 h fasted-gilthead seabream through similar mechanisms to those observed in higher mammals, by stimulating bile acid secretion, bile salt-activated lipase activity, and through promotion of fatty acid oxidation (Platel and Srinivasan, 2004; Srinivasan, 2005; Westerterp-Plantenga et al., 2006). Regarding the blend of bile salts and the SPICY feed additive composed of capsicum, black pepper, ginger, and cinnamaldehyde, it is difficult to establish the molecular mechanisms of action that regulate lipid metabolism due to the limited number of genes from the PCR-array that exhibited differential expression compared to the control group. However, what is undeniable is that both bile salts and pungent spices have hypolipidemic effects and, similar to the SO additive, they increase bile acid secretion and bile salt-activated lipase activity, which ultimately reduces the levels of hepatic, intestinal, and perivisceral fat (Chapter I; Ruiz et al., 2023a; Chapter III; Ruiz et al., 2023c). In conclusion, the three additives tested in the present thesis can be used as a strategy to reduce fat accumulation and lipid digestion problems without compromising the nutritional quality of fish, and when tied to the use of fish oil alternatives could be used in the aquaculture industry to implement more cost-effective, available, and sustainable alternatives to fish oil, as well as simply to alleviate lipid metabolism disorders through the above-explained mechanisms.

3. Dietary modulation of the blend of bile salts and combinations of spices on the immune condition in fish

The substitution of fish oil with alternative ingredients in aquafeeds not only poses a potential risk to the fish growth and lipid metabolism, but it may also compromise the immune status of fish (Montero et al., 2010; Tan et al., 2016; Tan et al., 2017). Therefore, we also assessed the effect of the tested feed additives on the regulation of the intestinal immune condition in gilthead seabream through the study of the expression of a panel of selected biomarkers related to epithelial integrity, barrier function, and immune response (Chapter II; Ruiz et al., 2023b; Chapter III; Ruiz et al., 2023c; Chapter IV; Ruiz et al., 2024a).

Regarding the effect of bile salts, it is important to consider that in mammals it is well-known that bile acids have the ability to modulate innate immunity through the activation of FXR and G protein-coupled bile acid receptor 1 (GPBAR1/TGR5) (Schubert et al., 2017). For instance, many studies in mammals have shown that activation of FXR reduces intestinal epithelial permeability (Gadaleta et al., 2011; Stojancevic et al., 2012). Thus, under current experimental conditions, the down-regulation of *fxr* in the liver and the reduced content of CDCA, the main agonist of FXR, in the gallbladder of gilthead seabream fed the diet supplemented with the blend of bile salts (Chapter I; Ruiz et al., 2023a), may be related to the down-regulation of cadherin-17 (*cdh17*), and gap junction Cx32.2 protein (*cx32.2*) in the intestine (Chapter II; Ruiz et al., 2023b). Similarly, there was a down-regulation of mucin 13 (*muc13*). These results *a priori* might suggest a lower turnover rate of the epithelial tissue and mucus, and consequently a higher intestinal permeability. In this sense, in mammals, an excessive content of bile salts can lead to a loss of tight junctions between epithelial cells, resulting in a higher epithelial permeability and cell death (Keating and Keely, 2009). Similarly, Fuentes et al. (2024) reported that the addition of a mixture containing 3% CA and 97% DCA to the intestine of gilthead seabream *in vitro* at high concentrations (500 µg/ml in the intestinal fluid) increased the epithelial permeability of the intestine. However, when supplementing gilthead seabream diet with the tested blend of bile salts we observed no changes in the expression of the remaining tight junction proteins that were assayed (Chapter II; Ruiz et al., 2023b), nor on the histological values of villus height, enterocyte height, and number of goblet cells (Chapter I; Ruiz et al., 2023a). Thus, our results indicated that despite the down-regulation of *cdh17*, *cx32.2*, and *muc13*, the inclusion of the blend of bile salts at a dietary level of 0.06% did not compromise the epithelial integrity in gilthead seabream intestine. In addition, we observed a down-regulation of proliferating cell nuclear antigen (*pcna*). Previous studies have correlated a higher proliferative activity associated to *pcna* up-regulation with a decreased cell life (Van Der Hulst, 1998). Thus, the down-regulation of *pcna* in fish fed the $BS_{0.06\%}$ diet may be attributed to an extended cell life, a lower epithelial turnover rate, and an improved health condition of the enterocytes (Gisbert et al., 2017).

Many studies in fish have also demonstrated that dietary supplementation of bile salts can induce an innate immune response. This was the case of striped catfish, which showed an improvement in the rates of lysozyme activity, phagocytic activity, and phagocytic index in

serum when supplementing the diet with a blend of bile salts at a dietary inclusion level of 0.025-0.15% (Adam et al., 2023). Analogously, Jin et al. (2019) described an enhanced lysozyme activity in the liver, but not in serum, of black seabream fed a high-fat diet under supplementation with bile salts at a dietary inclusion levels of 0.02%. In tongue sole fed a diet supplemented with a blend of bile acids at inclusion levels of 0.03 and 0.09%, an improved intestinal lysozyme activity was also reported, as well as a higher content of complement 3, suggesting an improved non-specific immune response (Li et al., 2021b). Zhang et al. (2022a) also suggested an enhanced immune response in Chinese perch when its diet was supplemented with bile acids at an inclusion level of 0.09% due to an increase in the levels of globulin in plasma. Nonetheless, the results of the studies presented in this thesis are not comparable to many of the above-mentioned results since we evaluated the transcriptomic profiling of a panel of local immune biomarkers in the gut, but we did not target the systemic immune response. In this sense, the local immune response affects the host vulnerability and success of infection by potential pathogens in a particular tissue, in this case the gut. On the other hand, the systemic response impacts the host susceptibility to coinfection of various tissues and organs by potential pathogens and involves circulating immune factors in the blood and the coordination of a general response to infection (Rynkiewicz et al., 2019). In addition, the gut microbiota can regulate the local immune response though production of molecular metabolites, such as SCFAs, tryptophan metabolites, and secondary bile acids. Although the gut microbiota can also regulate the systemic immune response, the mechanisms differ with respect to those for regulation of local immunity (*i.e.*, translocation into the circulation of microbial soluble products which influence the activation of immune cells in the periphery; Wiertsema et al., 2021). Thus, while the majority of fish studies examining the effects of bile acids or spices have focused on systemic immunity, it is important to note that these findings cannot be extrapolated to those assessing local immunity or conflated as being the same as the results presented in this thesis.

Teleost fish have a specialized and complex gut immune system in which the intestinal mucosal layer, comprising the mucus, its commensal bacteria and epithelial cells, forms a crucial physical and biochemical defense barrier against exogenous bacteria, toxins, and allergens (Firmino et al., 2021b). It also contributes to the local immune response by recognizing and processing antigens, recruiting innate and adaptive immune cells, and secreting cytokines, chemokines, antimicrobial peptides, and mucins through the gut-associated lymphoid tissue (GALT) (Firmino et al., 2021a; Firmino et al., 2021b). In this sense, the innate immunity is the first line of defense against exogenous substances, and it responds in a non-specific manner before triggering the specific adaptive immune response. The innate immune response is induced by the recognition of pathogen-associated or damage-associated molecular patterns (PAMPs/DAMPs) by pathogen recognition receptors (PRRs). On the other hand, the adaptive responses entail the specific activation of lymphocytes through the interaction of their antigen receptors with specific foreign antigens (Secombes and Wang, 2012). Regarding the expression results of the genes included in the PCR-array of gilthead seabream intestine, they may suggest the induction of an innate immune response by bile salts when supplemented at a dietary inclusion of 0.06% (Chapter III; Ruiz et al., 2023c). This was demonstrated by an up-regulation of C-C chemokine receptor type 3 (*ccr3*), which promotes the chemotaxis and activation of eosinophils and mast cells (Heath et al., 1997; Oliveira et al.,

2003). In line with *ccr3* up-regulation, bile salts also induced an up-regulation of C-C motif chemokine 20 (*ccl20*). This chemokine stimulates the migration of lymphocytes, and to a lesser extent, granulocytes (Hieshima et al., 1997), and also works as an agonist of C-C chemokine receptor type 6 (CCR6; Bird and Tafalla, 2015). These C-C chemokine receptors are involved in the recruitment of B and T cells, and the differentiation of $CD4^+$ regulatory T cells (T_{reg} cells), promoting inflammation (Williams, 2006; Kulkarni et al., 2018). In this sense, an adaptative immune response may also be expected in the intestine of gilthead seabream fed the $BS_{0.06\%}$, considering the up-regulation of cluster of differentiation 4-1 (*cd4-1*) and C-C chemokine receptor type 9 (*ccr9*). The cell marker CD4 can be found on a wide range of immune cells, including T cells, monocytes, macrophages, and dendritic cells, and drives their migration (Ashfaq et al., 2019). These same immune cells, together with B cells, express CCR9, which induce the migration of immune cells to the gut, where they control intestinal inflammation (Pathak and Lal, 2020).

Additionally, dietary supplementation with bile salts also induced an up-regulation of immunoglobulin T membrane-bound form (*igt-m*) in the intestine of gilthead seabream. This immunoglobulin is produced by B cells mainly located in the *lamina propria* of the gut and is specialized in protecting the mucosal surfaces of teleost fish by maintaining microbiota homeostasis and clearing pathogens, similar to the role of immunoglobulin A in mammals (Yu et al., 2020). The absence of changes in the expression of immunoglobulin M (*igm*) are in line with the differential regulation of IgT and IgM described in gilthead seabream by Piazzon et al. (2016). On the other hand, contrary to our results, Li et al. (2021b) observed an increase in IgM levels in the intestine of tongue sole under bile salt dietary supplementation at 0.03 and 0.09%, indicating that the immune response is also dependent on the fish species, additive composition and origin, and dietary inclusion level, among other experimental conditions.

Regarding the differential expression of interleukins and PRRs, as stated in Chapter II of this thesis (Ruiz et al., 2023b), the up-regulation of the PRR macrophage mannose receptor 1 (*mrc1*) and of interleukin-8 (*il-8*) that we observed in the intestine of gilthead seabream fed the $BS_{0.06\%}$ diet may indicate a pro-inflammatory response (Gazi and Martinez-Pomares, 2009; Zou and Secombes, 2016), together with the up-regulation of *ccr3*, *ccl20*, *cd4-1*, and *ccr9*, which may be attributed to a migration of immune cells towards the GALT. On the other hand, bile salt dietary supplementation also induced an up-regulation of the PRR galectin-1 (*lgals1*), which reduces the synthesis of pro-inflammatory cytokines (Seropian et al., 2018), and a down-regulation of the PRRs toll-like receptor 9 (*tlr9*) and galectin-8 (*lgals8*), which promotes the production of pro-inflammatory cytokines (Kumagai et al., 2008; Cattaneo et al., 2014, respectively), suggesting the induction of an anti-inflammatory response. In this sense, Jin et al. (2019) reported an anti-inflammatory response to bile salt dietary supplementation in black seabream, indicated by the down-regulation of tumor necrosis factor-alpha (*tnf-a*) and interleukin-1 beta (*il-1β*) in the intestine. Wang et al. (2022) found a down-regulation of the proinflammatory cytokines *tnf-a* and *il-8*, but an up-regulation of transforming growth factor-β1 (*tgf-β1*), also pro-inflammatory, in the liver of tongue sole under bile salt dietary supplementation. Additionally, Yu et al. (2019) found an up-regulation of some pro-inflammatory cytokines (*tnf-a* and *il-8*), but also an up-regulation of anti-inflammatory cytokines [interleukin-10 (*il-10*), interleukin-11 (*il-11*), and *tgf-β1*] in the liver of largemouth bass when feeding it with a diet supplemented with bile salts, which is in line with the results

of the present thesis, even though the liver primarily serves as a mediator of the systemic immune response (Kubes and Jenne, 2018) and in this doctoral thesis the results pertain to the local immune response in the gut. The induction of the expression of both pro- and anti-inflammatory related genes may be a mechanism to stimulate a sort of immunocompetence priming without compromising the fish physiology (Salomón et al., 2020; Firmino et al., 2021b; Firmino et al., 2021c; Salomón et al., 2021), since no signs of inflammation or morphological differences aside from differential accumulation of fat deposits, were observed in the intestine of gilthead seabream fed the different diets (Chapter I; Ruiz et al., 2023a).

Interestingly, the regulation of many of the above-mentioned genes was shared between the tested blend of bile salts and the SO feed additive, as it was the case for *il-8*, *ccr3*, *lgals1*, and *mrc1*, which were up-regulated, and *tlr9*, which was down-regulated in fish fed the SO diet with respect to their congeners fed the control diet. In addition, this combination of spices induced an up-regulation of *il-1β*, and a down-regulation of the PRR fucolectin (*fcl*) (Chapter IV; Ruiz et al., 2024a). In this sense, Il-1β is a cytokine which activates various immune cells, mainly lymphocytes and phagocytic cells, and is one of the primary mediators of the inflammatory response (Sakai et al., 2021). The PRR FCL is a fucose-binding lectin, and it has been described to induce phagocytosis of potential pathogens in an *in vitro* study in the macrophages of European sea bass (Salerno et al., 2009). Thus, the down-regulation of *fcl*, together with the down-regulation of *tlr9*, which recognizes unmethylated CpG motifs of bacterial genomic DNA (Hemmi et al., 2000), may indicate a lower exposure to potential pathogens, maybe induced by the antimicrobial effect of turmeric, capsicum, black pepper, and ginger (Pundir and Jain, 2010; Jiang, 2019), as will be further discussed in the following section. However, further studies would be needed to confirm this hypothesis, since the PRRs *lgals1* and *mrc1* were up-regulated in gilthead seabream fed the SO diet.

Overall, the above-mentioned results indicated that, similar to the tested blend of bile salts, the SO additive induced a state of immunocompetence, improving the intestinal health of gilthead seabream. In agreement with the results of the present thesis, many studies have shown the role of such spices in the stimulation of fish immune response at the systemic level, whereas our studies were one of the few focused on local immunity of the gut. For instance, turmeric and curcumin have been reported to enhance the phagocytic, bactericidal, and lysozyme activities, phagocytic index, and/or resistance against infectious pathogens in many fish species, including gilthead seabream (Ashry et al., 2021), common carp (Abdel-Tawwab and Abbass, 2017), grass carp (Ming et al., 2020), rainbow trout (Yonar et al., 2019), and Nile tilapia (Diab et al., 2014). Although fewer studies have focused on the immunomodulatory properties of capsicum in fish, Ibrahim et al. (2024) observed a higher content of complement 3, increased phagocytic and bactericidal activities in serum, and an up-regulation of *il-1β*, interleukin-6 (*il-6*), *il-8*, *il-10*, and *tgf-β* in the head kidney of Nile tilapia fed with a diet supplemented with capsicum. Similarly, an increase in lysozyme activity, higher levels of IgM, and an activation of the complement system, including components C3 and C4, have been observed in rainbow trout when including capsicum in the diet (Firouzbakhsh et al., 2019; Shamsaie Mehrjan et al., 2020).

Under piperine dietary supplementation many studies have also described a stimulation of the lysozyme and phagocytic activities and/or an increased resistance to infectious pathogens

in rohu (Nilavan et al., 2017), olive flounder (Malintha et al., 2023), and common carp (Giri et al., 2023). Some of the studies testing piperine supplementation in aquafeeds have also described increased immunoglobulin levels (Malintha et al., 2023), and an induction of the alternative complement pathway and respiratory burst activities in serum (Giri et al., 2023).

Moreover, when supplementing ginger in fish diets, an increase in the activities of lysozyme and alternative complement pathways, as well as total immunoglobulin levels, have been found in common carp (Fazelan et al., 2020) and striped catfish (Mohammadi et al., 2020). In addition to the increased lysozyme activity, improved bactericidal action, anti-protease, and respiratory burst activities in serum, and phagocytic activity in head kidney, have been shown in Asian sea bass fed a diet supplemented with ginger (Talpur et al., 2013). Similarly, Sukumaran et al. (2016) reported an enhancement in lysozyme activity and total immunoglobulin levels in skin mucus, and an up-regulation of the anti-inflammatory cytokines *il-10*, and *tgf-β*, and down-regulation of pro-inflammatory cytokines *il-1β* and *tnf-a* in the head kidney, hepatopancreas and intestine of rohu when fed a diet supplemented with ginger. In addition, these authors observed a higher resistance to *Aeromonas hydrophila* infection and antimicrobial activity in skin mucus against potential pathogens. This is consistent with the higher resistance against *Streptococcus agalactiae* of Nile tilapia (Brum et al., 2017) and increased survival rate and antibacterial capacity against *Vibrio* spp. and fecal coliforms in the intestine of striped catfish (Ashry et al., 2023) when their diets were supplemented with ginger. However, under the current experimental approaches considered within this doctoral thesis, there was no evaluation of the potential disease-resistance effects of the tested additives in fish; thus, this approach deserves further attention once their lipotropic effect has been demonstrated (Chapter I; Ruiz et al., 2023a; Chapter II; Ruiz et al., 2023b; Chapter III; Ruiz et al., 2023c; Chapter IV; Ruiz et al., 2024a).

Regarding the spices present in the SPICY feed additive, apart from the above-mentioned immunostimulatory properties of capsicum, black pepper, and ginger, many authors have observed an immune response under supplementation of cinnamaldehyde in aquafeeds. For instance, in Nile tilapia and fat greenling, an increased serum content of immunoglobulin G (IgG) has been shown under dietary supplementation of cinnamaldehyde (Amer et al., 2018; Gu et al., 2022). In tongue sole, an immune response has also been suggested by the use of dietary supplementation with cinnamaldehyde based on the increased lysozyme activity of liver and muscle, but not in serum, mid kidney, or spleen (Wang et al., 2021a). On the other hand, Abd El-Hamid et al. (2021) reported an increase in serum lysozyme and alternative complement activities, IgM content, and in the expression of *il-1β*, *il-8*, *il-10*, and *tnf-a* in the spleen in Nile tilapia, as well as a higher resistance against *S. agalactiae* infection under cinnamaldehyde dietary supplementation. These results were partly in contradiction to those observed by Amer et al. (2018), who reported no significant differences in lysozyme activity and IgM content in serum in Nile tilapia. Such different findings among studies reveal once again, that the mechanisms of immune response induced by an additive can vary depending on its format, origin, composition, inclusion level, and many other experimental conditions, even among fish from the same species (Firmino et al., 2021a). However, the results of such studies only targeted the immune response at a systemic level, which as mentioned above cannot be extrapolated to the immune response found at a local level, as was our case.

Concerning the immune effect of the SPICY additive on the gut, under current experimental conditions, the expression of only two genes was up-regulated in gilthead seabream at the 2-h postprandial state: *il-1β*, which was up-regulated, and *ccr9*, which was down-regulated (Chapter III; Ruiz et al., 2023c). On the other hand, a clear anti-inflammatory immune response was observed in the liver of postprandial gilthead seabream when, in our previous assay, a diet with partial fish oil replacement by mammalian-rendered fat was supplemented with the SPICY additive at 0.15% (Ruiz et al., 2024c). Such results are quite different with respect to those reported herein in which gilthead seabream diets containing poultry fat as the main lipid source were supplemented with the SPICY additive at 0.1% led to mild intestinal immune modulation of *il-1β* and *ccr9* in 2 h-postprandial gilthead seabream when supplementing the SPICY additive at 0.1% in the diet with partial fish oil replacement by poultry fat and soybean oil (Chapter III; Ruiz et al., 2023c). In this context, the liver primarily serves as a mediator of systemic immune responses (Kubes and Jenne, 2018; Secombes and Wang, 2012). It is also important to acknowledge the complexity of these results, considering that, as explained above, a higher gene expression does not necessarily involve a higher translation into proteins and/or a higher enzymatic activity. The decoupling of gene expression from enzyme activity can be due to post-transcriptional or post-translational modifications that were not part of the present study, so further validation of the gene expression results with complete proteomic analyses could provide more clarity. In addition, only 44 genes related to intestinal permeability, barrier function, and immunity were studied in the current thesis, whereas in Ruiz et al. (2024c) a microarray-based transcriptomic analysis coupled with an interactomic approach was employed to study the effect of the SPICY additive on thousands of genes, many of which were associated with biological processes related to immunity.

Nonetheless, it is important to highlight that in 48 h fasted-gilthead seabream the number of genes regulated by both spice additives, SPICY and SO, significantly increased, while inversely only three genes were affected by the blend of bile salts in the fasting state. In this sense, the tested blend of bile salts induced an up-regulation of two genes encoding for tight junction proteins: claudin-15 (*cldn15*), and coxsackievirus and adenovirus receptor homolog (*cxadr*). These results may indicate a decrease in epithelial permeability (Raschperger et al., 2008; Tipsmark et al., 2010) and subsequently an increasing gut barrier function induced by the blend of bile salts in the fasting state. On the other hand, the blend of bile salts only down-regulated the cluster of differentiation 8 beta (*cd8b*), which encodes for one of the two chains of the cell marker CD8 expressed on the surface of cytotoxic T cells, which acts as a signal transduction molecule and protects against intracellular bacterial infection (Nakanishi et al., 2015). In some mammalian species, CD8β can also be expressed in NK cells, macrophages, and mast cells (Gibbings and Befus, 2009). In addition, studies in mammals have correlated the down-regulation of *cd8* with the differentiation of helper and cytotoxic T cells (Nomura and Taniuchi, 2020). However, it is difficult to interpret the significance of the down-regulation of *cd8b* considering that no other immune-related genes were modulated by the blend of bile salts in 48 h fasted-fish (Chapter II; Ruiz et al., 2023b). In addition, previous studies have reported changes in the cell surface secretion of CD8 despite the sustained expression of this gene (Xiao et al., 2007), which is in line with the above-mentioned hypothesis that gene expression does not necessarily correlate with the translation rate and/or enzymatic activity.

On the other hand, opposite to the results obtained at 2-h postprandial state, the immunostimulatory effect of the SPICY feed additive was very evident in 48 h fasted-fish. As explained in the Chapter III of the present thesis (Ruiz et al., 2023c), the up-regulation of *pcna* and down-regulation of *cxadr* might suggest a lower permeability and higher epithelial integrity. In line with the up-regulation of *pcna*, the rate of cell proliferation seems to be inversely correlated with the expression of *cxadr* in mammals (Raschperger et al., 2006). Furthermore, the presumably decrease in permeability induced by the pungent spices may be the reason for the subsequent reduced expression of the PRR CD302 antigen (*cd302*), which is a C-type lectin receptor that plays a multifaceted role in immunity, contributing to the recognition and clearance of pathogens through endocytosis and phagocytosis, and to cell adhesion and migration (Kato et al., 2007). Several studies in fish have confirmed the role of CD302 in phagocytosis and bactericidal activity (Chen et al., 2016; Zhang et al., 2022c; Peng et al., 2023), which suggested that *cd302* down-regulation may indicate a lower exposure to PAMPs due to the presumably higher gut epithelial integrity of 48 h fasted-fish fed the $SPICY_{0.1\%}$ diet (Chapter III; Ruiz et al., 2023c). Additionally, Peng et al. (2023) found that the up-regulation of *cd302* was associated with an up-regulation of the pro-inflammatory cytokines *il-1β*, *il-6*, interferon-γ (*ifn- γ*), and *tnf-a* in the mid kidney of yellow drum (*Nibea albiflora*) infected with *Vibrio harveyi*, suggesting the induction of a pro-inflammatory immune response. Thus, the down-regulation of *cd302* that we observed in the intestine of 48 h fasted-gilthead seabream fed with the $SPICY_{0.1\%}$ diet may be associated with the down-regulation of the cell markers *cd4-1*, and *cd8b*, suggesting a lower accumulation of immune cells in the gut, and with the down-regulation of the cytokines, interleukin-15 (*il-15*) and interleukin-34 (*il-34*), which are pro-inflammatory (Zou and Secombes, 2016). Overall, these results may suggest an anti-inflammatory immune response in the intestine of 48 h fasted-gilthead seabream fed with the diet supplemented with the SPICY feed additive at an inclusion level of 0.1% (Chapter III; Ruiz et al., 2023c).

When supplementing the basal diet with the SO additive, a similar down-regulation to that caused by the SPICY additive was observed for *cd4-1*, *cd8b*, *il-15*, and *il-34* in the 48-h fasting state (Chapter IV; Ruiz et al., 2024a). In agreement with these results, a down-regulation of *il-6*, interleukin-12 subunit beta (*il-12b*), and *tnf-a*, which encode pro-inflammatory cytokines (Zou and Secombes, 2016; Sakai et al., 2021), was also observed in 48 h fasted-fish fed with the SO diet. On the other hand, the expression of the gene encoding for the anti-inflammatory cytokine *il-10* was also down-regulated by the SO additive. In fish, IL-10 can inhibit the expression of pro-inflammatory cytokines, such as *il-1β*, *il-6*, *il-8* and *tnf-a* (Grayfer et al., 2011; Piazzon et al., 2015). In addition, it has been reported that IL-10 induces the development of T_{reg} cells and proliferation of $CD8^+$ memory T cells in cultures from immunized European common carp (*C. carpio carpio* L.), as well as promoting the proliferation of IgM^+ B cells and production of IgM in cultures from naive and immunized fish (Piazzon et al., 2015). In this sense, even though under current conditions *il-10* down-regulation might be in line with the down-regulation of *cd4-1* and *cd8b*, there were no changes in the expression of other immune biomarkers like *igm*, *il-1β*, *il-8*, and *tnf-a*; and *il-6* was down-regulated, contrary to what may be expected considering the results of the above-mentioned studies. Thus, these results suggested that the down-regulation of *il-10* was not correlated with a significant modulation of IL-10 production and/or that the effects induced by *il-10* down-regulation did not

completely counteract the coordinated anti-inflammatory immune response induced by the down-regulation of pro-inflammatory cytokines (*il-6*, *il-12b*, *il-15*, *il-34*, *tnf-a*).

Furthermore, the down-regulation of the immune cell markers *ccr9*, and C-C chemokine receptor type 11 (*ccr11*) may also be an indicator of the anti-inflammatory immune response induced by the SO feed additive in 48 h fasted-gilthead seabream. For instance, previous studies have correlated the up-regulation of C-C chemokine receptors, including *ccr11*, with the presence of proinflammatory cytokines, PAMPs, and bacterial infection in rainbow trout (Qi et al., 2017). Considering the above information, one of the potential causes of the down-regulation of such C-C chemokine receptors and pro-inflammatory cytokines may be the presumably lower recognition of PAMPs by the PRR toll-like receptor 2 (TLR2) associated to *tlr2* down-regulation. This PRR plays an essential role in the detection of a wide range of fungal, bacterial, and viral PAMPs, including chitin, lipoproteins, lipopeptides, lipoteichoic acid, and lipoarabinomannan (Palti, 2011; Oliveira-Nascimento et al., 2012; Zhang et al., 2014). To what extend the anti-inflammatory immune response herein observed in 48 h fasted-fish may be associated to a lower exposure to potential pathogens deserves further investigation, and a deeper focus on the fish gut autochthonous microbiota, which will be addressed in the following section.

Overall, it is clear that the combinations of spices included in both the SPICY and SO feed additives induced an intestinal anti-inflammatory immune response in gilthead seabream, which was particularly evident at the 48-h fasting state (Chapter III; Ruiz et al., 2023c; Chapter IV; Ruiz et al., 2024a). Although under current conditions no visible signs of inflammation were observed in the intestine of gilthead seabream, the anti-inflammatory properties of the tested spices have been well-described in mammals (Srinivasan, 2005; Jiang, 2019) and they have also been reported in some fish species, in concordance with our results of immune-gene expression. For instance, the anti-inflammatory effects of ginger, turmeric, and cinnamaldehyde have been reported in rohu (Sukumaran et al., 2016), grass carp (Ming et al., 2020), and zebrafish (Faikoh et al., 2014) respectively, through an up-regulation of anti-inflammatory cytokines, such as *il-10* and *tgf*-β, and a down-regulation of pro-inflammatory cytokines, such as *il-1β*, *il-6*, *il-8*, *il-15*, and *tnf-a*. In this sense, similar to mammals, the terpenes and organosulfur compounds present in phytogenics have been proposed as being responsible for the antimicrobial, antioxidant, anti-inflammatory, and immunomodulatory properties of herbs and spices in fish (Firmino et al., 2021a).

In summary, different immunomodulatory effects of the tested additives were observed in the present thesis depending on the time passed since the last feeding. At the 2 h-postprandial state, the blend of bile salts and the SO feed additive showed an intestinal immune modulation in fish which was characterized by the regulation of both pro- and anti-inflammatory genes, likely resulting in a state of immunocompetence against potential threats. On the other hand, after a 48-h fasting period, a clear anti-inflammatory pattern was observed in gilthead seabream fed the diet supplemented with the SPICY and SO feed additives, which may potentially improve intestinal motility, feed digestion, nutrient absorption, and ultimately feed utilization and fish growth (Serna-Duque and Esteban, 2020). Therefore, these results indicate that the three additives tested in the present thesis may enhance the health status of gilthead seabream, which is in agreement with the above-mentioned studies in other fish

species and higher vertebrates, even though the evaluation of their activity in promoting disease resistance mechanisms when the host is exposed to a pathogen, deserves further attention and needs to be addressed in future studies.

4. Dietary modulation of the blend of bile salts and combinations of spices, and effect of the intestinal microbiota transplant (IMT) on the fish gut microbiota

It is well-known that the gut microbiota has a crucial role in fish growth and health, through the regulation of multiple physiological functions, such as feed and nutrient utilization, metabolism, development, mucosal integrity, and immune system modulation (Egerton et al., 2018; Yu et al., 2021). Among this wide range and pleiotropic functions under regulation by the gut microbiota, its involvement in the host's lipid metabolism is highly remarkable, as it has been well-demonstrated in mammals. In this sense, obesity has been correlated with changes in the composition, diversity, and gene regulation profile in the gut microbiota of mammals (Ley et al., 2006; Turnbaugh et al., 2019). Particularly, these studies have associated a leaner phenotype with a higher microbial diversity and an increased abundance of Bacteroidota in humans and mice. In mice, it has also been shown that the gut microbiota of an obese individual has higher capacity to harvest energy from the diet compared to the microbiota of a lean mouse, based on predictive metagenomics and on biochemical analyses (Turnbaugh et al., 2006). It has been postulated that in higher vertebrates there is cross-talk between the gut microbiota and the adipose tissue of the host that is able to regulate energy metabolism via adipokines and metabolites, such as bile acids and short-chain fatty acids (SCFAs) (Wu et al., 2022). In addition, some studies performing fecal microbiota transplants (FMTs) between mice with different fatty phenotypes have shown an establishment of the donor phenotype after the FMT (Turnbaugh et al., 2006; Yan et al., 2023). Similar results have been obtained when performing inter-specific FMTs from humans (Ridaura et al., 2013; Tremaroli et al., 2015) or pigs (Yang et al., 2018) to germ-free mice. These findings highlight the relevance of the microbiota in defining the levels of fat accumulation in mammals. Similarly, many studies in fish have shown that the gut microbiota is able to regulate intestinal absorption of fatty acids, lipid deposition in digestive tissues, and the expression of genes related to lipid metabolism (Semova et al., 2012; Ni et al., 2014; Sheng et al., 2018).

There are reports that have demonstrated and proposed different mechanisms by which the fish gut microbiota can regulate lipid metabolism and promote fat mobilization, that are very similar to the mechanisms found in mammals (Wu et al., 2024). The most well-demonstrated mechanism in fish is the production and secretion of microbial lipases. Bacterial lipases are normally extracellular and versatile in terms of temperature and pH, even though they are more functional under the alkaline environment typical of the intestinal lumen (Gupta et al., 2004). Bacterial lipases have been documented in a diverse range of aquaculture species comprising freshwater, brackish water, and marine fish (Ray et al., 2012). In addition, as in mammals, some members of the fish gut microbiota are able to metabolize non-starch carbohydrates that are indigestible for the host into short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate (Hao et al., 2017). Subsequently, it has been demonstrated that such SCFAs, especially acetate (Xu et al., 2022c; Yu et al., 2022; Zhou et al., 2023) and butyrate (Zhou et al., 2019; Chen et al., 2022), improve hepatic lipid metabolism, reducing lipid

accumulation in fish as well as improving the condition of the intestinal epithelium (Estensoro et al., 2016; Liu et al., 2023; Xun et al., 2023; Zhao et al., 2024). Another mechanism of regulation of lipid metabolism by the gut microbiota may be through the gut-liver axis, transforming primary bile acids into secondary bile acids, which are then reabsorbed in the gut and transported to the liver via the hepatic portal vein (Romano et al., 2020; Introduction, Figure 3).

Considering the above-mentioned mechanisms, it is evident that the intestinal microbiota has a major role on fish lipid metabolism and on the regulation of fat accumulation, and consequently in the overall fish performance and health condition. However, in nutritional studies it is usually difficult to determine to what extent the observed changes in gut microbial communities are a cause or a consequence of the modulation of the fish health by aquafeeds. Thus, in the first part of this section, we will compare the changes observed in the gut microbiota of gilthead seabream when supplementing its diet with the tested additives and intend to explain the potential relationships between such changes and the lipid metabolism and overall health condition of fish based on the literature. For this purpose, we will also establish a correlation between the gut microbial composition and the fish performance parameters measured in the articles of the present thesis.

As mentioned above, the gut microbiota is involved in lipid metabolism through regulation of the bile salt profile, but this modulation is bidirectional since bile salts can also regulate the gut microbiota profile. In this sense, bile salts can be toxic for the host fish, or higher vertebrates, and for some bacterial strains when they are found at high concentrations in the intestinal lumen because the detergent activity of bile salts can disrupt membrane integrity (Schubert et al., 2017; Fuentes et al., 2024). In addition, the hydrophobic nature of bile acids has been correlated with toxicity, with bile salts being more hydrophobic in their deconjugated form, and after metabolization by the gut microbiota, namely, secondary bile salts (Hofmann, 1999; Ridlon et al., 2014). This may explain the lower observed (number of ASVs) and estimated richness values (Chao1 and ACE indices), which were observed in the anterior intestine of gilthead seabream when supplementing its diet with the tested blend of bile salts (Chapter II; Ruiz et al., 2023b). The increased content of the secondary bile salt T-DCA in the anterior intestine of gilthead seabream fed the $BS_{0.06\%}$ diet (Chapter I; Ruiz et al., 2023a), which have more toxicity than primary bile acids (Schubert et al., 2017), was in line with such decrease in the richness of the gut microbiota from the anterior intestine.

On the other hand, there were no significant differences in Chao1 and ACE values between fish fed the control diet and that supplemented with bile salts in the posterior intestine, where production of secondary bile acids mainly takes place (Hagey et al., 2010). However, paradoxically, by synthesizing different types of secondary bile salts, the gut microbiota also prevents the toxicity that may be caused by the accumulation of primary bile salts synthesized by the host (Schubert et al., 2017). Fortunately, the decrease in bacterial richness observed in the anterior intestine of gilthead seabream was not associated to changes in its diversity (Shannon and Simpson indices) neither were there differences in inter-individual diversity among dietary treatments (beta diversity; Bray-Curtis). In this sense, a loss of microbial diversity is usually associated with a loss of microbial functionality, leading to reduced digestive capacity, lower energy production, and increased susceptibility to diseases (Infante-

Villamil et al., 2021). A decreased microbial diversity has been usually associated with an inflammatory condition in higher vertebrates (Sekirov et al., 2010). In addition, the decreased resource competition in an environment with low microbial diversity leads to a higher risk of colonization by potential pathogens, causing infectious diseases (Sekirov et al., 2010). These results indicated that the administration of the blend of bile salts as a dietary additive did not pose a risk of dysbiosis in gilthead seabream.

Similar to our study, no differences in diversity (Shannon and Simpson indices) were found in the posterior intestine of Chinese perch when its diet was supplemented with a blend of bile salts at 0.09% (Zhang et al., 2022a). Neither were there significant differences in observed richness, even though the number of observed OTUs was higher in fish fed the diet supplemented with bile salts than in those fed the control diet (Zhang et al., 2022a). On the other hand, different results have been obtained in tongue sole (Li et al., 2021b) and grass carp (Zhou et al., 2018a). In the mid intestine of tongue sole, a decrease in the number of observed OTUs was observed under dietary supplementation of a blend of bile salts of porcine origin at both tested inclusion levels, 0.03 and 0.09% (Li et al., 2021b). Such results were in agreement with the decreased number of ASVs that we observed in both anterior and posterior intestinal regions of gilthead seabream when supplementing its diet with bile salts. However, Li et al. (2021b) reported no changes in estimated richness (Chao1 and ACE indices), as well as an increase in diversity (Shannon and Simpson indices), which was suggested to improve the general health and the ability to resist infection. Such increase in diversity was also correlated with the separation among all groups observed in the PCoA for beta diversity based on Bray-Curtis distances, indicating a different structure of the gut bacterial communities (Li et al., 2021b). In the intestine of grass carp, a diminishment in the number of OTUs was also found under dietary supplementation with bile salts at an inclusion level of 0.006% (Zhou et al., 2018a). Nonetheless, an increase in estimated richness as shown by Chao1 and ACE indices and a reduction in the values of Shannon index were also reported. In this sense, the specific type of bile salt tested has a pivotal role on microbial diversity and richness, as demonstrated by Xiong et al. (2018). These authors evaluated the specific effect of different primary and secondary bile salts at an inclusion level of 0.2 mmol kg^{-1} on the microbial communities of the posterior intestine of grass carp. Particularly, only T-CDCA and taurolithocholic acid (T-LCA) were able to increase bacterial richness (Chao1 index), while L-TCA and tauroursodeoxycholic acid (T-UDCA) increased the values of Shannon and Simpson indices. Overall, the differential results in microbial richness and diversity found among studies were likely dependent on the type of bile acids/salts tested, as well as on the specific conditions of the nutritional study, such as the feeding period, the fish species, the diet composition, the inclusion levels and origin of the additive, and the intestinal region sampled, among other factors (Xiong et al., 2018; Li et al., 2021b).

Regarding spices, previous studies in mammals have shown that many spices, including capsicum, cinnamon, and turmeric, have antimicrobial properties against potential pathogens, resulting in beneficial effects for the host health (Jiang, 2019). Similarly, the antimicrobial properties of spices have also been reported in fish. In particular, antibacterial activities for capsicum and turmeric have been described in Nile tilapia (Ibrahim et al., 2024) and rainbow trout (Yonar et al., 2019), respectively. The antimicrobial activity of ginger has been reported in Asian sea bass (Talpur et al., 2013), and common carp (Fazelan et al., 2020). However, these

studies only evaluated the antimicrobial activities of such species in serum, which is not transposable to the fish gut. At an intestinal level, the antimicrobial activity of some of the tested spices has also been described, such as black pepper in rohu (Ullah et al., 2021), ginger in striped catfish (Ashry et al., 2023), and turmeric in gilthead seabream (Ashry et al., 2021). Nonetheless, the above-mentioned studies evaluated the antibacterial activity of such species by means of traditional microbiological methodologies like the comparison of the number of colony forming units (CFUs) on culture media; thus, only considering the viable culturable bacteria.

Very few studies have evaluated the effect of the herein tested spices in the gut microbial communities of fish using 16S rRNA gene sequencing. Among these studies, Yılmaz et al. (2024) reported no significant differences in observed (number of OTUs) and estimated richness (Chao1 index), neither on the diversity and structure of the bacterial communities in the intestine of rainbow trout when supplementing its diet with capsicum at different inclusion levels (0.7-2.8%). On the other hand, an increase in the values of observed (number of OTUs) and estimated richness (Chao1 index), as well as in diversity (Shannon index), and phylogenetic diversity (Faith index), was observed in tongue sole under dietary supplementation with cinnamaldehyde at an inclusion level of 0.1% (Wang et al., 2021a). A different structure (beta diversity) in tongue sole fed the diet supplemented with cinnamaldehyde was also observed with respect to tongue sole fed the control diet (Wang et al., 2021a). In Ruiz et al. (2023c; Chapter III), no differences in estimated richness (ACE index) were found in the intestine of gilthead seabream fed the diet supplemented with the SPCIY additive at 0.1%, containing capsicum, black pepper, ginger, and cinnamaldehyde, with respect to their congeners fed the control diet. Regarding bacterial diversity indices, no differences were observed either for the values of Shannon or Faith phylogenetic diversity, but the supplementation of the SPICY additive induced a significant increase in the values of the Simpson index in the posterior intestine. Shannon and Simpson indices are both estimators of diversity, but while the Shannon index puts more weight on richness, the Simpson index puts more weight on evenness, which is the degree of homogeneity in the distribution of species abundances (Kim et al., 2017b). Consequently, the increase in the values of the Simpson index that we observed may be associated with a higher bacterial diversity due to a higher evenness of the population rather than to a higher richness, since there were no differences in the values of ACE and Shannon indices. In this sense, an increase in bacterial diversity is typically regarded as a beneficial aspect since it is typically linked to an improvement in the condition of the host health (Terova et al., 2019). On the other hand, no differences in estimated richness (ACE index), diversity (Shannon and Simpson indices), or phylogenetic diversity (Faith index) were observed in gilthead seabream when the diet was supplemented with the SO additive at 0.2%, containing turmeric, capsicum, black pepper, and ginger (Chapter IV; Ruiz et al., 2024a). In addition, no inter-individual phylogenetic differences (beta diversity based on weighted UniFrac distances) were found in fish fed the $SPICY_{0.1\%}$ nor the SO diet with respect to the control group, indicating the absence of risks of dysbiosis when the additives based on spices were administered to gilthead seabream.

Overall, the similar diversity that we observed in all the microbiota samples may be explained by the existence of a core microbiota common to all individuals, which is stable and persistent regardless of changing factors and is normally composed of highly abundant microbial

members (Astudillo-García et al., 2017). In this sense, previous studies have demonstrated that, as in mammals, fish also have a core gut microbiota which does not change regardless of external factors, such as the feeding of different diets or distinct environmental conditions (Roeselers et al., 2011; Mente et al., 2018; Nikouli et al., 2018; Rudi et al., 2018; Kokou et al., 2019). In our case, only 350 out of 19,829 ASVs were shared among the four dietary groups (control diet, $BS_{0.06\%}$ diet, $SPICY_{0.1\%}$ diet, SO diet) in the anterior intestine, and 319 out of 13,996 ASVs were common to all groups in the posterior intestine (Figure 1). The majority of ASVs were unique to each dietary treatment. Despite the relatively low number of ASVs common to all dietary treatments, these represented an abundance of 40.7% in the anterior intestine, and of 45.3% in the posterior intestine. Related to this, a recent study has reported the presence of a core gut microbiome in gilthead seabream which was maintained in all individuals during the nutritional trial after a change of diet (Ruiz et al., 2024d). Although the diet plays a paramount role in defining the fish gut microbial diversity, structure, and composition (Silva et al., 2011; Ghanbari et al., 2015), there are other biotic and abiotic factors, which were common to all individuals used in the nutritional trials of the present thesis, and that may contribute to the shaping of a core microbiome. For instance, it has been shown that the fish microbiota is influenced by host inherent factors, such as the genetic background (Navarrete et al., 2012) and the origin of the fish (Dhanasiri et al., 2011), and by environmental factors, including the water temperature, salinity (Rudi et al., 2018) and the microbial composition of the environment (Roeselers et al., 2011). Moreover, the nutritional trials evaluating the effects of the dietary supplementation of the tested additives on gilthead seabream were carried out in parallel in different tanks from the same recirculating aquaculture system (RAS, IRTAmar™), under the same environmental conditions, and all the fish come from the same commercial hatchery. Thus, the common origin, genetic background, and environmental conditions in the four nutritional studies included in this thesis were the main determinants of the common ASVs found among studies.

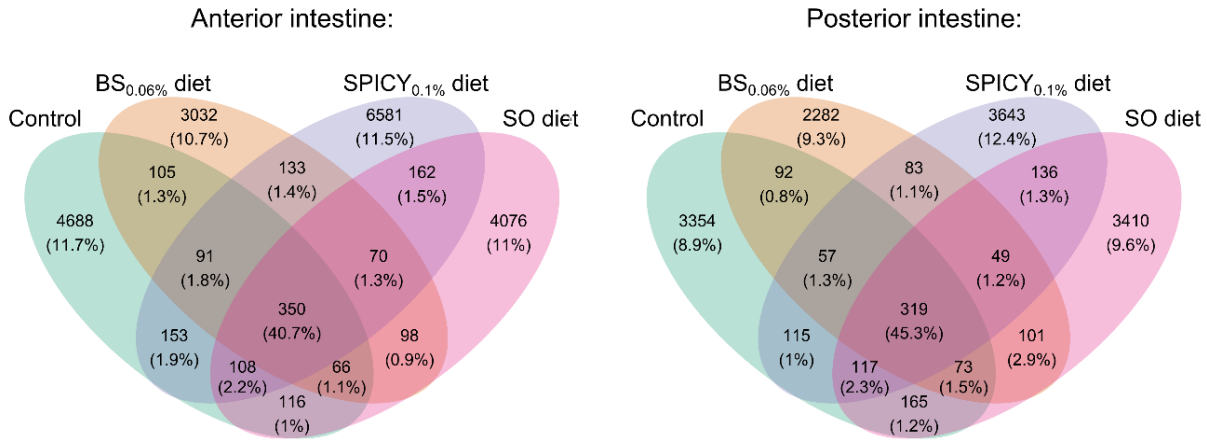

Figure 1. Venn diagram plotting the number of unique and shared ASVs (and relative abundance % with respect to the total abundance of ASVs) in gilthead seabream (*Sparus aurata*) fed the control diet and the diets supplemented with bile salts at an inclusion level of 0.06% ($BS_{0.06\%}$ diet), a combination of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde at 0.1% ($SPICY_{0.1\%}$ diet), and a combination of turmeric, capsicum, black pepper, and ginger oleoresins at 0.2% (SO diet).

The similar microbial diversity and structure of the gut microbial communities among different dietary treatments was associated to a similar bacterial composition. At the level of phylum, significant differences with respect to the control diet ($P \leq 0.05$) were only found in fish fed the $BS_{0.06\%}$ and $SPICY_{0.1\%}$ diet. In this regard, an increase of 2.2% in the relative abundance of Desulfobacterota was observed in the anterior intestine of gilthead seabream fed the $BS_{0.06\%}$ diet with respect to the control group (Chapter II; Ruiz et al., 2023b), which was attributed to the increased abundance of the genus *Desulfovibrio*, whose importance will be further discussed below. In addition, under dietary bile salt supplementation, an increase of 13.6% in the relative abundance of Firmicutes, and a decrease of 14.3% in the relative abundance of Proteobacteria were also observed in the posterior intestine (Chapter II; Ruiz et al., 2023b). In agreement with our study, Zhang et al. (2022a) reported an increase in the abundance of Firmicutes in the posterior intestine of Chinese perch when its diet was supplemented with bile salts at an inclusion level of 0.09%. These authors associated the increased abundance of this phylum with a higher stress tolerance. Additionally, in higher vertebrates it has been shown that members of the phylum Firmicutes are able to tolerate and metabolize primary bile salts, thus having a higher survival in the gut than non-tolerant bacteria (Islam et al., 2011; Joyce and Gahan, 2017). In this sense, it is well-known that the presence of bile acids in the intestine usually inhibits the growth of bacteria sensitive to them, while promotes a higher growth of bacteria able to tolerate and/or metabolize bile acids (Ridlon et al., 2014). On the other hand, Zhou et al. (2018a) observed a significant reduction in the abundance of Firmicutes in the intestine of grass carp when fed a diet supplemented with bile salts at 0.006%. However, the above-mentioned studies are not directly comparable, since it is important to consider that Zhou et al. (2018a) homogenized the whole gut, while Zhang et al. (2022a) only sampled the posterior intestine, which is the region where we found differences in the abundance of Firmicutes. In addition, to ensure the collection of autochthonous microbiota, we fasted the fish for 48 h before the sampling and Zhang et al. (2022a) removed the intestinal digesta, whereas Zhou et al. (2018a) apparently targeted all the microbial content. Thus, such differences in the sampling method regarding the intestinal region and type of targeted microbiota probably influenced the results obtained from each study, which reinforces the idea of the importance of standardizing sample collection and processing for studies focused on the host's microbiota (Kashinskaya et al., 2017; Legrand et al., 2020a; Ruiz et al., 2024e). Nonetheless, Zhou et al. (2018a) also observed a decrease in the relative abundance of Proteobacteria, which is in line with the effect that the tested blend of bile salts caused in the posterior intestine of gilthead seabream. Further, in fish fed the $SPICY_{0.1\%}$ diet, an increase of 0.9% in the relative abundance of Chloroflexi was found in the posterior intestine, with respect to the individuals fed the control diet (Chapter III; Ruiz et al., 2023c). Although this widespread and metabolic diverse phylum has been found in the intestine of many fish species (Fan et al., 2017; Bereded et al., 2020; Liu et al., 2021b; Naya-Català et al., 2021b; Nikouli et al., 2021), it has not yet been correlated with the metabolism of lipids nor bile acids, nor is there strong evidence of its role on fish intestinal health.

Some studies have used different ratios at the level of phylum as markers of the animal health and condition, with the ratio Firmicutes/Bacteroidota (F/B) being the most commonly used. In humans and mice, a reduction in the F/B ratio has been usually associated to obesity (Turnbaugh et al., 2006; Indiani et al., 2018), while in fish it is used as a biomarker of intestinal

dysbiosis (Mougin and Joyce, 2023). On the other hand, the ratio Bacteroidota/Proteobacteria (B/P) has been correlated with inflammation, but its significance can vary depending on the animal (Brugman et al., 2018). In mammals, decreased values of the B/P ratio have been associated to inflammation, whereas in fish an increase in the values of this ratio has been observed upon inflammation (Brugman et al., 2018). In the studies presented in this thesis, no significant differences in the values of the ratios F/B and B/P were found in the anterior nor posterior intestine of gilthead seabream when the control diet was supplemented with the tested additives (Table 2), which is in line with the similarities in gut microbial composition observed at the level of phylum. Thus, the absence of differences in the values of the ratio F/B may indicate no risk of dysbiosis in gilthead seabream when supplementing its diet with the blend of bile salts and both additives based on spices. The numerical though not statistically significant decrease in the ratio B/P in the posterior intestine of fish fed the $BS_{0.06\%}$ diet, with respect to the control group, may be related to the anti-inflammatory potential of bile salts in the fish gut (Iwashita et al., 2009; Kortner et al., 2016). Nonetheless, such a decrease in B/P values was not significant, and no signs of inflammation were found in the intestine of gilthead seabream under current conditions (Chapter I; Ruiz et al., 2023a; Chapter II; Ruiz et al., 2023b).

Table 2. Values of the ratios Firmicutes/Bacteroidota (F/B) and Bacteroidota/Proteobacteria (B/P) in the anterior and posterior intestine of gilthead seabream (*Sparus aurata*) fed the control diet and the diets supplemented with bile salts at an inclusion level of 0.06% ($BS_{0.06\%}$), a combination of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde at 0.1% ($SPICY_{0.1\%}$), and a combination of turmeric, capsicum, black pepper, and ginger oleoresins at 0.2% (SO).

	Control	**$BS_{0.06\%}$**	**$SPICY_{0.1\%}$**	**SO**	***P*-value**
Anterior intestine					
F/B ratio	1.65 ± 0.13	1.77 ± 0.07	1.70 ± 0.29	1.61 ± 0.12	0.852
B/P ratio	1.34 ± 0.17	1.17 ± 0.21	1.33 ± 0.14	1.06 ± 0.13	0.436
Posterior intestine					
F/B ratio	2.13 ± 0.16	2.01 ± 0.25	1.92 ± 0.21	2.20 ± 0.12	0.582
B/P ratio	1.67 ± 0.42	0.91 ± 0.12	1.35 ± 0.33	1.54 ± 0.25	0.162

Values are represented as mean ± SEM (n = 12 fish per dietary group). There were no significant differences among groups (Kruskal-Wallis; $P > 0.05$).

Considering the above-mentioned role of the gut microbial communities in fish performance, in order to facilitate the interpretation of the results, we tested the correlation between the results of growth and feed performance and the relative abundances of the predominant genera (≥ 1%) in the anterior and posterior intestine of gilthead seabream (Spearman's correlation, $P \leq 0.05$; Figure 2). However, it should be noted that a significant correlation does not necessarily imply a cause-effect relationship.

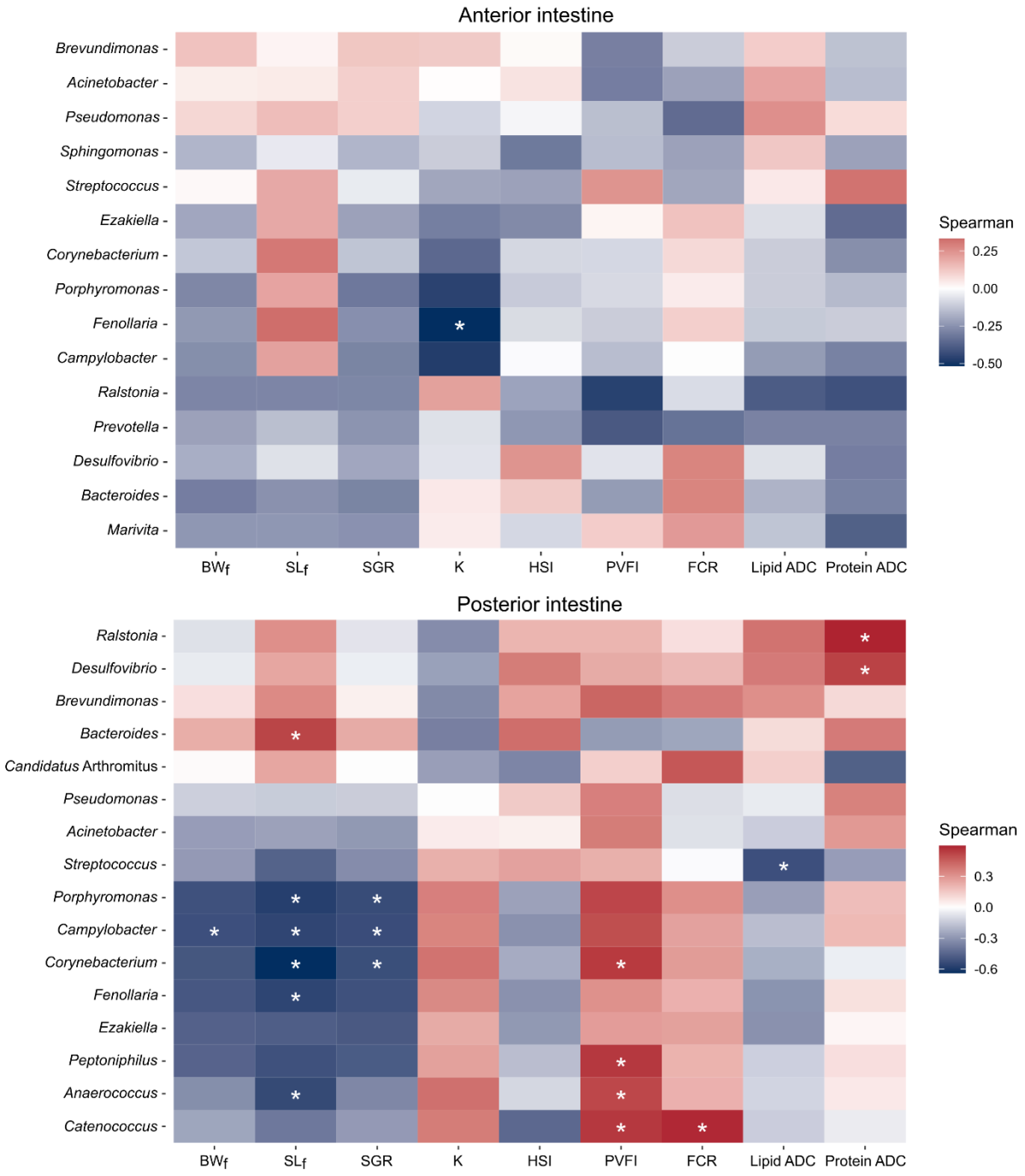

Figure 2. Spearman's correlation among the relative abundance of the most abundant bacteria (≥ 1%) in the anterior and posterior intestine and the values of the fish performance indicators measured in gilthead seabream (*Sparus aurata*) fed the control diet and the diets supplemented with bile salts at an inclusion level of 0.06% ($BS_{0.06\%}$ diet), a combination of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde at 0.1% ($SPICY_{0.1\%}$ diet), and a combination of turmeric, capsicum, black pepper, and ginger oleoresins at 0.2% (SO diet). The correlation was tested among the mean values per tank of each parameter (n = 16 tanks). Spearman's correlation coefficients (r_s) are represented using a color gradient (dark red: positive correlation – dark blue: negative correlation) and significant correlations ($P \leq 0.05$) are marked with an asterisk. Abbreviations: BW_f: final body weight; SL_f: final standard length; SGR: specific growth rate; K: Fulton's condition factor; HSI: hepatosomatic index; PVFI: perivisceral fat index; FCR: feed conversion ratio; ADC: apparent digestibility coefficient.

Considering the genera with a relative abundance ≥ 1%, the tested blend of bile salts had an effect in the relative abundance of bacterial taxa from both the anterior and posterior intestine (Chapter II; Ruiz et al., 2023b). Interestingly, on the other hand, the dietary effect of the SPICY additive was only detected in the posterior intestine (Chapter III; Ruiz et al., 2023c), while the effect of the SO additive was observed in the anterior intestine (Chapter IV; Ruiz et al., 2024a). Furthermore, it was striking to find common patterns of regulation of the relative abundance of some genera between the blend of bile salts used and the two tested combinations of spices. For instance, a significant increase in the levels of representatives of the genera *Bacteroides*, *Desulfovibrio* and *Ralstonia* was observed in the anterior intestine of gilthead seabream fed with both the $BS_{0.06\%}$ diet and the SO diet, with respect to the control group.

In concordance with our results, Zhou et al. (2018a) also observed an increase in the abundance of *Bacteroides* in grass carp under dietary supplementation with bile salts. As discussed in Ruiz et al. (2023b, 2024a; Chapter II; Chapter IV), it is well-demonstrated that members of both genera *Bacteroides* and *Desulfovibrio* are involved in bile acid metabolism. In this sense, some species belonging to *Bacteroides* have been reported to possess bile salt hydrolase (BSH) activity, which is necessary for the deconjugation of primary bile acids, the first step in the metabolization of primary bile salts into secondary bile acids (Kawamoto et al., 1989). Furthermore, some members belonging to this Gram-negative anaerobic genus have enzymatic activities for 7α-dehydroxylase, 7α-hydroxysteroid dehydrogenase (7α-HSD) and 7β-hydroxysteroid dehydrogenase (7β-HSD), which catalyze the epimerization and oxidation or reduction of bile acids, necessary to complete their transformation into secondary bile acids (Chattopadhyay et al., 2022). However, species belonging to *Bacteroides* do not only contribute to lipid digestion and metabolism through their role in the metabolization of bile salts, but also through the production of SCFAs. In this sense, *Bacteroides* spp. are involved in the synthesis of propionate and acetate (Chattopadhyay et al., 2022), which can promote lipid catabolism and digestion, preventing fat accumulation in fish (Yu et al., 2022; Yousefi et al., 2024). In addition, some species belonging to the genera *Desulfovibrio* are capable of generating hydrogen sulfide (H_2S) from taurine (Hu et al., 2022a; Rausch, 2023), which is released from deconjugated bile acids. Further, it has been shown that the bile acids CA and DCA induce the growth of *Desulfovibrio* (Chattopadhyay et al., 2022). Thus, in our studies, the increased relative abundance of *Desulfovibrio* may be attributed to the higher content of T-DCA in the intestine of gilthead seabream fed the $BS_{0.06\%}$ diet (Chapter I; Ruiz et al., 2023a), and to the numerical increase in the levels of T-CA in the intestine of fish fed the SO diet (Chapter IV; Ruiz et al., 2024a). Moreover, it has been reported that both Gram-negative anaerobic genera, *Bacteroides* and *Desulfovibrio*, promote the growth of bacteria with 7α-dehydroxylase activities, including *Bacteroides* (Hirano and Masuda, 1982; Hu et al., 2022a).

While the role of *Ralstonia* in the fish gut remains unknown, considering the metabolic versatility of some species from this genus (Lu et al., 2013; Riedel et al., 2014), previous studies have hypothesized that they might biosynthesize bioactive compounds and secondary metabolites beneficial for the fish (Cerezo-Ortega et al., 2021). However, further studies are needed to confirm this hypothesis. Using the data of the studies included in this thesis, it was observed that the relative abundance of *Ralstonia* in the anterior intestine tended to negatively correlate, even though not significantly (Spearman's correlations coefficient r_s = -0.46, n = 16, P = 0.071), with the values of the PVFI. Thus, the increased abundance of this genus may be

associated the reduction in the levels of perivisceral fat found in gilthead seabream fed the $BS_{0.06\%}$ diet (Chapter I; Ruiz et al., 2023a), and the SO diet (Chapter IV; Ruiz et al., 2024a).

In the posterior intestine of gilthead seabream, we found common patterns in the regulation of the relative abundance of some genera by the tested blend of bile salts and the SPICY feed additive. Indeed, fish fed the $BS_{0.06\%}$ and the $SPICY_{0.1\%}$ diets showed a decrease in the relative abundance of the genera *Campylobacter*, *Corynebacterium*, and *Peptoniphilus* in the posterior intestine, with respect to their congeners fed the control diet (Chapter II; Ruiz et al., 2023b; Chapter III; Ruiz et al., 2023c). Such reductions in bacterial abundance may be consequence of the above-mentioned antimicrobial effect of bile salts (Schubert et al., 2017) and of spices (Jiang, 2019). Although in higher vertebrates some bacterial species belonging to these genera are considered potential pathogens (Sahin et al., 2002; Spier, 2008; Brown et al., 2014), in fish they have been found as part of the commensal microbiota of the intestine (Estruch et al., 2015; Zhou et al., 2018b; Liu et al., 2022b). Nonetheless, to date their role in fish health remains largely unexplored.

When testing the correlation between the relative abundance of the genera from the posterior intestine of gilthead seabream and the fish performance indicators, significant correlations were found for the three genera. In particular, the abundance of *Campylobacter* was negatively correlated with the values BW_f (r_s = -0.52, n = 16, P = 0.049), SL_f (r_s = -0.56, P = 0.034), and SGR (r_s = -0.53, P = 0.045), and the abundance of *Corynebacterium* was negatively correlated with the values of SL_f (r_s = -0.64, P = 0.012) and SGR (r_s = -0.53, P = 0.047; Figure 2). Thus, the improved growth performance of fish fed the $BS_{0.06\%}$ and $SPICY_{0.1\%}$ diets may be associated to the lower levels of these genera in the posterior intestine in presence of such additives, or *vice versa*. The relative abundance of *Porphyromonas*, which decreased in fish fed the $BS_{0.06\%}$ diet, also showed a negative correlation with SL_f (r_s = -0.58, P = 0.026) and SGR (r_s = -0.54, P = 0.042). In addition, a positive correlation was found between the relative abundance of *Corynebacterium* and *Peptoniphilus* and the values of PVFI (r_s = 0.54, P = 0.039; r_s = 0.55, P = 0.036, respectively), indicating that there was an association between the decreased abundance of both genera in the posterior intestine and the lower levels of perivisceral fat in fish the $BS_{0.06\%}$ and the $SPICY_{0.1\%}$ diets.

Overall, these results showed that the blend of bile salts and both combinations of spices were able to modulate the composition of the gut microbial communities of gilthead seabream without compromising their diversity and structure. In addition, the common patterns found between the blend of bile salts and the SO additive in the anterior intestine, and the SPICY additive in the posterior intestine, suggested that the modulatory effect of both, bile salts and spices, on the gut microbiota was in part due to a higher secretion rate of bile salts into the intestine. This is congruent with reports that supplementation of spices can induce a higher secretion of bile salts to the intestine (Platel and Srinivasan, 2004), which promotes the growth of bile salt-tolerant bacteria over bacteria which are not able to metabolize these compounds (Ridlon et al., 2014). Thus, the numerical increase of total bile salts in the intestine of gilthead seabream fed the $SPICY_{0.1\%}$ and SO diets may partly be the cause of the microbial modulation induced by the tested spices (Chapter III; Ruiz et al., 2023c; Chapter IV; Ruiz et al., 2024a).

Changing the subject, another objective of this thesis was to assess the viability of performing an intestinal microbiota transplant (IMT) from Atlantic salmon to gilthead seabream (Chapter

V; Ruiz et al., 2024b). The two fish species selected were marine carnivores that thrive at different environmental conditions, in order to be able to discriminate the microbial composition coming from the donor and receptor fish, and this assay was designed as a conceptual approach for future IMTs aimed at reducing fat accumulation by modulating the gut microbial communities of fish. In this sense, if we had observed a significant change in gut microbial diversity, structure, and composition under the dietary supplementation of any of the tested additives that might support the role of gut microbiota on body and perivisceral adiposity, the protocol developed in Ruiz et al. (2024b; Chapter V) could have been applied from gilthead seabreams fed one of the supplemented diets to their congeners that were fed the control diet to try to modulate their gut microbial communities and body fat levels. However, in the nutritional assays presented in this thesis, we did not observe such a radical change in the gut microbial communities as to expect to discern the establishment and/or maintenance of a new intestinal microbiota capable of contributing to the modulation of fat accumulation in gilthead seabream (Chapter II; Ruiz et al., 2023b; Chapter III; Ruiz et al., 2023c; Chapter IV; Ruiz et al., 2024a). Nonetheless, in the hypothetical case that this trial had been carried out, a point to address would have been whether gilthead seabream may still require the dietary additive supplementation to maintain its novel intestinal microbiota over time after the IMT. Therefore, the influence of the diet was also taken into account in our conceptual approach. Hence, one group of gilthead seabreams was fed with the typical gilthead seabream diet provided during the 36-day trial (GSB diet), while another group was fed with the diet of the Atlantic salmon after the IMT (Chapter V; Ruiz et al., 2024b).

To date, very few studies evaluating the efficiency of FMTs and IMTs on the gut microbial modulation have been carried out in fish. Concerning fish from the same species, Hu et al. (2022b) performed a FMT in adult zebrafish via supplementation of the diet with fecal content from young donors, but the gut microbial communities after the transplantation were not evaluated. On the other hand, Zhang et al. (2023) performed a FMT in large yellow croaker from 1-year old individuals to larvae by oral administration through the diet for 30 days. In this case, the authors compared the gut microbiota of fish after the FMT with their congeners that were not administered the FMT, but not with the microbiota from the donors. Zhang et (2023) observed a significant increase in the observed (number of OTUs) and estimated richness (ACE and Chao1 indices) with respect to their congeners. No differences in Shannon and Simpson diversity indices were found, but there was a significant increase in Faith phylogenetic diversity index. Regarding beta diversity, in terms of unweighted UniFrac distances, the structure of the control group and the fish that received the FMT was different, with the latter having a much higher dispersion. The FMT also had an effect in the composition of the gut microbiota of large yellow croaker, increasing the relative abundance of the classes Gammaproteobacteria, Bacilli, Actinobacteria and Nitrososphaeria, and decreasing the relative abundance of the classes Alphaproteobacteria and unidentified Planctomycetes (Zhang et al., 2023). Thus, it is evident that the FMT performed by these authors was able to induce a modulation in the richness, structure, and composition of the gut microbial communities in large yellow croaker. Nonetheless, considering that the microbiota of the donor fish was not studied, it is difficult to determine to what extent the richness, structure, and composition of the donor was maintained.

Interestingly, Smith et al. (2017) and Legrand et al. (2020b) assessed the effect of FMT taking into account the variable "time", to check if the microbial changes were maintained over time. In this sense, Legrand et al. (2020b) performed FMTs via oral gavage and through the water to yellowtail kingfish and compared the microbial profile of the inoculum from the donor fish and the gut microbiota of the receptors with the microbial communities of the individuals submitted to the transplant at 2, 8 and 15 days after the FMT. At 2 days post-FMT, these authors observed an increase in microbial richness (observed ASVs) and diversity (Shannon index) in 2 out of 4 fish submitted to the FMT via oral gavage, reaching values similar to those of the inoculum from the donor fish, while the other 2 transplanted fish showed values more similar to the recipient fish before the transplant. A few of the transplanted individuals, specifically 2 fish submitted to FMT via gavage and one submitted to FMT through water exposure, also showed a more similar beta diversity, based on Bray-Curtis distances, to the donor inoculum than to the recipient individuals. In addition, 17 out of the 79 ASVs found in the donor inoculum were observed in yellowtail kingfish at 2 days post-FMT, belonging mainly to the genera *Aliivibrio* and *Lactobacillus*. Nonetheless, at 8 and 15 days after the FMT, the microbial richness and diversity were reduced, beta diversity was similar to the recipient individuals, and the above-mentioned ASVs were not reported in the gut of transplanted fish, meaning that the microbial modulation induced by the FMT was not persistent over time (Legrand et al., 2020b). Additionally, Smith et al. (2017) performed an IMT through water exposure from young-age (6 weeks) African turquoise killifish (*Nothobranchius furzeri*) to middle-age (9.5 week) individuals. One week after the IMT, a similar richness (observed OTUs) to that of the donor and recipient fish was found, but the microbial structure (Bray-Curtis and unweighted Unifrac distances) was different among these groups of fish. At 7 weeks post-transplant, the richness of fish submitted to the FMT was lower than for the donor fish, but higher than for their congeners not submitted to FMT. In addition, at 7 weeks post-IMT the gut microbial communities were more enriched with members of the phyla Bacteroidota and Firmicutes and with several genera which were highly abundant in the young donors, showing that in this case, gut colonization by the donors' microbiota was a progressive process over time (Smith et al., 2017).

Inter-specific FMT and IMT have also been performed, between different animal species and zebrafish, or reciprocally. Focusing on inter-specific transplantations with fish as receptors, Rawls et al. (2006) transferred the cecal content of adult female mice to germ-free zebrafish larvae through water exposure. At 14 days post-transplantation, the authors observed a decrease in bacterial richness (Chao1 and ACE indices) and diversity (abundance of total sequences), and a separation in beta diversity based on weighted UniFrac distances with respect to the host before the IMT, clustering closer to the donor group. In addition, the phylum Firmicutes, which was highly abundant in the mice, colonized up to 65% of the gut microbial communities of zebrafish after the IMT. However, the majority of such Firmicutes belonged to the class Bacilli, which was more common in recipient zebrafish than in donor mice (Rawls et al., 2006). On the other hand, Valenzuela et al. (2018) performed a FMT from human to zebrafish larvae through exposure to an inoculum of the fecal sample. As a result, in terms of beta diversity (weighted UniFrac), the gut microbiota of zebrafish larvae at 2.5h after the transplant clustered together with that of recipient zebrafish before the FMT, but separately to the fecal sample. Indeed, only 6 of the 74 genera identified in the human sample

were transferred to the fish, corresponding to the genera *Bacillus*, *Roseburia*, *Oscillospira*, *Prevotella*, and two unassigned genera of the families Ruminococcaceae and Enterobacteriaceae (Valenzuela et al., 2018). These results were in line with the attempt of Toh et al. (2013) to perform an inoculation of bacteria isolated from a human fecal sample into zebrafish larvae through static immersion or microinjection. Larvae were sampled at 3, 5, and 7 days post-inoculation, but only the species *Lactobacillus paracasei* and *Eubacterium limosum*, out of the 22 species and 30 strains inoculated, were observed at 3 days post-inoculation. At 7 days, only *L. paracasei* was maintained when microinjection was used, but none of the species persisted when static immersion was used for inoculation (Toh et al., 2013).

To our knowledge, the IMT presented in this thesis is the first attempt of IMT between two different fish species with aquaculture interest (Chapter V; Ruiz et al., 2024b). As main results, at the first sampling time after the transplantation (2 days post-IMT), we found no differences in richness (ACE index), diversity (Shannon index) nor phylogenetic diversity (Faith index). This was likely because donor (Atlantic salmon) and receptor (gilthead seabream) groups displayed similar values for such indices. In addition, at 2 days post-IMT all gilthead seabream displayed a similar microbial structure (weighted UniFrac distances) regardless of the feeding, which was very different to the microbial structure of the diets. The predominant phyla of the fish submitted to the IMT were Proteobacteria and Firmicutes, maintaining similar abundances to the donor Atlantic salmon and receptor gilthead seabream before the IMT. At 2 days post-IMT we also observed many genera common to both donor and receptor fish, such as *Photobacterium*, *Vibrio*, and *Escherichia-Shigella*, while there were other genera exclusive from the donors, like *Aliivibrio*, or from the host, like *Catenococcus*.

In addition, except for Ruiz et al. (2024b; Chapter V), none of the above-mentioned trials considered the variable diet, which may be used as a complementary factor to attempt to prolongate the persistence of the gut microbial modulation induced by FMTs and IMTs, considering its ability to shape the fish gut microbiota (Silva et al., 2011; Ghanbari et al., 2015). In this sense, in our trial we observed that at the final sampling time (36 days post-IMT), the richness (ACE index) of gilthead seabream fed the GSB diet was similar to the richness of gilthead seabream before the IMT, while the richness of gilthead seabream fed the salmon diet was similar to that of Atlantic salmon. Additionally, the microbial structure, based on weighted UniFrac distances, of fish fed each diet was different, with the microbial structure of gilthead seabream fed the salmon diet being similar to this diet, while fish fed the GSB diet clustered together with the GSB diet in the PCoA, despite being significantly different. The diet also showed a high influence on the gut microbial composition. In this sense, high abundances of the phylum Firmicutes, and the genera *Lactobacillus* and *Ligilactobacillus*, which were the predominant taxa found in the GSB diet, were observed in the gut of gilthead seabream fed the GSB diet at 36 days post-IMT. On the other hand, the feeding of the salmon diet led to the development of a new unique microbiota profile, which showed a decrease in the phylum Proteobacteria, and an increase in Firmicutes, Actinobacteriota and Bacteroidota with respect to the fish at 2 days post-IMT. There was also an increase in the abundance of the genera *Escherichia-Shigella*, *Acinetobacter*, and *Cutibacterium*, and an emergence of new genera such as *Alloiococcus*, *Asinibacterium*, *Bacillus*, and *Turicella*, while *Photobacterium* and *Vibrio* decreased (Chapter V; Ruiz et al., 2024b).

Summarizing, even though *a priori* the IMT performed herein seemed to work and the diet also contributed to a gut microbial modulation over time, the microbial changes observed after the 2 days post-MT were not as expected. In this sense, there exist some factors affecting the efficiency of the IMT that may be improved in further studies with regard to the application of the experimental protocol described in Ruiz et al. (2024b; Chapter V). For instance, one of the main constraining factors in our trial was the change of temperature that the microbial species suffered after the IMT, considering that the temperature can shape the fish microbial diversity, structure, and composition (Soriano et al., 2018; Sepulveda and Moeller, 2020; Liu et al., 2022b). Under current conditions, the water temperature at which donor Atlantic salmons were reared was 12 ºC, while the water temperature of gilthead seabream tanks was 20 ºC. Thus, considering fish as ectothermic organisms, it can be expected a decrease in the relative abundance of psychrophilic microorganisms, which are those species with an optimal growth temperature of 15 ºC or lower, and with a maximal growth temperature of 20 ºC (Moyer and Morita, 2007). This may be the case for some *Aliivibrio* species, since many of them have an optimal growth rate within the range of 12-18 ºC and lose growth capacity at temperatures of approximately 20 ºC (Colquhoun et al., 2002; Khrulnova et al., 2011; Söderberg et al., 2019), and in our trial the relative abundance of this genus decreased to near zero after 2 days post-IMT (Chapter V; Ruiz et al., 2024b).

Another potential weakness of our study may be the inability to confirm that no further changes occurred after the final sampling time. In his sense, we are confident that the mixture of antimicrobials (AMs) employed to obliterate the host basal microbiota did not have an effect on the results of gilthead seabream at the 36 days post-IMT, since the values microbial richness (ACE index) and diversity (Shannon and Faith indices), and weighted UniFrac distances at 17 days post-AMs were already similar to those of gilthead seabream before the application of AMs. In addition, microbial composition was also very similar at 17 days post-AMs with respect to the one of fish pre-AMs, with the only exception of the phylum *Cyanobacteria* and the genera *Vibrio* and *Escherichia-Shigella* (Chapter V; Ruiz et al., 2024b). Nonetheless, considering the high number of underlying factors that may influence the gut microbial communities after the IMT, such as the microbial adaptation to the new environmental conditions or the resource competition among bacteria (Neuman et al., 2016; Scheuring et al., 2022), it is not possible to ensure that after 36 days no further changes in microbial communities would occur, especially in the case of the group that was submitted to a change of diet to the salmon diet after the IMT. In this sense, a study in Atlantic cod (*Gadus morhua*) reported that the microbial modulation induced by the dietary supplementation of egg wrack (*Ascophyllum nodosum*) extended over a long period, finding differences in microbial diversity, structure, and composition between the weeks 8 and 12 of the trial (Keating et al., 2021). Indeed, these authors observed a trend towards convergence of the gut microbial communities of all fish over time irrespective of their dietary treatment. In agreement with these results, in a previous trial we observed that the gut microbial modulation of gilthead seabream after a change of diet was extended up to the 60 days that the trial lasted, also showing a convergence towards an increasing core microbiota over time in all fish (Ruiz et al., 2024d). Such results may be in line with the existence of an increasing core microbiota that we found in gilthead seabream at 7 days post-IMT (8 days post-AMs) and at 16 days post-IMT (17 days post-AMs)

irrespective of the treatment in Ruiz et al. (2024b; Chapter V), and whose growth could potentially have continued over time if we had extended the trial for a longer period.

In addition to the aforementioned factors that can be improved when applying the proposed IMT protocol, there are also many other factors, not only specific to our protocol but also at a more generic level, that should be considered in the hypothetical scenario of IMTs being used as a strategy for productive purposes on an industrial scale. A significant flaw in many protocols of IMTs, including ours, is the use of large quantities of antibiotics to purge the existing microbiota. To reduce the application of antibiotics, this approach might be modified to include alternatives such as the use of anti-microbial peptides (Cheng et al., 2014), a stress-induced dysbiosis (Uren Webster et al., 2021), or an extended fasting period to deplete the existing host basal microbiota (Viver et al., 2023). It is also important to consider that another constraint of IMTs in fish is the high number of individuals managed in aquaculture (FAO, 2022), which economically and functionally hinders their massive application on an industrial scale.

In view of the above-mentioned information, nowadays IMTs may only be used in an experimental context, to test their effects on animal performance, as has been done in higher vertebrates, such as ruminants (Ribeiro et al., 2017), swine (Hu et al., 2018), and poultry (Siegerstetter et al., 2018), and to help elucidate the role on fish performance of the gut microbial communities which are modulated by the IMTs. In this sense, studies in African turquoise killifish, zebrafish, and large yellow croaker have shown that FMTs and IMTs can be used to improve fish performance and physiology, in terms of growth, digestive capacity, reproductive performance, intestinal health, gut microbial diversity, and life longevity, among other parameters (Hu et al., 2022b; Smith et al., 2017; Zhang et al., 2023). Likewise, the effect of the gut microbial changes induced by an IMT on the levels of fat accumulation in fish may be tested in future studies. In this sense, studies performing intra- (Turnbaugh et al., 2006) and inter-specific microbial transplants (Ridaura et al., 2013; Tremaroli et al., 2015; Yang et al., 2018) in mice have shown that FMTs and IMTs are able to modulate the levels of body fat accumulation in association with changes in the gut microbial composition. Similarly, studies in poultry have shown that FMTs can modulate the chicken body weight and up-regulate genes involved in fat metabolism (Zhang et al., 2022b). Thus, the application of IMTs in future studies in fish may lead to promising results in terms of an improved fish performance and/or regulation of fat accumulation. However, with the existing knowledge of today, the use of IMTs as a feasible strategy in the industrial sector still seems implausible.

Overall, the results of gut microbiota of the present thesis show that both tested strategies, the supplementation of aquafeeds with supplements based on bile salts or spices and the performance of an IMT followed by a dietary treatment, were able to modulate gut microbial composition of gilthead seabream. In addition, the IMT and posterior dietary treatment was able to modulate bacterial richness, diversity, and structure. However, even though the effect of the IMT was more pronounced, the supplementation of aquafeeds with the tested additives is currently closer to being used as a strategy to modulate the gut microbial communities, and fat accumulation levels, in the aquaculture sector.

CONCLUSIONS

Conclusions

1. The dietary supplementation of a blend of bile salts containing sodium cholate, sodium deoxycholate, and sodium taurocholate hydrate at an inclusion level of 0.06% promoted somatic growth and did not affect feed performance in gilthead seabream (*Sparus aurata*).

2. The administration of a combination of capsicum, black pepper, and ginger oleoresins, and cinnamaldehyde (SPICY additive) at inclusion levels of 0.1% and 0.15% in the diet improved somatic growth, whereas at 0.1% it also reduced feed conversion ratio values in gilthead seabream.

3. The inclusion of a combination of turmeric, capsicum, black pepper, and ginger oleoresins (SO additive) at 0.2% in the diet did not affect the growth and feed performances in gilthead seabream.

4. The tested blend of bile salts at a dietary inclusion level of 0.06%, the SPICY additive at 0.1% and the SO additive at 0.2% reduced the values of perivisceral fat in gilthead seabream without compromising the proximate composition and fatty acid profile of the fillet, whereas the SO additive at the dietary inclusion of 0.2% also reduced the lipid content in the liver.

5. The blend of bile salts at 0.12% in the diet, increased lipid apparent digestibility, in agreement with the higher activity of the bile salt-activate lipase and increased content of the bile salt taurodeoxycholic acid in the intestine, whereas the administration of a blend of bile salts at 0.06% and 0.12% reduced the accumulation of fat deposits in digestive organs like the liver and intestine.

6. The supplementation of the SPICY additive at 0.1% and 0.15%, and of the SO additive at 0.2% in the diet reduced the accumulation of fat deposits in the liver and the intestine, in line with the increased activity of the bile salt-activate lipase in the intestine.

7. Based on the results of gene expression from the liver, the SO additive at 0.2% in the diet promoted fatty acid oxidation in 48 h fasted-gilthead seabream.

8. Regarding the results of gene expression in the intestine, the blend of bile salts at 0.06% and the SO additive at 0.2% stimulated an immunocompetence priming at 2-h postprandial, whereas in fish fasted for 48 h the SPICY additive at 0.1% and SO additive at 0.2% induced an intestinal anti-inflammatory immune response, which may improve the intestinal health of the fish.

9. The supplementation of the tested blend of bile salts and combinations of spices in aquafeeds are a safe strategy to improve the health and condition in farmed fish as well as modulate body fat accumulation without affecting the nutritional quality of the fillet.

10. The dietary supplementation of the blend of bile salts at 0.06% decreased the bacterial richness (observed ASVs, Chao1 and ACE indices) in the anterior intestine of gilthead seabream without affecting the diversity (Shannon and Simpson diversity indices) or structure (Bray-Curtis distances), increased the abundance of genera containing members able to metabolize bile acids (*Bacteroides*, *Desulfovibrio*, and *Brevundimonas*) and decreased the abundance of genera which were positively correlated with the levels of perivisceral fat and negatively correlated with growth performance (*Porphyromonas*, *Campylobacter*, *Corynebacterium*, and *Peptoniphilus*).

11. The inclusion of the SPCIY additive at 0.1% in the diet increased the bacterial diversity (Simpson index) and modulated the composition of the bacterial communities in the posterior intestine, resulting in a decrease of some genera (*Campylobacter*, *Corynebacterium*, and *Peptoniphilus*) which were positively correlated with the levels of perivisceral fat and negatively correlated with growth performance, without compromising the gut bacterial structure (weighted UniFrac distances).

12. The administration of the SO additive at 0.2% in the diet modulated the bacterial composition at the level of genus in the anterior intestine, favouring an increase in the abundance of genera containing members able to metabolize bile acids (*Bacteroides* and *Desulfovibrio*), without affecting bacterial richness (ACE index), diversity (Shannon, Simpson and Faith phylogenetic diversity indices), and structure (unweighted UniFrac and weighted UniFrac distances).

13. Although some genera of bacteria were significantly correlated with the levels of perivisceral fat, a robust pattern in microbial diversity, structure and composition was not observed in the individuals with different levels of fat accumulation in the perivisceral cavity, liver, and intestine regardless of the tested additives administered.

14. After performing an intestinal microbiota transplant (IMT) from donor Atlantic salmon (*Salmo salar*) to recipient gilthead seabream, at 2 days post-IMT the values of gut bacterial richness (ACE index) and diversity (Shannon and Faith phylogenetic diversity) of gilthead seabream were similar to those of donor and recipient fish, and many bacterial phyla and genera from donor and recipient fish were also observed in gilthead seabream at 2 days post-IMT.

15. After the IMT the diet played a paramount role in defining the gut microbiota of gilthead seabream, modulating the bacterial richness (ACE index), diversity (Shannon and Faith phylogenetic diversity), structure (Bray-Curtis and weighted UniFrac distances), and composition in a distinct manner depending on the type of diet that was fed.

16. As a conceptual approach, the IMT is a feasible strategy to modulate the gut bacterial composition of the receptor fish, even though confirmation of the maintenance of the regulation induced by the IMT over the diet in long-term results can be improved with to use individuals reared at similar environmental conditions, or from the same species.

REFERENCES

References

Abd El-Hack, M. E., Alagawany, M., Shaheen, H., Samak, D., Othman, S. I., Allam, A. A., et al. (2020). Ginger and its derivatives as promising alternatives to antibiotics in poultry feed. Animals, 10, 452.

Abd El-Hamid, M. I., Ibrahim, S. M., Eldemery, F., El-Mandrawy, S. A., Metwally, A. S., Khalifa, E., et al. (2021). Dietary cinnamaldehyde nanoemulsion boosts growth and transcriptomes of antioxidant and immune related genes to fight *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*). Fish & Shellfish Immunology, 113, 96-105.

Abdel-Tawwab, M., and Abbass, F. E. (2017). Turmeric powder, *Curcuma longa* L., in common carp, *Cyprinus carpio* L., diets: growth performance, innate immunity, and challenge against pathogenic *Aeromonas hydrophila* infection. Journal of the World Aquaculture Society, 48, 303-312.

Adam, A. H., Verdegem, M., Soliman, A. A., Zaki, M., Khalil, R. H., Nour, A. E. M., et al. (2023). Effect of dietary bile acids: Growth performance, immune response, genes expression of fatty acid metabolism, intestinal, and liver morphology of striped catfish (*Pangasianodon hypophthalmus*). Aquaculture Reports, 29, 101510.

Adewale, A., Ifeoluwa, A. P., Olusoji, O. A., Bukunmi, A. A., and Simeon, O. O. (2021). Historical, botanical and medicinal perspectives on ginger (*Zingiber officinale*). Journal of Complementary and Alternative Medical Research, 15, 44-67.

Akram, M. (2014). Citric acid cycle and role of its intermediates in metabolism. Cell Biochemistry and Biophysics, 68, 475-478.

Al-Kassie, G. A., Al-Nasrawi, M. A., and Ajeena, S. J. (2011). Use of black pepper (*Piper nigrum*) as feed additive in broilers diet. Research Opinions in Animal & Veterinary Sciences, 1, 169-173.

Alagawany, M., Farag, M. R., Abdelnour, S. A., Dawood, M. A., Elnesr, S. S., and Dhama, K. (2021). Curcumin and its different forms: A review on fish nutrition. Aquaculture, 532, 736030.

Alagbe, J. O. (2017). Growth performance and blood parameters of weaner pigs fed diets supplemented with turmeric powder. Scholarly Journal of Agricultural Science, 7:57-61.

Alzawqari, M. H., Al-Baadani, H. H., Alhidary, I. B., Al-Owaimer, A. N., and Abudabos, A. M. (2016). Effect of taurine and bile acid supplementation and their interaction on performance, serum components, ileal viscosity and carcass characteristics of broiler chickens. South African Journal of Animal Science, 46, 448-457.

Amer, S. A., Metwally, A. E., and Ahmed, S. A. (2018). The influence of dietary supplementation of cinnamaldehyde and thymol on the growth performance, immunity and antioxidant status of monosex Nile tilapia fingerlings (*Oreochromis niloticus*). The Egyptian Journal of Aquatic Research, 44, 251-256.

APROMAR (2023). La Acuicultura en España 2023, Asociación de Empresas de Acuicultura (APROMAR).

Aqmasjed, S. B., Sajjadi, M. M., Falahatkar, B., and Safari, R. (2023). Effects of dietary ginger (*Zingiber officinale*) extract and curcumin on growth, hematology, immunity, and antioxidant status in rainbow trout (*Oncorhynchus mykiss*). Aquaculture Reports, 32, 101714.

Arshad, M. A., Bhatti, S. A., Rehman, M. S. U., Yousaf, W., Younus, G., Sizmaz, O., et al. (2021). Supplementation of bile acids and lipase in broiler diets for better nutrient utilization and performance: Potential effects and future implications-A review. Annals of Animal Science, 21, 757-787.

Ashfaq, H., Soliman, H., Saleh, M., and El-Matbouli, M. (2019). CD4: a vital player in the teleost fish immune system. Veterinary Research, 50, 1.

Ashry, A. M., Habiba, M. M., El-Zayat, A. M., Badreldeen, A. H., Younis, N. A., Ahmed, H. A., et al. (2023). Effects of ginger (*Zingiber officinale*) on the growth performance, digestive enzyme activity, antioxidative response, and antibacterial capacity of striped catfish (*Pangasianodon hypophthalmus*) reared in outdoor conditions. Aquaculture Reports, 33, 101760.

Ashry, A. M., Hassan, A. M., Habiba, M. M., El-Zayat, A., El-Sharnouby, M. E., Sewilam, H., et al. (2021). The impact of dietary curcumin on the growth performance, intestinal antibacterial capacity, and haemato-biochemical parameters of gilthead seabream (*Sparus aurata*). Animals, 11, 1779.

Astudillo-García, C., Bell, J. J., Webster, N. S., Glasl, B., Jompa, J., Montoya, J. M., et al. (2017). Evaluating the core microbiota in complex communities: a systematic investigation. Environmental Microbiology, 19, 1450-1462.

Basurco, B., Lovatelli, A., and García, B. (2011). Current status of Sparidae aquaculture. In: Pavlidis, M. A., and Mylonas, C. C. (Eds.), Sparidae: Biology and Aquaculture of Gilthead Sea Bream and Other Species (pp. 1–50), Blackwell Publishing Ltd., West Sussex, UK.

Bazarsadueva, S. V., Radnaeva, L. D., Shiretorova, V. G., and Dylenova, E. P. (2021). The comparison of fatty acid composition and lipid quality indices of roach, perch, and pike of Lake Gusinoe (Western Transbaikalia). International Journal of Environmental Research and Public Health, 18, 9032.

Benedito-Palos, L., Navarro, J. C., Kaushik, S., and Pérez-Sánchez, J. (2010). Tissue-specific robustness of fatty acid signatures in cultured gilthead sea bream (*Sparus aurata* L.) fed practical diets with a combined high replacement of fish meal and fish oil. Journal of Animal Science, 88, 1759-1770.

Bereded, N. K., Curto, M., Domig, K. J., Abebe, G. B., Fanta, S. W., Waidbacher, H., et al. (2020). Metabarcoding analyses of gut microbiota of Nile tilapia (*Oreochromis niloticus*) from Lake Awassa and Lake Chamo, Ethiopia. Microorganisms, 8, 1040.

Bermúdez, M. A., Pereira, L., Fraile, C., Valerio, L., Balboa, M. A., and Balsinde, J. (2022). Roles of palmitoleic acid and its positional isomers, hypogeic and sapienic acids, in inflammation, metabolic diseases and cancer. Cells, 11, 2146.

Bhusare, S., Sardar, P., Sahu, N. P., Shamna, N., Kumar, P., Paul, M., et al. (2023). Bile acid improves growth, lipid utilization and antioxidative status of genetically improved farmed tilapia (*Oreochromis niloticus*) fed with varying protein-lipid diets reared in inland saline water. Animal Feed Science and Technology, 303, 115677.

Bird, S., and Tafalla, C. (2015). Teleost chemokines and their receptors. Biology, 4, 756-784.

Boyd, C. E., D'Abramo, L. R., Glencross, B. D., Huyben, D. C., Juarez, L. M., Lockwood, G. S., et al. (2020). Achieving sustainable aquaculture: Historical and current perspectives and future needs and challenges. Journal of the World Aquaculture Society, 51, 578-633.

Breck, J. E. (2014). Body composition in fishes: body size matters. Aquaculture, 433, 40-49.

Brown, K., Church, D., Lynch, T., and Gregson, D. (2014). Bloodstream infections due to *Peptoniphilus* spp.: report of 15 cases. Clinical Microbiology and Infection, 20, O857-O860.

Brown, P. B., and S. D. Hart. (2011). Soybean oil and other n-6 polyunsaturated fatty acid-rich vegetable oils. In: Turchini, G. M., Ng, W. K., and Tocher, D. R. (Eds.), Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds (pp. 133–160), CRC Press, Boca Raton, USA.

Brugman, S., Ikeda-Ohtsubo, W., Braber, S., Folkerts, G., Pieterse, C. M., and Bakker, P. A. (2018). A comparative review on microbiota manipulation: lessons from fish, plants, livestock, and human research. Frontiers in Nutrition, 5, 80.

Brum, A., Pereira, S. A., Owatari, M. S., Chagas, E. C., Chaves, F. C. M., Mouriño, J. L. P., et al. (2017). Effect of dietary essential oils of clove basil and ginger on Nile tilapia (*Oreochromis niloticus*) following challenge with *Streptococcus agalactiae*. Aquaculture, 468, 235-243.

Bruslé, J., and Gonzàlez i Anadon, G. (1996). The structure and function of fish liver. In: Munshi, J.S.D., and Dutta, H.M. (Eds.), Fish Morphology (pp. 77–93), Science Publishers Inc., New Hampshire, USA.

Buchinger, T. J., Li, W., and Johnson, N. S. (2014). Bile salts as semiochemicals in fish. Chemical Senses, 39, 647-654.

Caballero, M. J., Obach, A., Rosenlund, G., Montero, D., Gisvold, M., and Izquierdo, M. S. (2002). Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*. Aquaculture, 214, 253-271.

Campos, I., Matos, E., Maia, M. R., Marques, A., and Valente, L. M. (2019a). Partial and total replacement of fish oil by poultry fat in diets for European seabass (*Dicentrarchus labrax*) juveniles: Effects on nutrient utilization, growth performance, tissue composition and lipid metabolism. Aquaculture, 502, 107-120.

Campos, I., Matos, E., and Valente, L. M. (2019b). Ability of European seabass (*Dicentrarchus labrax*) to digest rendered animal fats from fish, poultry and mammals. Aquaculture Nutrition, 25, 729-736.

Campoy, C., Escolano-Margarit, M. V., Anjos, T., Szajewska, H., and Uauy, R. (2012). Omega 3 fatty acids on child growth, visual acuity and neurodevelopment. British Journal of Nutrition, 107, S85-S106.

Canfora, E. E., Jocken, J. W., and Blaak, E. E. (2015). Short-chain fatty acids in control of body weight and insulin sensitivity. Nature Reviews Endocrinology, 11, 577-591.

Cao, A. Z., Lai, W. Q., Zhang, W. W., Dong, B., Lou, Q. Q., Han, M. M., et al. (2021). Effects of porcine bile acids on growth performance, antioxidant capacity, blood metabolites and nutrient digestibility of weaned pigs. Animal Feed Science and Technology, 276, 114931.

Carey, M. C., and Small, D. M. (1970). The characteristics of mixed micellar solutions with particular reference to bile. The American Journal of Medicine, 49, 590-608.

Carr, I., Glencross, B., and Santigosa, E. (2023). The importance of essential fatty acids and their ratios in aquafeeds to enhance salmonid production, welfare, and human health. Frontiers in Animal Science, 4, 1147081.

Carvalho, M., Montero, D., Torrecillas, S., Castro, P., Zamorano, M. J., and Izquierdo, M. (2021). Hepatic biochemical, morphological and molecular effects of feeding microalgae and poultry oils to gilthead sea bream (*Sparus aurata*). Aquaculture, 532, 736073.

Cattaneo, V., Tribulatti, M. V., Carabelli, J., Carestia, A., Schattner, M., and Campetella, O. (2014). Galectin-8 elicits pro-inflammatory activities in the endothelium. Glycobiology, 24, 966-973.

Cerezo-Ortega, I. M., Di Zeo-Sánchez, D. E., García-Márquez, J., Ruiz-Jarabo, I., Sáez-Casado, M. I., Balebona, M. C., et al. (2021). Microbiota composition and intestinal integrity remain unaltered after the inclusion of hydrolysed *Nannochloropsis gaditana* in *Sparus aurata* diet. Scientific Reports, 11, 18779.

Chattopadhyay, I., Gundamaraju, R., Jha, N. K., Gupta, P. K., Dey, A., Mandal, C. C., et al. (2022). Interplay between dysbiosis of gut microbiome, lipid metabolism, and tumorigenesis: can gut dysbiosis stand as a prognostic marker in cancer?. Disease Markers, 2022, 2941248.

Chen, J., and Liu, H. (2020). Nutritional indices for assessing fatty acids: A mini-review. International Journal of Molecular Sciences. 21, 5695.

Chen, S. X., Ma, H. L., Shi, Y. H., Li, M. Y., and Chen, J. (2016). Molecular and functional characterization of a novel CD302 gene from ayu (*Plecoglossus altivelis*). Fish & Shellfish Immunology, 55, 140-148.

Chen, W., Gao, S., Sun, P., Sun, X., Wei, Y., Chen, J., et al. (2022). Dietary sodium butyrate supplementation alleviates high-fat diet-induced liver injury by activating Nrf2 in common carp (*Cyprinus carpio*). Pakistan Journal of Zoology, 55, 2085-2094.

Chen, Y., Yuan, C., Yang, T., Song, H., Zhan, K., and Zhao, G. (2024). Effects of bile acid supplementation on lactation performance, nutrient intake, antioxidative status, and serum biochemistry in mid-lactation dairy cows. Animals, 14, 290.

Cheng, G., Hao, H., Xie, S., Wang, X., Dai, M., Huang, L., et al. (2014). Antibiotic alternatives: the substitution of antibiotics in animal husbandry?. Frontiers in Microbiology, 5, 217.

Chiang, J. Y., and Ferrell, J. M. (2022). Discovery of farnesoid X receptor and its role in bile acid metabolism. Molecular and Cellular Endocrinology, 548, 111618.

Chiang, J. Y., and Ferrell, J. M. (2020). Up to date on cholesterol 7 alpha-hydroxylase (CYP7A1) in bile acid synthesis. Liver Research, 4, 47-63.

Chopra, R. N., and Nayar, S. L. (1956). Glossary of Indian medicinal plants. Council of Scientific And Industrial Research, New Delhi, India.

Chowdhury, D. K., Sahu, N. P., Sardar, P., Deo, A. D., Bedekar, M. K., Singha, K. P., et al. (2021). Feeding turmeric in combination with ginger or garlic enhances the digestive enzyme activities, growth and immunity in *Labeo rohita* fingerlings. Animal Feed Science and Technology, 277, 114964.

Christaki, E., Giannenas, I., Bonos, E., and Florou-Paneri, P. (2020). Innovative uses of aromatic plants as natural supplements in nutrition. In: Florou-Paneri, P., Christaki, E., and Giannenas, I. (Eds.), Feed Additives. Aromatic Plants and Herbs in Animal Nutrition and Health (pp. 19-34), Elsevier Academic Press, London, UK.

Coccia, E., Varricchio, E., Vito, P., Turchini, G.M., Francis, D.S., and Paolucci, M. (2014). Fatty acid-specific alterations in leptin, PPARα, and CPT-1 gene expression in the rainbow trout. Lipids, 49, 1033-1046.

Colquhoun, D. J., Alvheim, K., Dommarsnes, K., Syvertsen, C., and Sørum, H. (2002). Relevance of incubation temperature for *Vibrio salmonicida* vaccine production. Journal of Applied Microbiology, 92, 1087-1096.

Dalby, M. J. (2023). Questioning the foundations of the gut microbiota and obesity. Philosophical Transactions of the Royal Society B, 378, 20220221.

Dawood, M. A. (2021). Nutritional immunity of fish intestines: Important insights for sustainable aquaculture. Reviews in Aquaculture, 13, 642-663.

Deleu, S., Machiels, K., Raes, J., Verbeke, K., and Vermeire, S. (2021). Short chain fatty acids and its producing organisms: An overlooked therapy for IBD?. EBioMedicine, 66: 103293.

DePeters, E. J., and George, L. W. (2014). Rumen transfaunation. Immunology Letters, 162, 69-76.

Dhanasiri, A. K., Brunvold, L., Brinchmann, M. F., Korsnes, K., Bergh, Ø., and Kiron, V. (2011). Changes in the intestinal microbiota of wild Atlantic cod *Gadus morhua* L. upon captive rearing. Microbial Ecology, 61, 20-30.

Diab, A. M., Saker, O., Eldakroury, M., and Elseify, M. (2014). Effects of garlic (*Allium sativum*) and curcumin (Turmeric, *Curcuma longa* Linn) on Nile tilapia immunity. Veterinary Medical Journal-Giza, 60, C1-C19.

Ding, T., Xu, N., Liu, Y., Du, J., Xiang, X., Xu, D., et al. (2020). Effect of dietary bile acid (BA) on the growth performance, body composition, antioxidant responses and expression of lipid metabolism-related genes of juvenile large yellow croaker (*Larimichthys crocea*) fed high-lipid diets. Aquaculture, 518, 734768.

EFPRA (2021). The facts about aquafeed. Available at: https://efpra.eu/wp-content/uploads/2021/07/The-facts-about-aguafeed.pdf (accessed 30 April 2024).

Egerton, S., Culloty, S., Whooley, J., Stanton, C., and Ross, R. P. (2018). The gut microbiota of marine fish. Frontiers in Microbiology, 9, 873.

El-Houseiny, W., Khalil, A. A., Abd-Elhakim, Y. M., and Badr, H. A. (2019). The potential role of turmeric and black pepper powder diet supplements in reversing cadmium-induced growth retardation, ATP depletion, hepatorenal damage, and testicular toxicity in *Clarias gariepinus*. Aquaculture, 510, 109-121.

Ellis, J. L., Bove, K. E., Schuetz, E. G., Leino, D., Valencia, C. A., Schuetz, J. D., et al. (2018). Zebrafish *abcb11b* mutant reveals strategies to restore bile excretion impaired by bile salt export pump deficiency. Hepatology, 67, 1531-1545.

Elwan, H., Abdelhakeam, M., El-Shafei, S., El-Rahman, A. A., Ismail, Z., Zanouny, A., et al. (2020). Efficacy of dietary supplementation with *Capsicum annum* L on performance, hematology, blood biochemistry and hepatic antioxidant status of growing rabbits. Animals, 10, 2045.

Emery, J.A., Norambuena, F., Trushenski, J., and Turchini, G.M. (2016). Uncoupling EPA and DHA in fish nutrition: dietary demand is limited in Atlantic salmon and effectively met by DHA alone. Lipids, 51, 399-412.

Estensoro, I., Ballester-Lozano, G., Benedito-Palos, L., Grammes, F., Martos-Sitcha, J. A., Mydland, L. T., et al. (2016). Dietary butyrate helps to restore the intestinal status of a marine teleost (*Sparus aurata*) fed extreme diets low in fish meal and fish oil. PLoS One, 11, e0166564.

Estruch, G., Collado, M. C., Peñaranda, D. S., Tomás Vidal, A., Jover Cerdá, M., Pérez Martínez, G., et al. (2015). Impact of fishmeal replacement in diets for gilthead sea bream (*Sparus aurata*) on the gastrointestinal microbiota determined by pyrosequencing the 16S rRNA gene. PloS One, 10, e0136389.

Faikoh, E. N., Hong, Y. H., and Hu, S. Y. (2014). Liposome-encapsulated cinnamaldehyde enhances zebrafish (*Danio rerio*) immunity and survival when challenged with *Vibrio vulnificus* and *Streptococcus agalactiae*. Fish & Shellfish immunology, 38, 15-24.

Fan, L., Chen, J., Meng, S., Song, C., Qiu, L., Hu, G., et al. (2017). Characterization of microbial communities in intensive GIFT tilapia (*Oreochromis niloticus*) pond systems during the peak period of breeding. Aquaculture Research, 48, 459-472.

FAO (2022). The State of World Fisheries and Aquaculture 2022. Towards Blue Transformation. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

FAO (2024). Cultured aquatic species information programme: *Sparus aurata*. In: Colloca, F., and Cerasi, S. (Eds.), Cultured Aquatic Species Information Programme, Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

Fazelan, Z., Vatnikov, Y. A., Kulikov, E. V., Plushikov, V. G., and Yousefi, M. (2020). Effects of dietary ginger (*Zingiber officinale*) administration on growth performance and stress, immunological, and antioxidant responses of common carp (*Cyprinus carpio*) reared under high stocking density. Aquaculture, 518, 734833.

Feng, D., Huang, Q. Y., Liu, K., Zhang, S. C., and Liu, Z. H. (2014). Comparative studies of zebrafish *Danio rerio* lipoprotein lipase (*lpl*) and hepatic lipase (*lipc*) genes belonging to the lipase gene family: evolution and expression pattern. Journal of Fish Biology, 85, 329-342.

Ferreira, M., Costa, J., and Reis-Henriques, M. A. (2014). ABC transporters in fish species: a review. Frontiers in Physiology, 5, 266.

Fiorella, K. J., Okronipa, H., Baker, K., and Heilpern, S. (2021). Contemporary aquaculture: implications for human nutrition. Current Opinion in Biotechnology, 70, 83-90.

Fiorucci, S., Carino, A., Baldoni, M., Santucci, L., Costanzi, E., Graziosi, L., et al. (2021). Bile acid signaling in inflammatory bowel diseases. Digestive Diseases and Sciences, 66, 674-693.

Firmino, J. P., Galindo-Villegas, J., Reyes-López, F. E., and Gisbert, E. (2021a). Phytogenic bioactive compounds shape fish mucosal immunity. Frontiers in Immunology, 12, 695973.

Firmino, J. P., Vallejos-Vidal, E., Balebona, M. C., Ramayo-Caldas, Y., Cerezo, I. M., Salomón, R., et al. (2021b). Diet, immunity, and microbiota interactions: an integrative analysis of the intestine transcriptional response and microbiota modulation in gilthead seabream (*Sparus aurata*) fed an essential oils-based functional diet. Frontiers in Immunology, 12, 625297.

Firmino, J. P., Fernández-Alacid, L., Vallejos-Vidal, E., Salomón, R., Sanahuja, I., Tort, L., et al. (2021c). Carvacrol, thymol, and garlic essential oil promote skin innate immunity in gilthead seabream (*Sparus aurata*) through the multifactorial modulation of the secretory pathway and enhancement of mucus protective capacity. Frontiers in Immunology, 12, 633621.

Firouzbakhsh, F., Hghparast, S., and Memarzadeh, M. R. (2019). Effects of chili pepper extract (*Caspicum annum*) on immune responses, hematology and growth of rainbow trout (*Oncorhynchus mykiss*). Journal of Aquatic Ecology, 9, 9-20.

Flachs, P., Rossmeisl, M., Bryhn, M., and Kopecky, J. (2009). Cellular and molecular effects of n− 3 polyunsaturated fatty acids on adipose tissue biology and metabolism. Clinical Science, 116, 1-16.

Fonseca-Madrigal, J., Karalazos, V., Campbell, P. J., Bell, J. G., and Tocher, D. R. (2005). Influence of dietary palm oil on growth, tissue fatty acid compositions, and fatty acid metabolism in liver and intestine in rainbow trout (*Oncorhynchus mykiss*). Aquaculture Nutrition, 11, 241-250.

Forsythe, P. (2013). Gut microbes as modulators of the neuro-immuno-endocrine system. PharmaNutrition, 1, 115-122.

Fountoulaki, E., Vasilaki, A., Hurtado, R., Grigorakis, K., Karacostas, I., Nengas, I., et al. (2009). Fish oil substitution by vegetable oils in commercial diets for gilthead sea bream (*Sparus aurata* L.); effects on growth performance, flesh quality and fillet fatty acid profile: Recovery of fatty acid profiles by a fish oil finishing diet under fluctuating water temperatures. Aquaculture, 289, 317-326.

Francis, D. S., Turchini, G. M., Jones, P. L., and De Silva, S. S. (2007). Effects of fish oil substitution with a mix blend vegetable oil on nutrient digestibility in Murray cod, *Maccullochella peelii peelii*. Aquaculture, 269, 447-455.

Frisch, K., and Alstrup, A. K. O. (2018). On the evolution of bile salts and the farnesoid X receptor in vertebrates. Physiological and Biochemical Zoology, 91, 797-813.

Fuentes-Quesada, J. P., Viana, M. T., Rombenso, A. N., Guerrero-Rentería, Y., Nomura-Solís, M., Gomez-Calle, V., et al. (2018). Enteritis induction by soybean meal in *Totoaba macdonaldi* diets: Effects on growth performance, digestive capacity, immune response and distal intestine integrity. Aquaculture, 495, 78-89.

Furmanski, S., and Mor, T. (2017). First case report of fecal microbiota transplantation in a cat in Israel. Israel Journal of Veterinary Medicine, 72, 35-41.

Fusco, W., Lorenzo, M. B., Cintoni, M., Porcari, S., Rinninella, E., Kaitsas, F., et al. (2023). Short-chain fatty-acid-producing bacteria: key components of the human gut microbiota. Nutrients, 15, 2211.

Gadaleta, R. M., Van Erpecum, K. J., Oldenburg, B., Willemsen, E. C., Renooij, W., Murzilli, S., et al. (2011). Farnesoid X receptor activation inhibits inflammation and preserves the intestinal barrier in inflammatory bowel disease. Gut, 60, 463-472.

Galanakis, C. M. (2024). The future of food. Foods, 13, 506.

Gao, Y., Yao, Y., Huang, J., Sun, Y., Wu, Q., Guo, D., et al. (2023). Effect of dietary bile acids supplementation on growth performance, feed utilization, intestinal digestive enzyme activity and fatty acid transporters gene expression in juvenile leopard coral grouper (*Plectropomus leopardus*). Frontiers in Marine Science, 10, 1171344.

García-Escobar, E., Soriguer, F., García-Serrano, S., Gómez-Zumaquero, J. M., Morcillo, S., Haro, J., et al. (2008). Dietary oleic acid and adipocyte lipolytic activity in culture. The Journal of Nutritional Biochemistry, 19, 727-731.

Gazi, U., and Martinez-Pomares, L. (2009). Influence of the mannose receptor in host immune responses. Immunobiology, 214, 554-561.

Ge, X. K., Wang, A. A., Ying, Z. X., Zhang, L. G., Su, W. P., Cheng, K., et al. (2019). Effects of diets with different energy and bile acids levels on growth performance and lipid metabolism in broilers. Poultry science, 98, 887-895.

Geng, S., Zhang, Y., Cao, A., Liu, Y., Di, Y., Li, J., et al. (2022). Effects of fat type and exogenous bile acids on growth performance, nutrient digestibility, lipid metabolism and breast muscle fatty acid composition in broiler chickens. Animals, 12, 1258.

Ghanbari, M., Kneifel, W., and Domig, K. J. (2015). A new view of the fish gut microbiome: advances from next-generation sequencing. Aquaculture, 448, 464-475.

Gibbings, D., and Befus, A. D. (2009). CD4 and CD8: an inside-out coreceptor model for innate immune cells. Journal of Leukocyte Biology, 86, 251-259.

Giri, S. S., Kim, S. G., Saha, S., Kim, H. J., Jun, J. W., Chi, C., et al. (2023). Impact of dietary piperine on growth performance, immune response, antioxidant status, and immune-related gene expression in pathogen-aggravated *Cyprinus carpio*. Fish & Shellfish Immunology, 141, 109081.

Gisbert, E., Andree, K. B., Quintela, J. C., Calduch-Giner, J. A., Ipharraguerre, I. R., and Pérez-Sánchez, J. (2017). Olive oil bioactive compounds increase body weight, and improve gut health and integrity in gilthead sea bream (*Sparus aurata*). British Journal of Nutrition, 117, 351-363.

Gisbert, E., Ortiz-Delgado, J. B., and Sarasquete, C. (2008). Nutritional cellular biomarkers in early life stages of fish. Histology and histopathology, 23, 1525-1539.

González-Félix, M. L., Santana-Bejarano, E. B., Perez-Velazquez, M., and Villalba-Villalba, A. G. (2018). Partial characterization, quantification and activity of pancreatic lipase in the gastrointestinal tract of *Totoaba macdonaldi*. Archives of Biological Sciences, 70, 489-496.

Grant, K. L., and Lutz, R. B. (2000). Alternative therapies: ginger. American Journal of Health-System Pharmacy, 57, 945-947.

Grayfer, L., Hodgkinson, J. W., Hitchen, S. J., and Belosevic, M. (2011). Characterization and functional analysis of goldfish (*Carassius auratus* L.) interleukin-10. Molecular Immunology, 48, 563-571.

Grigorakis, K. (2007). Compositional and organoleptic quality of farmed and wild gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) and factors affecting it: A review. Aquaculture, 272, 55-75.

Grigorakis, K., and Alexis, M. N. (2005). Effects of fasting on the meat quality and fat deposition of commercial-size farmed gilthead sea bream (*Sparus aurata*, L.) fed different dietary regimes. Aquaculture Nutrition, 11, 341-344.

Grigorakis, K., Taylor, K. D. A., and Alexis, M. N. (2003). Organoleptic and volatile aroma compounds comparison of wild and cultured gilthead sea bream (*Sparus aurata*): sensory differences and possible chemical basis. Aquaculture, 225, 109-119.

Gu, M., Bai, N., and Kortner, T. M. (2017). Taurocholate supplementation attenuates the changes in growth performance, feed utilization, lipid digestion, liver abnormality and sterol metabolism in turbot (*Scophthalmus maximus*) fed high level of plant protein. Aquaculture, 468, 597-604.

Gu, M., Bai, N., Zhang, Y., and Krogdahl, Å. (2016). Soybean meal induces enteritis in turbot *Scophthalmus maximus* at high supplementation levels. Aquaculture, 464, 286-295.

Gu, M., Kortner, T. M., Penn, M., Hansen, A. K., & Krogdahl, Å. (2014). Effects of dietary plant meal and soya-saponin supplementation on intestinal and hepatic lipid droplet accumulation and lipoprotein and sterol metabolism in Atlantic salmon (*Salmo salar* L.). British Journal of Nutrition, 111, 432-444.

Gu, Y., Han, J., Wang, W., Zhan, Y., Wang, H., Hua, W., et al. (2022). Dietary cinnamaldehyde enhances growth performance, digestion, immunity, and lipid metabolism in juvenile fat greenling (*Hexagrammos otakii*). Aquaculture Nutrition, 2022.

Guldiken, B., Ozkan, G., Catalkaya, G., Ceylan, F. D., Yalcinkaya, I. E., and Capanoglu, E. (2018). Phytochemicals of herbs and spices: Health versus toxicological effects. Food and Chemical Toxicology, 119, 37-49.

Gunstone, F.D., 2011. The world's oils and fats. In: Turchini, G. M., Ng, W. K., and Tocher, D. R. (Eds.), Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds (pp. 61–98), CRC Press, Boca Raton, USA.

Gupta, A., Naraniwal, M., and Kothari, V. (2012). Modern extraction methods for preparation of bioactive plant extracts. International Journal of Applied and Natural Sciences, 1, 8-26.

Gupta, R., Gupta, N., and Rathi, P. J. A. M. (2004). Bacterial lipases: an overview of production, purification and biochemical properties. Applied Microbiology and Biotechnology, 64, 763-781.

Gupta, S., Allen-Vercoe, E., and Petrof, E. O. (2016). Fecal microbiota transplantation: in perspective. Therapeutic Advances in Gastroenterology, 9, 229-239.

Hagey, L. R., Møller, P. R., Hofmann, A. F., and Krasowski, M. D. (2010). Diversity of bile salts in fish and amphibians: evolution of a complex biochemical pathway. Physiological and Biochemical Zoology, 83, 308-321.

Han, L. K., Morimoto, C., Zheng, Y. N., Li, W., Asami, E., Okuda, H., et al. (2008). Effects of zingerone on fat storage in ovariectomized rats. Yakugaku zasshi: Journal of the Pharmaceutical Society of Japan, 128, 1195-1201.

Hao, Y. T., Wu, S. G., Xiong, F., Tran, N. T., Jakovlić, I., Zou, H., et al. (2017). Succession and fermentation products of grass carp (*Ctenopharyngodon idellus*) hindgut microbiota in response to an extreme dietary shift. Frontiers in Microbiology, 8, 1585.

Hardy, R. W., 2012. The nutritional pathology of teleosts. In: Roberts, R.J. (Ed.), Fish Pathology (pp. 421–422), Wiley-Blackwell, Chichester, UK.

Hasan, N., and Yang, H. (2019). Factors affecting the composition of the gut microbiota, and its modulation. PeerJ, 7, e7502.

Hattori, J. F. D. A., Alves, D. R. S., Oliveira, S. R. D., Almeida, A. A. D. S., and Boscolo, W. R. (2021). Attractiveness and palatability of liquid hydrolysates for Dourado (*Salminus brasiliensis*) fingerlings. Aquaculture Research, 52, 5682-5690.

Heath, H., Qin, S., Rao, P., Wu, L., LaRosa, G., Kassam, N., et al. (1997). Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. The Journal of Clinical Investigation, 99, 178-184.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., et al. (2000). A Toll-like receptor recognizes bacterial DNA. Nature, 408, 740-745.

Henderson, R. J. (1996). Fatty acid metabolism in freshwater fish with particular reference to polyunsaturated fatty acids. Archives of Animal Nutrition, 49, 5-22.

Herrero-Encinas, J., Huerta, A., Blanch, M., Pastor, J. J., Morais, S., and Menoyo, D. (2023). Impact of dietary supplementation of spice extracts on growth performance, nutrient digestibility and antioxidant response in broiler chickens. Animals, 13, 250.

Herve, T., Raphaël, K. J., Ferdinand, N., Victor Herman, N., Willy Marvel, N. M., Cyril D'Alex, T., et al. (2019). Effects of ginger (*Zingiber officinale*, Roscoe) essential oil on growth and laying performances, serum metabolites, and egg yolk antioxidant and cholesterol status in laying Japanese quail. Journal of Veterinary Medicine, 2019, 7857504.

Hieshima, K., Imai, T., Opdenakker, G., Van Damme, J., Kusuda, J., Tei, H., et al. (1997). Molecular cloning of a novel human CC chemokine liver and activation-regulated chemokine (LARC) expressed in liver: chemotactic activity for lymphocytes and gene localization on chromosome 2. Journal of Biological Chemistry, 272, 5846-5853.

Hirano, S., and Masuda, N. (1982). Enhancement of the 7 alpha-dehydroxylase activity of a gram-positive intestinal anaerobe by *Bacteroides* and its significance in the 7-dehydroxylation of ursodeoxycholic acid. Journal of Lipid Research, 23, 1152-1158.

Hofmann, A. F. (1999). The continuing importance of bile acids in liver and intestinal disease. Archives of Internal Medicine, 159, 2647-2658.

Hofmann, A. F., Hagey, L. R., and Krasowski, M. D. (2010). Bile salts of vertebrates: Structural variation and possible evolutionary significance. Journal of Lipid Research, 51, 226-246.

Holen, E., Araujo, P., Sissener, N. H., Rosenlund, G., and Waagbø, R. (2018). A comparative study: Difference in omega-6/omega-3 balance and saturated fat in diets for Atlantic salmon (*Salmo salar*) affect immune-, fat metabolism-, oxidative and apoptotic-gene expression, and eicosanoid secretion in head kidney leukocytes. Fish & Shellfish Immunology, 72, 57-68.

Horrocks, L. A., and Yeo, Y. K. (1999). Health benefits of docosahexaenoic acid (DHA). Pharmacological research, 40, 211-225.

Hoseinifar, S. H., Dadar, M., and Ringø, E. (2017). Modulation of nutrient digestibility and digestive enzyme activities in aquatic animals: the functional feed additives scenario. Aquaculture Research, 48, 3987-4000.

Houston, S. J., Karalazos, V., Tinsley, J., Betancor, M. B., Martin, S. A., Tocher, D. R., et al. (2017). The compositional and metabolic responses of gilthead seabream (*Sparus aurata*) to a gradient of dietary fish oil and associated n-3 long-chain PUFA content. British Journal of Nutrition, 118, 1010-1022.

Hsieh, R. J., and Kinsella, J. E. (1989). Oxidation of polyunsaturated fatty acids: mechanisms, products, and inhibition with emphasis on fish. Advances in Food and Nutrition Research, 33, 233-341.

Hu, D., Hou, M., Song, P., Chen, Q., Feng, Y., Wu, X., et al. (2024). Dietary bile acids supplementation improves the growth performance and alleviates fatty liver in broilers fed a high-fat diet via improving the gut microbiota. Poultry Science, 103, 103270.

Hu, D., Zhao, J., Zhang, H., Wang, G., and Gu, Z. (2023). Fecal microbiota transplantation for weight and glycemic control of obesity as well as the associated metabolic diseases: meta-analysis and comprehensive assessment. Life, 13, 1488.

Hu, H., Shao, W., Liu, Q., Liu, N., Wang, Q., Xu, J., et al. (2022a). Gut microbiota promotes cholesterol gallstone formation by modulating bile acid composition and biliary cholesterol secretion. Nature Communications, 13, 252.

Hu, C., Liu, M., Sun, B., Tang, L., Zhou, X., and Chen, L. (2022b). Young fecal transplantation mitigates the toxicity of perfluorobutanesulfonate and potently refreshes the reproductive endocrine system in aged recipients. Environment International, 167, 107418.

Hu, L., Geng, S., Li, Y., Cheng, S., Fu, X., Yue, X., et al. (2018). Exogenous fecal microbiota transplantation from local adult pigs to crossbred newborn piglets. Frontiers in Microbiology, 8, 2663.

Huang, H., Zhang, Y., Cao, M., Xue, L., and Shen, W. (2018). Effects of fasting on the activities and mRNA expression levels of lipoprotein lipase (LPL), hormone-sensitive lipase (HSL) and fatty acid synthetase (FAS) in spotted seabass *Lateolabrax maculatus*. Fish Physiology and Biochemistry, 44, 387-400.

Ibeas, C., Izquierdo, M. S., and Lorenzo, A. (1994). Effect of different levels of n-3 highly unsaturated fatty acids on growth and fatty acid composition of juvenile gilthead seabream (*Sparus aurata*). Aquaculture, 127, 177-188.

Ibrahim, R. E., Rhouma, N. R., Elbealy, M. A., Abdelwarith, A. A., Younis, E. M., Khalil, S. S., et al. (2024). Effect of dietary intervention with *Capsicum annuum* extract on growth performance, physiological status, innate immune response, and related gene expression in Nile tilapia. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 270, 110914.

Ikeda-Ohtsubo, W., Brugman, S., Warden, C. H., Rebel, J. M., Folkerts, G., and Pieterse, C. M. (2018). How can we define "optimal microbiota?": A comparative review of structure and functions of microbiota of animals, fish, and plants in agriculture. Frontiers in Nutrition, 5, 90.

Iijima, N., Tanaka, S., and Ota, Y. (1998). Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiology and Biochemistry, 18, 59-69.

Indiani, C. M. D. S. P., Rizzardi, K. F., Castelo, P. M., Ferraz, L. F. C., Darrieux, M., and Parisotto, T. M. (2018). Childhood obesity and Firmicutes/Bacteroidetes ratio in the gut microbiota: a systematic review. Childhood Obesity, 14, 501-509.

Infante-Villamil, S., Huerlimann, R., and Jerry, D. R. (2021). Microbiome diversity and dysbiosis in aquaculture. Reviews in Aquaculture, 13, 1077-1096.

Islam, K. S., Fukiya, S., Hagio, M., Fujii, N., Ishizuka, S., Ooka, T., et al. (2011). Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. Gastroenterology, 141, 1773-1781.

Iwashita, Y., Suzuki, N., Matsunari, H., Sugita, T., and Yamamoto, T. (2009). Influence of soya saponin, soya lectin, and cholyltaurine supplemented to a casein-based semipurified diet on intestinal morphology and biliary bile status in fingerling rainbow trout *Oncorhynchus mykiss*. Fisheries Science, 75, 1307-1315.

Iwashita, Y., Suzuki, N., Yamamoto, T., Shibata, J. I., Isokawa, K., Soon, A. H., et al. (2008). Supplemental effect of cholyltaurine and soybean lecithin to a soybean meal-based fish meal-free diet on hepatic and intestinal morphology of rainbow trout *Oncorhynchus mykiss*. Fisheries Science, 74, 1083-1095.

Izquierdo, M. S., Montero, D., Robaina, L., Caballero, M. J., Rosenlund, G., and Ginés, R. (2005). Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding. Aquaculture, 250, 431-444.

Fuentes, J., Gregório, S. F., Fonseca, F., Robles, R., Martos-Sitcha, J.A., and Moyano, F. J. (2024). Effect of bile salts on intestinal epithelial function in gilthead seabream (*Sparus aurata*). Fish Physiology and Biochemistry. In press.

Jakhetia, V., Patel, R., Khatri, P., Pahuja, N., Garg, S., Pandey, A., et al. (2010). Cinnamon: a pharmacological review. Journal of Advanced Scientific Research, 1, 19-23.

Jaya-Ram, A., Kuah, M. K., Lim, P. S., Kolkovski, S., and Shu-Chien, A. C. (2008). Influence of dietary HUFA levels on reproductive performance, tissue fatty acid profile and desaturase and elongase mRNAs expression in female zebrafish *Danio rerio*. Aquaculture, 277, 275-281.

Ji, R., Xiang, X., Li, X., Mai, K., and Ai, Q. (2021). Effects of dietary curcumin on growth, antioxidant capacity, fatty acid composition and expression of lipid metabolism-related genes of large yellow croaker fed a high-fat diet. British Journal of Nutrition, 126, 345-354.

Jiang, J., Wu, X. Y., Zhou, X. Q., Feng, L., Liu, Y., Jiang, W. D., et al. (2016). Effects of dietary curcumin supplementation on growth performance, intestinal digestive enzyme activities and antioxidant capacity of crucian carp *Carassius auratus*. Aquaculture, 463, 174-180.

Jiang, M., Wen, H., Gou, G. W., Liu, T. L., Lu, X., and Deng, D. F. (2018). Preliminary study to evaluate the effects of dietary bile acids on growth performance and lipid metabolism of juvenile genetically improved farmed tilapia (*Oreochromis niloticus*) fed plant ingredient-based diets. Aquaculture Nutrition, 24, 1175-1183.

Jiang, T. A. (2019). Health benefits of culinary herbs and spices. Journal of AOAC International, 102, 395-411.

Jin, M., Pan, T., Cheng, X., Zhu, T. T., Sun, P., Zhou, F., et al. (2019). Effects of supplemental dietary L-carnitine and bile acids on growth performance, antioxidant and immune ability, histopathological changes and inflammatory response in juvenile black seabream (*Acanthopagrus schlegelii*) fed high-fat diet. Aquaculture, 504, 199-209.

Joyce, S. A., and Gahan, C. G. (2017). Disease-associated changes in bile acid profiles and links to altered gut microbiota. Digestive diseases, 35, 169-177.

Jump, D. B. (2004). Fatty acid regulation of gene transcription. Critical Reviews in Clinical Laboratory Sciences, 41, 41-78.

Kartikasari, L. R., Hughes, R. J., Geier, M. S., Makrides, M., and Gibson, R. A. (2012). Dietary alpha-linolenic acid enhances omega-3 long chain polyunsaturated fatty acid levels in chicken tissues. Prostaglandins, Leukotrienes and Essential Fatty Acids, 87, 103-109.

Kashinskaya, E. N., Andree, K. B., Simonov, E. P., and Solovyev, M. M. (2017). DNA extraction protocols may influence biodiversity detected in the intestinal microbiome: a case study from wild Prussian carp, *Carassius gibelio*. FEMS Microbiology Ecology, 93, fiw240.

Kasumyan, A. O., and Vinogradskaya, M. I. (2019). Palatability of bile substances for fish. Journal of Ichthyology, 59, 610-618.

Kato, M., Khan, S., d'Aniello, E., McDonald, K. J., and Hart, D. N. (2007). The novel endocytic and phagocytic C-Type lectin receptor DCL-1/CD302 on macrophages is colocalized with F-

actin, suggesting a role in cell adhesion and migration. The Journal of Immunology, 179, 6052-6063.

Kaushik, S. J. (2013). Feed management and on-farm feeding practices of temperate fish with special reference to salmonids. In: Hasan, M., New, and M. B. (Eds.), FAO fisheries and aquaculture Technical paper (pp. 519-551), Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

Kawamoto, K., Horibe, I., and Uchida, K. (1989). Purification and characterization of a new hydrolase for conjugated bile acids, chenodeoxycholyltaurine hydrolase, from *Bacteroides vulgatus*. The Journal of Biochemistry, 106, 1049-1053.

Kawamura, G., Yong, A. S. K., Au, H. L., Doison, A., Ooi, S. Y., and Lim, L. S. L. S. (2019). Malaysian herbs as feeding attractants and enhancers for the giant freshwater prawn (*Macrobrachium rosenbergii*) and the whiteleg shrimp (*Litopenaeus vannamei*). Borneo Journal of Marine Science and Aquaculture, 3, 57-67.

Keating, C., Bolton-Warberg, M., Hinchcliffe, J., Davies, R., Whelan, S., Wan, A. H. L., et al. (2021). Temporal changes in the gut microbiota in farmed Atlantic cod (*Gadus morhua*) outweigh the response to diet supplementation with macroalgae. Animal Microbiome, 3, 7.

Keating, N., and Keely, S. J. (2009). Bile acids in regulation of intestinal physiology. Current Gastroenterology Reports, 11, 375-382.

Kenari, A. A., Mozanzadeh, M. T., and Pourgholam, R. (2011). Effects of total fish oil replacement to vegetable oils at two dietary lipid levels on the growth, body composition, haemato-immunological and serum biochemical parameters in caspian brown trout (*Salmo trutta caspius* Kessler, 1877). Aquaculture research, 42, 1131-1144.

Khan, R. U., Naz, S., Javdani, M., Nikousefat, Z., Selvaggi, M., Tufarelli, V., et al. (2012). The use of turmeric (*Curcuma longa*) in poultry feed. World's Poultry Science Journal, 68, 97-103.

Khrulnova, S. A., Manukhov, I. V., and Zavil'gel'skii, G. B. (2011). "Quorum sensing" regulation and the structure of *lux* the operon in marine bacteria *Aliivibrio logei*. Russian Journal of Genetics, 47, 1415-1421.

Kidani, Y., and Bensinger, S. J. (2012). Liver X receptor and peroxisome proliferator-activated receptor as integrators of lipid homeostasis and immunity. Immunological Reviews, 249, 72-83.

Kim, J. S., Ha, T. Y., Kim, S., Lee, S. J., and Ahn, J. (2017a). Red paprika (*Capsicum annuum* L.) and its main carotenoid capsanthin ameliorate impaired lipid metabolism in the liver and adipose tissue of high-fat diet-induced obese mice. Journal of Functional Foods, 31, 131-140.

Kim, B. R., Shin, J., Guevarra, R. B., Lee, J. H., Kim, D. W., Seol, K. H., et al. (2017b). Deciphering diversity indices for a better understanding of microbial communities. Journal of Microbiology and Biotechnology, 27, 2089-2093.

Kim, M. H., Park, S. J., Kim, J. H., Seong, J. B., Kim, K. M., Woo, H. A., et al. (2018). Peroxiredoxin 5 regulates adipogenesis-attenuating oxidative stress in obese mouse models induced by a high-fat diet. Free Radical Biology and Medicine, 123, 27-38.

Kim, M. H., Seong, J. B., Huh, J. W., Bae, Y. C., Lee, H. S., & Lee, D. S. (2020). Peroxiredoxin 5 ameliorates obesity-induced non-alcoholic fatty liver disease through the regulation of oxidative stress and AMP-activated protein kinase signaling. Redox Biology, 28, 101315.

Kim, S. K., Kim, K. G., Kim, K. D., Kim, K. W., Son, M. H., Rust, M., et al. (2015). Effect of dietary taurine levels on the conjugated bile acid composition and growth of juvenile Korean rockfish *Sebastes schlegeli* (Hilgendorf). Aquaculture Research, 46, 2768-2775.

Kokou, F., Sasson, G., Friedman, J., Eyal, S., Ovadia, O., Harpaz, S., et al. (2019). Core gut microbial communities are maintained by beneficial interactions and strain variability in fish. Nature Microbiology, 4, 2456-2465.

Kortner, T. M., Björkhem, I., Krasnov, A., Timmerhaus, G., and Krogdahl, Å. (2014). Dietary cholesterol supplementation to a plant-based diet suppresses the complete pathway of cholesterol synthesis and induces bile acid production in Atlantic salmon (*Salmo salar* L.). British Journal of Nutrition, 111, 2089-2103.

Kortner, T. M., Gu, J., Krogdahl, Å., and Bakke, A. M. (2013). Transcriptional regulation of cholesterol and bile acid metabolism after dietary soyabean meal treatment in Atlantic salmon (*Salmo salar* L.). British Journal of Nutrition, 109, 593-604.

Kortner, T. M., Penn, M. H., Björkhem, I., Måsøval, K., and Krogdahl, Å. (2016). Bile components and lecithin supplemented to plant based diets do not diminish diet related intestinal inflammation in Atlantic salmon. BMC Veterinary Research, 12, 1-12.

Kraugerud, O. F., Penn, M., Storebakken, T., Refstie, S., Krogdahl, Å., and Svihus, B. (2007). Nutrient digestibilities and gut function in Atlantic salmon (*Salmo salar*) fed diets with cellulose or non-starch polysaccharides from soy. Aquaculture, 273, 96-107.

Kubes, P., and Jenne, C. (2018). Immune responses in the liver. Annual Review of Immunology, 36, 247-277.

Kuipers, F., Claudel, T., Sturm, E., and Staels, B. (2004). The Farnesoid X Receptor (FXR) as modulator of bile acid metabolism. Reviews in Endocrine and Metabolic Disorders, 5, 319-326.

Kulkarni, N., Meitei, H. T., Sonar, S. A., Sharma, P. K., Mujeeb, V. R., Srivastava, S., et al. (2018). CCR6 signaling inhibits suppressor function of induced-Treg during gut inflammation. Journal of Autoimmunity, 88, 121-130.

Kumagai, Y., Takeuchi, O., and Akira, S. (2008). TLR9 as a key receptor for the recognition of DNA. Advanced drug delivery reviews, 60, 795-804.

Kurtovic, I., Marshall, S. N., Zhao, X., and Simpson, B. K. (2010). Purification and properties of digestive lipases from Chinook salmon (*Oncorhynchus tshawytscha*) and New Zealand hoki (*Macruronus novaezelandiae*). Fish Physiology and Biochemistry, 36, 1041-1060.

Lai, W., Huang, W., Dong, B., Cao, A., Zhang, W., Li, J., et al. (2018). Effects of dietary supplemental bile acids on performance, carcass characteristics, serum lipid metabolites and intestinal enzyme activities of broiler chickens. Poultry Science, 97, 196-202.

Le, H. T., Lie, K. K., Giroud-Argoud, J., Rønnestad, I., and Sæle, Ø. (2019). Effects of cholecystokinin (CCK) on gut motility in the stomachless fish ballan wrasse (*Labrus bergylta*). Frontiers in Neuroscience, 13, 553.

Leaver, M. J., Villeneuve, L. A., Obach, A., Jensen, L., Bron, J. E., Tocher, D. R., et al. (2008). Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*). BMC Genomics, 9, 299.

Legrand, T. P., Wynne, J. W., Weyrich, L. S., and Oxley, A. P. (2020a). A microbial sea of possibilities: current knowledge and prospects for an improved understanding of the fish microbiome. Reviews in Aquaculture, 12, 1101-1134.

Legrand, T. P., Catalano, S. R., Wos-Oxley, M. L., Wynne, J. W., Weyrich, L. S., and Oxley, A. P. (2020b). Antibiotic-induced alterations and repopulation dynamics of yellowtail kingfish microbiota. Animal Microbiome, 2, 26.

Lenas, D.S., Triantafillou, D.J., Chatziantoniou, S., and Nathanailides, C. (2011). Fatty acid profile of wild and farmed gilthead sea bream (*Sparus aurata*). Journal für Verbraucherschutz und Lebensmittelsicherheit, 6, 435-440.

Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I. (2006). Human gut microbes associated with obesity. Nature, 444, 1022-1023.

Li, J., Wang, Z., Cao, X., Wang, J., Gong, Y., Wang, X., et al. (2023). Effects of supplemental mixed bile acids on growth performance, body composition, digestive enzyme activities, skin color, and flesh quality of juvenile large yellow croaker (*Larimichthys crocea*) in soybean oil based diet. Frontiers in Marine Science, 10, 1149887.

Li, R., Andreu-Sánchez, S., Kuipers, F., and Fu, J. (2021a). Gut microbiome and bile acids in obesity-related diseases. Best Practice & Research Clinical Endocrinology & Metabolism, 35, 101493.

Li, Y., Wang, S., Hu, Y., Cheng, J., Cheng, X., Cheng, P., et al. (2021b). Dietary bile acid supplementation reveals beneficial effects on intestinal healthy status of tongue sole (*Cynoglossus semiliaevis*). Fish & Shellfish Immunology, 116, 52-60.

Liu, S., Zhang, S., Wang, Y., Lu, S., Han, S., Liu, Y., et al. (2023). Dietary sodium butyrate improves intestinal health of triploid *Oncorhynchus mykiss* fed a low fish meal diet. Biology, 12, 145.

Liu, S. J., Wang, J., He, T. F., Liu, H. S., and Piao, X. S. (2021a). Effects of natural capsicum extract on growth performance, nutrient utilization, antioxidant status, immune function, and meat quality in broilers. Poultry Science, 100, 101301.

Liu, Z. Y., Yang, H. L., Hu, L. H., Yang, W., Ai, C. X., and Sun, Y. Z. (2021b). Dose-dependent effects of histamine on growth, immunity and intestinal health in juvenile grouper (*Epinephelus coioides*). Frontiers in Marine Science, 8, 685720.

Liu, Y., Azad, M. A. K., Kong, X., Zhu, Q., and Yu, Z. (2022a). Dietary bile acids supplementation modulates immune response, antioxidant capacity, glucose, and lipid metabolism in normal and intrauterine growth retardation piglets. Frontiers in Nutrition, 9, 991812.

Liu, Y., Cheng, J., Xia, Y., Li, X., Liu, Y., and Liu, P. F. (2022b). Response mechanism of gut microbiome and metabolism of European seabass (*Dicentrarchus labrax*) to temperature stress. Science of the Total Environment, 813, 151786.

Liu, Y., Liu, J., and Zhang, Y. (2019). Research progress on chemical constituents of *Zingiber officinale* Roscoe. BioMed Research International, 2019, 5370823.

Long, S., Liu, S., Wang, J., Mahfuz, S., and Piao, X. (2021). Natural capsicum extract replacing chlortetracycline enhances performance via improving digestive enzyme activities, antioxidant capacity, anti-inflammatory function, and gut health in weaned pigs. Animal Nutrition, 7, 305-314.

Lorente-Cebrián, S., Costa, A. G., Navas-Carretero, S., Zabala, M., Martínez, J. A., and Moreno-Aliaga, M. J. (2013). Role of omega-3 fatty acids in obesity, metabolic syndrome, and cardiovascular diseases: a review of the evidence. Journal of Physiology and Biochemistry, 69, 633-651.

Lu, J., Brigham, C. J., Rha, C., and Sinskey, A. J. (2013). Characterization of an extracellular lipase and its chaperone from *Ralstonia eutropha* H16. Applied Microbiology and Biotechnology, 97, 2443-2454.

Luo, Q., Li, N., Zheng, Z., Chen, L., Mu, S., Chen, L., et al. (2020). Dietary cinnamaldehyde supplementation improves the growth performance, oxidative stability, immune function, and meat quality in finishing pigs. Livestock Science, 240, 104221.

Lupatsch, I., Kissil, G. W., Sklan, D., and Pfeffer, E. (1998). Energy and protein requirements for maintenance and growth in gilthead seabream (*Sparus aurata* L.). Aquaculture Nutrition, 4, 165-173.

Maldonado-Valderrama, J., Wilde, P., Macierzanka, A., and Mackie, A. (2011). The role of bile salts in digestion. Advances in Colloid and Interface Science, 165, 36-46.

Malintha, G. H. T., Jeong, J. B., Gunathilaka, B. E., Hasanthi, M., Yun, K. S., and Lee, K. J. (2023). Effects of dietary piperine supplementation on innate immunity, growth performance, feed utilization and intestinal morphology of olive flounder (*Paralichthys olivaceus*). Fish Physiology and Biochemistry, 49, 925-937.

Mancini, S., Secci, G., Preziuso, G., Parisi, G., and Paci, G. (2018). Ginger (*Zingiber officinale* Roscoe) powder as dietary supplementation in rabbit: life performances, carcass characteristics and meat quality. Italian Journal of Animal Science, 17, 867-872.

Marques, A., Matos, E., Aires, T., Melo, D., Oliveira, M. B. P., & Valente, L. M. (2022). Understanding the interaction between terrestrial animal fat sources and dietary emulsifier supplementation on muscle fatty acid profile and textural properties of European sea bass. Aquaculture, 560, 738547.

Matsuzaka, T., and Shimano, H. (2009). Elovl6: a new player in fatty acid metabolism and insulin sensitivity. Journal of Molecular Medicine, 87, 379-384.

Mente, E., Nikouli, E., Antonopoulou, E., Martin, S. A., and Kormas, K. A. (2018). Core versus diet-associated and postprandial bacterial communities of the rainbow trout (*Oncorhynchus mykiss*) midgut and faeces. Biology Open, 7, bio034397.

Mills, S., Stanton, C., Lane, J. A., Smith, G. J., and Ross, R. P. (2019). Precision nutrition and the microbiome, part I: current state of the science. Nutrients, 11, 923.

Ming, J., Ye, J., Zhang, Y., Xu, Q., Yang, X., Shao, X., et al. (2020). Optimal dietary curcumin improved growth performance, and modulated innate immunity, antioxidant capacity and related genes expression of NF-κB and Nrf2 signaling pathways in grass carp (*Ctenopharyngodon idella*) after infection with *Aeromonas hydrophila*. Fish & Shellfish Immunology, 97, 540-553.

Mohammad, S. M., and Hamed, H. K. (2012). Ginger (*Zingiber officinale*): A review. Journal of Medicinal Plants Research, 6, 4255-4258.

Mohammadi, G., Rashidian, G., Hoseinifar, S. H., Naserabad, S. S., and Van Doan, H. (2020). Ginger (*Zingiber officinale*) extract affects growth performance, body composition,

haematology, serum and mucosal immune parameters in common carp (*Cyprinus carpio*). Fish & Shellfish Immunology, 99, 267-273.

Molosse, V., Souza, C. F., Baldissera, M. D., Glombowsky, P., Campigotto, G., Cazaratto, C. J., et al. (2019). Diet supplemented with curcumin for nursing lambs improves animal growth, energetic metabolism, and performance of the antioxidant and immune systems. Small Ruminant Research, 170, 74-81.

Monteiro, M., Matos, E., Ramos, R., Campos, I., and Valente, L. M. (2018). A blend of land animal fats can replace up to 75% fish oil without affecting growth and nutrient utilization of European seabass. Aquaculture, 487, 22-31.

Montero, D., Mathlouthi, F., Tort, L., Afonso, J. M., Torrecillas, S., Fernández-Vaquero, A., et al. (2010). Replacement of dietary fish oil by vegetable oils affects humoral immunity and expression of pro-inflammatory cytokines genes in gilthead sea bream *Sparus aurata*. Fish & Shellfish Immunology, 29, 1073-1081.

Morais, S., Silva, T., Cordeiro, O., Rodrigues, P., Guy, D. R., Bron, J. E., et al. (2012). Effects of genotype and dietary fish oil replacement with vegetable oil on the intestinal transcriptome and proteome of Atlantic salmon (*Salmo salar*). BMC Genomics, 13, 448.

Morais, S., Torres, M., Hontoria, F., Monroig, Ó., Varó, I., Agulleiro, M.J., et al. (2020). Molecular and functional characterization of *Elovl4* genes in *Sparus aurata* and *Solea senegalensis* pointing to a critical role in very long-chain (>C_{24}) fatty acid synthesis during early neural development of fish. International Journal of Molecular Sciences, 21, 3514.

Mougin, J., and Joyce, A. (2023). Fish disease prevention via microbial dysbiosis-associated biomarkers in aquaculture. Reviews in Aquaculture, 15, 579-594.

Mourente, G., and Bell, J. G. (2006). Partial replacement of dietary fish oil with blends of vegetable oils (rapeseed, linseed and palm oils) in diets for European sea bass (*Dicentrarchus labrax* L.) over a long term growth study: effects on muscle and liver fatty acid composition and effectiveness of a fish oil finishing diet. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 145, 389-399.

Moyer, C. L., and Morita, R. Y. (2007). Psychrophiles and psychrotrophs. In: Morita, R. Y. (Ed.), Encyclopedia of Life Sciences (pp. 1-6), John Wiley & Sons Ltd., Chichester, UK.

Mozanzadeh, M.T., Hekmatpour, F., and Gisbert, E. (2021). Fish oil sparing and alternative lipid sources in aquafeeds. In: Lorenzo, J. M., and Simal-Gandara, J. (Eds.), Sustainable Aquafeeds: Technological Innovation and Novel Ingredients (pp. 185-292), CRC Press, London, UK.

Mozanzadeh, M. T., Agh, N., Yavari, V., Marammazi, J. G., Mohammadian, T., and Gisbert, E. (2016). Partial or total replacement of dietary fish oil with alternative lipid sources in silvery-black porgy (S*paridentex hasta*). Aquaculture, 451, 232-240.

Mullen, K. R., Yasuda, K., Divers, T. J., and Weese, J. S. (2018). Equine faecal microbiota transplant: Current knowledge, proposed guidelines and future directions. Equine Veterinary Education, 30, 151-160.

Murashita, K., Yoshiura, Y., Chisada, S. I., Furuita, H., Sugita, T., Matsunari, H., et al. (2014). Homologue gene of bile acid transporters *ntcp*, *asbt*, and *ost-alpha* in rainbow trout *Oncorhynchus mykiss*: tissue expression, effect of fasting, and response to bile acid administration. Fish Physiology and Biochemistry, 40, 511-525.

Naiel, M. A., Negm, S. S., Ghazanfar, S., Shukry, M., and Abdelnour, S. A. (2023). The risk assessment of high-fat diet in farmed fish and its mitigation approaches: A review. Journal of Animal Physiology and Animal Nutrition, 103, 948-969.

Nakanishi, T., Shibasaki, Y., and Matsuura, Y. (2015). T cells in fish. Biology, 4, 640-663.

Navarrete, P., Magne, F., Araneda, C., Fuentes, P., Barros, L., Opazo, R., et al. (2012). PCR-TTGE analysis of 16S rRNA from rainbow trout (*Oncorhynchus mykiss*) gut microbiota reveals host-specific communities of active bacteria. PloS One, 7, e31335.

Naya-Català, F., Wiggers, G. A., Piazzon, M. C., López-Martínez, M. I., Estensoro, I., Calduch-Giner, J. A., et al. (2021a). Modulation of gilthead sea bream gut microbiota by a bioactive egg white hydrolysate: Interactions between bacteria and host lipid metabolism. Frontiers in Marine Science, 8, 698484.

Naya-Català, F., do Vale Pereira, G., Piazzon, M. C., Fernandes, A. M., Calduch-Giner, J. A., Sitjà-Bobadilla, A., et al. (2021b). Cross-talk between intestinal microbiota and host gene expression in gilthead sea bream (*Sparus aurata*) juveniles: insights in fish feeds for increased circularity and resource utilization. Frontiers in Physiology, 12, 748265.

Naylor, R. L., Hardy, R. W., Buschmann, A. H., Bush, S. R., Cao, L., Klinger, D. H., et al. (2021). A 20-year retrospective review of global aquaculture. Nature, 591, 551-563.

Neto, J. G. O., Boechat, S. K., Romão, J. S., Pazos-Moura, C. C., and Oliveira, K. J. (2020). Treatment with cinnamaldehyde reduces the visceral adiposity and regulates lipid metabolism, autophagy and endoplasmic reticulum stress in the liver of a rat model of early obesity. The Journal of Nutritional Biochemistry, 77, 108321.

Neuman, C., Hatje, E., Zarkasi, K. Z., Smullen, R., Bowman, J. P., and Katouli, M. (2016). The effect of diet and environmental temperature on the faecal microbiota of farmed Tasmanian Atlantic Salmon (*Salmo salar* L.). Aquaculture Research, 47, 660-672.

Ng, W. K., Sigholt, T., and Gordon Bell, J. (2004). The influence of environmental temperature on the apparent nutrient and fatty acid digestibility in Atlantic salmon (*Salmo salar* L.) fed finishing diets containing different blends of fish oil, rapeseed oil and palm oil. Aquaculture Research, 35, 1228-1237.

Ni, J., Yan, Q., Yu, Y., and Zhang, T. (2014). Factors influencing the grass carp gut microbiome and its effect on metabolism. FEMS Microbiology Ecology, 87, 704-714.

Niederwerder, M. C., Constance, L. A., Rowland, R. R., Abbas, W., Fernando, S. C., Potter, M. L., et al. (2018). Fecal microbiota transplantation is associated with reduced morbidity and mortality in porcine circovirus associated disease. Frontiers in Microbiology, 9, 1631.

Nikouli, E., Kormas, K. A., Jin, Y., Olsen, Y., Bakke, I., and Vadstein, O. (2021). Dietary lipid effects on gut microbiota of first feeding Atlantic salmon (*Salmo salar* L.). Frontiers in Marine Science, 8, 665576.

Nikouli, E., Meziti, A., Antonopoulou, E., Mente, E., and Kormas, K. A. (2018). Gut bacterial communities in geographically distant populations of farmed sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). Microorganisms, 6, 92.

Nilavan, E. S., Raman, R. P., Srivastava, P. P., Basha, K. A., Kumar, K., Kumar, A., et al. (2017). Dietary piperine improves haemato-immunological parameters, growth profiles and resistance against *Aeromonas hydrophila* in *Labeo rohita* (Hamilton, 1822). Journal of Aquaculture in the Tropics, 32, 189-203.

Nilius, B., and Appendino, G. (2013). Spices: the savory and beneficial science of pungency. Reviews of Physiology, Biochemistry and Pharmacology, 164, 1-76.

Nolasco-Soria, H. (2023). Fish digestive lipase quantification methods used in aquaculture studies. Frontiers in Aquaculture, 2, 1225216.

Nomura, A., and Taniuchi, I. (2020). The role of CD8 downregulation during thymocyte differentiation. Trends in Immunology, 41, 972-981.

Ntambi, J. M., and Miyazaki, M. (2004). Regulation of stearoyl-CoA desaturases and role in metabolism. Progress in Lipid Research, 43, 91-104.

Odhaib, K. J., Ali, N. M. J., Abdulameer, H. A., and Khudhair, N. A. (2021). Influence of graded levels of turmeric (*Curcuma longa*) as feed additives alternatives to promote growth and enhance health status in lambs. Biochemical and Cellular Archives, 21, 3025-3032.

Oliva-Teles, A. (2012). Nutrition and health of aquaculture fish. Journal of Fish Diseases, 35, 83-108.

Oliveira, S. H. P. D., and Lukacs, N. W. (2003). The role of chemokines and chemokine receptors in eosinophil activation during inflammatory allergic reactions. Brazilian Journal of Medical and Biological Research, 36, 1455-1463.

Oliveira-Nascimento, L., Massari, P., and Wetzler, L. M. (2012). The role of TLR2 in infection and immunity. Frontiers in Immunology, 3, 79.

Olsen, R. E., Waagbø, R., Melle, W., Ringø, E., and Lall, S. P. (2011). Alternative marine resources. In: Turchini, G. M., Ng, W. K., and Tocher, D. R. (Eds.), Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds (pp. 267-324), CRC Press, Boca Raton, USA.

Orozco Colonia, B. S., de Melo Pereira, G. V., and Soccol, C. R. (2020). Omega-3 microbial oils from marine thraustochytrids as a sustainable and technological solution: A review and patent landscape. Trends in Food Science & Technology, 99, 244-256.

Palti, Y. (2011). Toll-like receptors in bony fish: from genomics to function. Developmental & Comparative Immunology, 35, 1263-1272.

Pandak, W. M., Hylemon, P. B., Ren, S., Marques, D., Gil, G., Redford, K., et al. (2002). Regulation of oxysterol 7α-hydroxylase (CYP7B1) in primary cultures of rat hepatocytes. Hepatology, 35, 1400-1408.

Panserat, S., Hortopan, G. A., Plagnes-Juan, E., Kolditz, C., Lansard, M., Skiba-Cassy, S., et al. (2009). Differential gene expression after total replacement of dietary fish meal and fish oil by plant products in rainbow trout (*Oncorhynchus mykiss*) liver. Aquaculture, 294, 123-131.

Parasuraman, S., Thing, G. S., and Dhanaraj, S. A. (2014). Polyherbal formulation: Concept of ayurveda. Pharmacognosy Reviews, 8, 73-80.

Parthasarathy, V. A., Sasikumar, B., Nair, R. R., and George, K. J. (2007). Black pepper: botany and horticulture. Horticultural Reviews - Westport Then New York, 33, 173-266.

Pathak, M., and Lal, G. (2020). The regulatory function of CCR9^{+} dendritic cells in inflammation and autoimmunity. Frontiers in Immunology, 11, 536326.

Peng, J., Li, J., Liang, J., Li, W., Yang, Y., Yang, Y., et al. (2023). A C-type lectin-like receptor CD302 in yellow drum (*Nibea albiflora*) functioning in antibacterial activity and innate immune signaling. International Journal of Biological Macromolecules, 247, 125734.

Peng, S., Shi, Z., Gao, Q., Zhang, C., and Wang, J. (2017). Dietary n-3 LC-PUFA s affect lipoprotein lipase (LPL) and fatty acid synthase (FAS) activities and mRNA expression during vitellogenesis and ovarian fatty acid composition of female silver pomfret (*Pampus argenteus*) broodstock. Aquaculture Nutrition, 23, 692-701.

Peng, S., Yue, Y., Gao, Q., Shi, Z., Yin, F., and Wang, J. (2014). Influence of dietary n-3 LC-PUFA on growth, nutritional composition and immune function in marine fish *Sebastiscus marmoratus*. Chinese Journal of Oceanology and Limnology, 32, 1000-1008.

Pereira, G. Q., Gomes, L. A., Santos, I. S., Alfieri, A. F., Weese, J. S., and Costa, M. C. (2018). Fecal microbiota transplantation in puppies with canine parvovirus infection. Journal of Veterinary Internal Medicine, 32, 707-711.

Piazzon, M. C., Galindo-Villegas, J., Pereiro, P., Estensoro, I., Calduch-Giner, J. A., Gómez-Casado, E., et al. (2016). Differential modulation of IgT and IgM upon parasitic, bacterial, viral, and dietary challenges in a perciform fish. Frontiers in Immunology, 7, 637.

Piazzon, M. C., Savelkoul, H. F., Pietretti, D., Wiegertjes, G. F., and Forlenza, M. (2015). Carp Il10 has anti-inflammatory activities on phagocytes, promotes proliferation of memory T cells, and regulates B cell differentiation and antibody secretion. The Journal of Immunology, 194, 187-199.

Piedecausa, M. A., Mazón, M. J., García, B. G., and Hernández, M. D. (2007). Effects of total replacement of fish oil by vegetable oils in the diets of sharpsnout seabream (*Diplodus puntazzo*). Aquaculture, 263, 211-219.

Platel, K., and Srinivasan, K. (2004). Digestive stimulant action of spices: a myth or reality?. Indian Journal of Medical Research, 119, 167-169.

Postler, T. S., and Ghosh, S. (2017). Understanding the holobiont: how microbial metabolites affect human health and shape the immune system. Cell Metabolism, 26, 110-130.

Pounden, W. D., and Hibbs, J. W. (1949). Rumen inoculations in young calves. Journal of the American Veterinary Medical Association, 114, 33-35.

Prasath, D., Kandiannan, K., Leela, N. K., Aarthi, S., Sasikumar, B., and Babu, K. N. (2018). Turmeric: Botany and production practices. Horticultural Reviews, 46, 99-184.

Proença, A. R., Sertié, R. A. L., Oliveira, A. C., Campaña, A. B., Caminhotto, R. O., Chimin, P., et al. (2014). New concepts in white adipose tissue physiology. Brazilian Journal of Medical and Biological Research, 47, 192-205.

Pundir, R. K., and Jain, P. (2010). Comparative studies on the antimicrobial activity of black pepper (*Piper nigrum*) and turmeric (*Curcuma longa*) extracts. International Journal of Applied Biology and Pharmaceutical Technology, 1, 492-500.

Qi, Z., Holland, J. W., Jiang, Y., Secombes, C. J., Nie, P., and Wang, T. (2017). Molecular characterization and expression analysis of four fish-specific CC chemokine receptors CCR4La, CCR4Lc1, CCR4Lc2 and CCR11 in rainbow trout (*Oncorhynchus mykiss*). Fish & Shellfish Immunology, 68, 411-427.

Qin, J., Wei, X., Cao, M., and Shi, B. (2023). Non-12α-hydroxylated bile acids improve piglet growth performance by improving intestinal flora, promoting intestinal development and bile acid synthesis. Animals, 13, 3380.

Rajput, N., Muhammad, N., Yan, R., Zhong, X., and Wang, T. (2013). Effect of dietary supplementation of curcumin on growth performance, intestinal morphology and nutrients utilization of broiler chicks. The Journal of Poultry Science, 50, 44-52.

Rantala, M., and Nurmi, E. (1973). Prevention of the growth of *Salmonella infantis* in chicks by the flora of the alimentary tract of chickens. British Poultry Science, 14, 627-630.

Raschperger, E., Neve, E. P., Wernerson, A., Hultenby, K., Pettersson, R. F., & Majumdar, A. (2008). The coxsackie and adenovirus receptor (CAR) is required for renal epithelial differentiation within the zebrafish pronephros. Developmental biology, 313(1), 455-464.

Raschperger, E., Thyberg, J., Pettersson, S., Philipson, L., Fuxe, J., & Pettersson, R. F. (2006). The coxsackie-and adenovirus receptor (CAR) is an in vivo marker for epithelial tight junctions, with a potential role in regulating permeability and tissue homeostasis. Experimental cell research, 312(9), 1566-1580.

Rathaur, P., Raja, W., Ramteke, P. W., and John, S. A. (2012). Turmeric: The golden spice of life. International Journal of Pharmaceutical Sciences and Research, 3, 1987.

Rausch, T. (2023). *Role of intestinal bacteria in the conversion of dietary sulfonates* (Doctoral dissertation), Universität Potsdam, Germany.

Ravindran, P. N., and Kallupurackal, J. A. (2012). Black pepper. In: Peter, K. V. (Ed.), Handbook of Herbs and Spices (pp. 86-115), Woodhead Publishing, Cambridge, UK.

Rawls, J. F., Mahowald, M. A., Ley, R. E., & Gordon, J. I. (2006). Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. Cell, 127(2), 423-433.

Ray, A. K., Ghosh, K., & Ringø, E. J. A. N. (2012). Enzyme-producing bacteria isolated from fish gut: a review. Aquaculture nutrition, 18(5), 465-492.

Recharla, N., Balasubramanian, B., Song, M., Puligundla, P., Kim, S. K., Jeong, J. Y., et al. (2021). Dietary turmeric (*Curcuma longa* L.) supplementation improves growth performance, short-chain fatty acid production, and modulates bacterial composition of weaned piglets. Journal of Animal Science and Technology, 63, 575-592.

Reis, B., Cabral, E. M., Fernandes, T. J., Castro-Cunha, M., Oliveira, M. B. P., Cunha, L. M., et al. (2014). Long-term feeding of vegetable oils to Senegalese sole until market size: Effects on growth and flesh quality. Recovery of fatty acid profiles by a fish oil finishing diet. Aquaculture, 434, 425-433.

Ribeiro, G. O., Oss, D. B., He, Z., Gruninger, R. J., Elekwachi, C., Forster, R. J., et al. (2017). Repeated inoculation of cattle rumen with bison rumen contents alters the rumen microbiome and improves nitrogen digestibility in cattle. Scientific Reports 7, 1276.

Ridaura, V. K., Faith, J. J., Rey, F. E., Cheng, J., Duncan, A. E., Kau, A. L., et al. (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science, 341, 1241214.

Ridlon, J. M., Kang, D. J., Hylemon, P. B., and Bajaj, J. S. (2014). Bile acids and the gut microbiome. Current Opinion in Gastroenterology, 30, 332-338.

Riedel, S. L., Lu, J., Stahl, U., and Brigham, C. J. (2014). Lipid and fatty acid metabolism in *Ralstonia eutropha*: relevance for the biotechnological production of value-added products. Applied Microbiology and Biotechnology, 98, 1469-1483.

Roeselers, G., Mittge, E. K., Stephens, W. Z., Parichy, D. M., Cavanaugh, C. M., Guillemin, K., et al. (2011). Evidence for a core gut microbiota in the zebrafish. The ISME journal, 5, 1595-1608.

Rogers, J., Urbina, S. L., Taylor, L. W., Wilborn, C. D., Purpura, M., Jäger, R., et al. (2018). Capsaicinoids supplementation decreases percent body fat and fat mass: adjustment using covariates in a post hoc analysis. BMC Obesity, 5, 22.

Rolen, S. H., and Caprio, J. (2008). Bile salts are effective taste stimuli in channel catfish. Journal of Experimental Biology, 211, 2786-2791.

Romano, N., Kumar, V., Yang, G., Kajbaf, K., Rubio, M. B., Overturf, K., et al. (2020). Bile acid metabolism in fish: disturbances caused by fishmeal alternatives and some mitigating effects from dietary bile inclusions. Reviews in Aquaculture, 12, 1792-1817.

Romarheim, O. H., Skrede, A., Penn, M., Mydland, L. T., Krogdahl, Å., and Storebakken, T. (2008). Lipid digestibility, bile drainage and development of morphological intestinal changes in rainbow trout (*Oncorhynchus mykiss*) fed diets containing defatted soybean meal. Aquaculture, 274, 329-338.

Rombenso, A. N., Turchini, G. M., and Trushenski, J. T. (2021). The omega-3 sparing effect of saturated fatty acids: A reason to reconsider common knowledge of fish oil replacement. Reviews in Aquaculture, 14, 213-217.

Rudi, K., Angell, I. L., Pope, P. B., Vik, J. O., Sandve, S. R., and Snipen, L. G. (2018). Stable core gut microbiota across the freshwater-to-saltwater transition for farmed Atlantic salmon. Applied and Environmental Microbiology, 84, e01974-17.

Ruiz, A., Andree, K. B., Sanahuja, I., Holhorea, P. G., Calduch-Giner, J. À., Morais, S., et al. (2023a). Bile salt dietary supplementation promotes growth and reduces body adiposity in gilthead seabream (*Sparus aurata*). Aquaculture, 566, 739203.

Ruiz, A., Andree, K. B., Furones, D., Holhorea, P. G., Calduch-Giner, J. À., Viñas, M., et al. (2023b). Modulation of gut microbiota and intestinal immune response in gilthead seabream (*Sparus aurata*) by dietary bile salt supplementation. Frontiers in Microbiology, 14, 1123716.

Ruiz, A., Sanahuja, I., Andree, K. B., Furones, D., Holhorea, P. G., Calduch-Giner, J. A., et al. (2023c). The potential of a combination of pungent spices as a novel supplement in gilthead seabream (*Sparus aurata*) diets to aid in the strategic use of fish oil in aquafeeds: a holistic perspective. Frontiers in Immunology, 14, 1222173.

Ruiz, A., Sanahuja, I., Andree, K. B., Furones, D., Holhorea, P. G., Calduch-Giner, J. A., et al. (2024a). Supplementation of gilthead seabream (*Sparus aurata*) diets with spices as a functional strategy to control excess adiposity through lipid, cholesterol and bile acid metabolism, and to induce an immunomodulatory intestinal regulation. Aquaculture, 581, 740378.

Ruiz, A., Gisbert, E., and Andree, K. B. (2024b). Impact of the diet in the gut microbiota after an inter-species microbial transplantation in fish. Scientific Reports, 14, 4007.

Ruiz, A., Gisbert, E., Estevez, A., Reyes-López, F.E., Vallejos-Vidal, E., Tort, L., et al. (2024c). Strategy combining mammalian fats with supplementation of pungent spices in aquafeeds, to mitigate negative impacts of fish oil replacement in fish performance, fillet quality and hepatic condition of gilthead seabream (*Sparus aurata*). Aquaculture. In press.

Ruiz, A., Alós, J., Gisbert, E., Furones, D., and Viver, T. (2024d). Resilience of gut microbial assemblages in farmed fish revealed by long-term dietary changes. Aquaculture Reports. In press.

Ruiz, A., Torrecillas, S., Kashinskaya, E., Andree, K. B., Solovyev, M., and Gisbert, E. (2024e). Comparative study of the gut microbial communities collected by scraping and swabbing in a fish model: a comprehensive guide to promote non-lethal procedures for gut microbial studies. Frontiers in Veterinary Science, 11, 1374803.

Ruiz-Lopez, N., Haslam, R. P., Napier, J. A., and Sayanova, O. (2014). Successful high-level accumulation of fish oil omega-3 long-chain polyunsaturated fatty acids in a transgenic oilseed crop. The Plant Journal, 77, 198-208.

Ruyter, B., Moya-Falcón, C., Rosenlund, G., and Vegusdal, A. (2006). Fat content and morphology of liver and intestine of Atlantic salmon (*Salmo salar*): effects of temperature and dietary soybean oil. Aquaculture, 252, 441-452.

Rynkiewicz, E. C., Clerc, M., Babayan, S. A., and Pedersen, A. B. (2019). Variation in local and systemic pro-inflammatory immune markers of wild wood mice after anthelmintic treatment. Integrative and Comparative Biology, 59, 1190-1202.

Sachan, A. K., Kumar, S., Kumari, K., and Singh, D. (2018). Medicinal uses of spices used in our traditional culture: World wide. Journal of Medicinal Plants Studies, 6, 116-122.

Saera-Vila, A., Calduch-Giner, J. A., Gómez-Requeni, P., Médale, F., Kaushik, S., and Pérez-Sánchez, J. (2005). Molecular characterization of gilthead sea bream (*Sparus aurata*) lipoprotein lipase. Transcriptional regulation by season and nutritional condition in skeletal muscle and fat storage tissues. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 142, 224-232.

Sahin, O., Morishita, T. Y., and Zhang, Q. (2002). *Campylobacter* colonization in poultry: sources of infection and modes of transmission. Animal Health Research Reviews, 3, 95-105.

Saini, R. K., and Keum, Y. S. (2018). Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance - A review. Life Sciences, 203, 255-267.

Sakai, M., Hikima, J. I., and Kono, T. (2021). Fish cytokines: current research and applications. Fisheries Science, 87, 1-9.

Salerno, G., Parisi, M. G., Parrinello, D., Benenati, G., Vizzini, A., Vazzana, M., et al. (2009). F-type lectin from the sea bass (*Dicentrarchus labrax*): purification, cDNA cloning, tissue expression and localization, and opsonic activity. Fish & Shellfish Immunology, 27, 143-153.

Salmerón, C. (2018). Adipogenesis in fish. Journal of Experimental Biology, 221, jeb161588.

Salomón, R., Firmino, J. P., Reyes-López, F. E., Andree, K. B., González-Silvera, D., Esteban, M. A., et al. (2020). The growth promoting and immunomodulatory effects of a medicinal plant leaf extract obtained from *Salvia officinalis* and *Lippia citriodora* in gilthead seabream (*Sparus aurata*). Aquaculture, 524, 735291.

Salomón, R., Furones, M. D., Reyes-López, F. E., Tort, L., Firmino, J. P., Esteban, M. A., et al. (2021). A bioactive extract rich in triterpenic acid and polyphenols from *Olea europaea* promotes systemic immunity and protects Atlantic salmon smolts against furunculosis. Frontiers in Immunology, 12, 737601.

Sampath, H., and Ntambi, J. M. (2005). The fate and intermediary metabolism of stearic acid. Lipids, 40, 1187-1191.

Sampath, V., Shanmugam, S., Park, J. H., and Kim, I. H. (2020). The effect of black pepper (*piperine*) extract supplementation on growth performance, nutrient digestibility, fecal microbial, fecal gas emission, and meat quality of finishing pigs. Animals, 10(11), 1965.

Sato, R. (2010). Sterol metabolism and SREBP activation. Archives of Biochemistry and Biophysics, 501, 177-181.

Scheuring, I., Rasmussen, J. A., Bozzi, D., and Limborg, M. T. (2022). A strategic model of a host-microbe-microbe system reveals the importance of a joint host-microbe immune response to combat stress-induced gut dysbiosis. Frontiers in Microbiology, 13, 912806.

Schonewille, M., de Boer, J. F., and Groen, A. K. (2016). Bile salts in control of lipid metabolism. Current Opinion in Lipidology, 27, 295-301.

Schubert, K., Olde Damink, S. W., von Bergen, M., and Schaap, F. G. (2017). Interactions between bile salts, gut microbiota, and hepatic innate immunity. Immunological Reviews, 279, 23-35.

Secombes, C. J., and Wang, T. (2012). The innate and adaptive immune system of fish. In: Austin, B. (Ed.), Infectious Disease in Aquaculture, Prevention and Control (pp. 3-68), Woodhead Publishing, Oxford, UK.

Sekirov, I., Russell, S. L., Antunes, L. C. M., and Finlay, B. B. (2010). Gut microbiota in health and disease. Physiological reviews, 90, 859-904.

Semova, I., Carten, J. D., Stombaugh, J., Mackey, L. C., Knight, R., Farber, S. A., et al. (2012). Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. Cell Host & Microbe, 12, 277-288.

Sepulveda, J., and Moeller, A. H. (2020). The effects of temperature on animal gut microbiomes. Frontiers in Microbiology, 11, 512773.

Serna-Duque, J. A., and Esteban, M. Á. (2020). Effects of inflammation and/or infection on the neuroendocrine control of fish intestinal motility: a review. Fish & Shellfish Immunology, 103, 342-356.

Seropian, I. M., González, G. E., Maller, S. M., Berrocal, D. H., Abbate, A., and Rabinovich, G. A. (2018). Galectin-1 as an emerging mediator of cardiovascular inflammation: mechanisms and therapeutic opportunities. Mediators of Inflammation, 2018, 8696543.

Shamsaie Mehrjan, M., Hosseini Shekarabi, S. P., Mahjob Zardest, M., and Mohammadi, N. (2020). Effect of dietary supplementation of red chili pepper (*Capsicum annuum*) powder on growth parameters, survival rate, hematological indices and some immunity responses of rainbow trout (*Oncorhynchus mykiss*) fry. Iranian Scientific Fisheries Journal, 29, 131-140.

Shaukat, M. N., Nazir, A., and Fallico, B. (2023). Ginger bioactives: a comprehensive review of health benefits and potential food applications. Antioxidants, 12, 2015.

Sheng, Y., Ren, H., Limbu, S. M., Sun, Y., Qiao, F., Zhai, W., et al. (2018). The presence or absence of intestinal microbiota affects lipid deposition and related genes expression in zebrafish (*Danio rerio*). Frontiers in Microbiology, 9, 1124.

Shepherd, J., and Bachis, E. (2014). Changing supply and demand for fish oil. Aquaculture Economics & Management, 18, 395-416.

Siegerstetter, S. C., Petri, R. M., Magowan, E., Lawlor, P. G., Zebeli, Q., O'Connell, N. E., et al. (2018). Fecal microbiota transplant from highly feed-efficient donors shows little effect on age-

related changes in feed-efficiency-associated fecal microbiota from chickens. Applied and Environmental Microbiology, 84, e02330-17.

Silva, F. C. D. P., Nicoli, J. R., Zambonino-Infante, J. L., Kaushik, S., and Gatesoupe, F. J. (2011). Influence of the diet on the microbial diversity of faecal and gastrointestinal contents in gilthead sea bream (*Sparus aurata*) and intestinal contents in goldfish (*Carassius auratus*). FEMS Microbiology Ecology, 78, 285-296.

Singletary, K. (2011). Red pepper: overview of potential health benefits. Nutrition Today, 46, 33-47.

Siyal, F. A., Babazadeh, D., Wang, C., Arain, M. A., Saeed, M., Ayasan, T., et al. (2017). Emulsifiers in the poultry industry. World's Poultry Science Journal, 73, 611-620.

Smith, P., Willemsen, D., Popkes, M., Metge, F., Gandiwa, E., Reichard, M., et al. (2017). Regulation of life span by the gut microbiota in the short-lived African turquoise killifish. Elife, 6, e27014.

Söderberg, J. J., Grgic, M., Hjerde, E., and Haugen, P. (2019). *Aliivibrio wodanis* as a production host: development of genetic tools for expression of cold-active enzymes. Microbial Cell Factories, 18, 197.

Song, J., Luo, C., Liu, Z., Liu, J., Xie, L., Zhang, X., et al. (2023). Early fecal microbiota transplantation from high abdominal fat chickens affects recipient cecal microbiome and metabolism. Frontiers in Microbiology, 14, 1332230.

Song, M., Zhang, F., Chen, L., Yang, Q., Su, H., Yang, X., et al. (2021). Dietary chenodeoxycholic acid improves growth performance and intestinal health by altering serum metabolic profiles and gut bacteria in weaned piglets. Animal Nutrition, 7, 365-375.

Soriano, E. L., Ramírez, D. T., Araujo, D. R., Gómez-Gil, B., Castro, L. I., and Sánchez, C. G. (2018). Effect of temperature and dietary lipid proportion on gut microbiota in yellowtail kingfish *Seriola lalandi* juveniles. Aquaculture, 497, 269-277.

Spier, S. J. (2008). *Corynebacterium pseudotuberculosis* infection in horses: An emerging disease associated with climate change?. Equine Veterinary Education, 20, 37-39.

Srinivasan, K. (2005). Spices as influencers of body metabolism: an overview of three decades of research. Food Research International, 38, 77-86.

Srinivasan, K. (2007). Black pepper and its pungent principle-piperine: a review of diverse physiological effects. Critical reviews in food science and nutrition, 47(8), 735-748.

Staessen, T. W. O., Verdegem, M. C. J., and Schrama, J. W. (2022). Effect of dietary NSP level and bile acid supplementation on nutrient digestibility and the bile acid metabolism in rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 561, 738724.

Stark, A. H., Reifen, R., and Crawford, M. A. (2016). Past and present insights on alpha-linolenic acid and the omega-3 fatty acid family. Critical Reviews in Food Science and Nutrition, 56, 2261-2267.

Stoev, T., and Zhelyazkov, G. (2021). Effect of black pepper (*Piper nigrum*) extract on productive traits, economic efficiency and blood biochemical parameters of rainbow trout (*Oncorhynchus mykiss* W.). Agricultural Science & Technology, 13, 266-271.

Stojancevic, M., Stankov, K., and Mikov, M. (2012). The impact of farnesoid X receptor activation on intestinal permeability in inflammatory bowel disease. Canadian Journal of Gastroenterology and Hepatology, 26, 631-637.

Sugiharto, S., Pratama, A. R., Yudiarti, T., Wahyuni, H. I., Widiastuti, E., and Sartono, T. A. (2020). Effect of acidified turmeric and/or black pepper on growth performance and meat quality of broiler chickens. International Journal of Veterinary Science and Medicine, 8, 85-92.

Sukumaran, V., Park, S. C., and Giri, S. S. (2016). Role of dietary ginger *Zingiber officinale* in improving growth performances and immune functions of *Labeo rohita* fingerlings. Fish & Shellfish Immunology, 57, 362-370.

Sun, L., Xin, Q., Jiao, H., Wang, X., Zhao, J., Li, H., et al. (2023). Effect of exogenous bile salts supplementation on the performance and hepatic lipid metabolism of aged laying hens. Journal of Animal Science, 101, skad334.

Taagepera, R., and Nemčok, M. (2023). World population growth over millennia: Ancient and present phases with a temporary halt in-between. The Anthropocene Review, 11, 163-183.

Talebi, M., Khara, H., Zoriehzahra, J., Ghobadi, S., Khodabandelo, A., and Mirrasooli, E. (2013). Study on effect of red bell pepper on growth, pigmentation and blood factors of rainbow trout (*Oncorhynchus mykiss*). World Journal of Zoology, 8, 17-23.

Talpur, A. D., Ikhwanuddin, M., and Bolong, A. M. A. (2013). Nutritional effects of ginger (*Zingiber officinale* Roscoe) on immune response of Asian sea bass, *Lates calcarifer* (Bloch) and disease resistance against *Vibrio harveyi*. Aquaculture, 400, 46-52.

Tan, P., Dong, X., Mai, K., Xu, W., and Ai, Q. (2016). Vegetable oil induced inflammatory response by altering TLR-NF-κB signalling, macrophages infiltration and polarization in adipose tissue of large yellow croaker (*Larimichthys crocea*). Fish & Shellfish Immunology, 59, 398-405.

Tan, P., Dong, X., Xu, H., Mai, K., & Ai, Q. (2017). Dietary vegetable oil suppressed non-specific immunity and liver antioxidant capacity but induced inflammatory response in Japanese sea bass (*Lateolabrax japonicus*). Fish & Shellfish Immunology, 63, 139-146.

Terova, G., Rimoldi, S., Ascione, C., Gini, E., Ceccotti, C., and Gasco, L. (2019). Rainbow trout (*Oncorhynchus mykiss*) gut microbiota is modulated by insect meal from *Hermetia illucens* prepupae in the diet. Reviews in Fish Biology and Fisheries, 29, 465-486.

Thampi, P. S. S. (2003). A glimpse of the world trade in *Capsicum*. In: De, A. K. (Ed.) *Capsicum*: the Genus *Capsicum* (pp. 16-24). Taylor and Francis, London, UK.

Thilsted, S. H., Thorne-Lyman, A., Webb, P., Bogard, J. R., Subasinghe, R., Phillips, M. J., et al. (2016). Sustaining healthy diets: The role of capture fisheries and aquaculture for improving nutrition in the post-2015 era. Food Policy, 61, 126-131.

Thomas, J., and Kuruvilla, K. M. (2012). Cinnamon. In: Peter, K. V. (Ed.), Handbook of Herbs and Spices (pp. 182-196), Woodhead Publishing, Cambridge, UK.

Thomas, M., Pasquet, A., Aubin, J., Nahon, S., and Lecocq, T. (2021). When more is more: taking advantage of species diversity to move towards sustainable aquaculture. Biological Reviews, 96, 767-784.

Tian, J., Wen, H., Zeng, L. B., Jiang, M., Wu, F., Liu, W., et al. (2013). Changes in the activities and mRNA expression levels of lipoprotein lipase (LPL), hormone-sensitive lipase (HSL) and

fatty acid synthetase (FAS) of Nile tilapia (*Oreochromis niloticus*) during fasting and re-feeding. Aquaculture, 400, 29-35.

Tipsmark, C. K., Sørensen, K. J., Hulgard, K., and Madsen, S. S. (2010). Claudin-15 and-25b expression in the intestinal tract of Atlantic salmon in response to seawater acclimation, smoltification and hormone treatment. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 155, 361-370.

Tocher, D. (2009). Issues surrounding fish as a source of omega-3 long-chain polyunsaturated fatty acids. Lipid Technology, 21, 13-16.

Tocher, D. R. (2003). Metabolism and functions of lipids and fatty acids in teleost fish. Reviews in Fisheries Science, 11, 107-184.

Tocher, D. R. (2015). Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. Aquaculture, 449, 94-107.

Toh, M. C., Goodyear, M., Daigneault, M., Allen-Vercoe, E., and Van Raay, T. J. (2013). Colonizing the embryonic zebrafish gut with anaerobic bacteria derived from the human gastrointestinal tract. Zebrafish, 10, 194-198.

Torrecillas, S., Mompel, D., Caballero, M. J., Montero, D., Merrifield, D., Rodiles, A., et al. (2017). Effect of fishmeal and fish oil replacement by vegetable meals and oils on gut health of European sea bass (*Dicentrarchus labrax*). Aquaculture, 468, 386-398.

Tremaroli, V., Karlsson, F., Werling, M., Ståhlman, M., Kovatcheva-Datchary, P., Olbers, T., et al. (2015). Roux-en-Y gastric bypass and vertical banded gastroplasty induce long-term changes on the human gut microbiome contributing to fat mass regulation. Cell metabolism, 22, 228-238.

Trushenski, J. T. (2009). Saturated lipid sources in feeds for sunshine bass: alterations in production performance and tissue fatty acid composition. North American Journal of Aquaculture, 71, 363-373.

Trushenski, J. T., and Lochmann, R. T. (2009). Potential, implications and solutions regarding the use of rendered animal fats in aquafeeds. American Journal of Animal and Veterinary Sciences, 4, 108-128.

Trushenski, J. T., Blaufuss, P., Mulligan, B., and Laporte, J. (2011). Growth performance and tissue fatty acid composition of rainbow trout reared on feeds containing fish oil or equal blends of fish oil and traditional or novel alternative lipids. North American Journal of Aquaculture, 73, 194-203.

Trushenski, J. T., Boesenberg, J., and Kohler, C. C. (2009). Influence of grow-out feed fatty acid composition on finishing success in Nile tilapia. North American journal of aquaculture, 71, 242-251.

Trushenski, J. T., Lewis, H. A., and Kohler, C. C. (2008). Fatty acid profile of sunshine bass: I. Profile change is affected by initial composition and differs among tissues. Lipids, 43, 629-641.

Tsikliras, A. C., and Polymeros, K. (2014). Fish market prices drive overfishing of the 'big ones'. PeerJ, 2, e638.

Turchini, G. M., and Mailer, R. J. (2011). Rapeseed (canola) oil and other monunsatruated fatty acid-rich vegetable oils. In: Turchini, G. M., Ng, W. K., and Tocher, D. R. (Eds.), Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds (pp. 161–209), CRC Press, Boca Raton, USA.

Turchini, G. M., Torstensen, B. E., and Ng, W. K. (2009). Fish oil replacement in finfish nutrition. Reviews in Aquaculture, 1, 10-57.

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., et al. (2009). A core gut microbiome in obese and lean twins. Nature, 457, 480-484.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., and Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. Nature, 444, 1027-1031.

Ullah, A., Azmat, H., Masood, Z., Arooj, F., Hussain, S. M., and Malik, A. (2021). Impact of dietary leaf extracts of Black pepper *Piper nigrum* L. on the growth, hematological and immunological parameters of *Labeo rohita* (Hamilton, 1822) cultured in glass aquaria. Brazilian Journal of Biology, 83, e246825.

Uren Webster, T. M., Consuegra, S., and de Leaniz, C. G. (2021). Early life stress causes persistent impacts on the microbiome of Atlantic salmon. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 40, 100888.

Valenzuela, M. J., Caruffo, M., Herrera, Y., Medina, D. A., Coronado, M., Feijóo, C. G., et al. (2018). Evaluating the capacity of human gut microorganisms to colonize the zebrafish larvae (*Danio rerio*). Frontiers in Microbiology, 9, 360080.

Van Der Hulst, R. R., Von Meyenfeldt, M. F., Van Kreel, B. K., Thunnissen, F. B., Brummer, R. J. M., Arends, J. W., et al. (1998). Gut permeability, intestinal morphology, and nutritional depletion. Nutrition, 14, 1-6.

Van Vo, B., Siddik, M. A., Fotedar, R., Chaklader, M. R., Hanif, M. A., Foysal, M. J., et al. (2020). Progressive replacement of fishmeal by raw and enzyme-treated alga, *Spirulina platensis* influences growth, intestinal micromorphology and stress response in juvenile barramundi, *Lates calcarifer*. Aquaculture, 529, 735741.

Vang, B., Pedersen, A. M., and Olsen, R. L. (2013). Oil extraction from the copepod *Calanus finmarchicus* using proteolytic enzymes. Journal of Aquatic Food Product Technology, 22, 619-628.

Vasala, P. A. (2012). Ginger. In: Peter, K. V. (Ed.), Handbook of Herbs and Spices (pp. 319-335), Woodhead Publishing, Cambridge, UK.

Verma, R. K., Kumari, P., Maurya, R. K., Kumar, V., Verma, R. B., and Singh, R. K. (2018). Medicinal properties of turmeric (*Curcuma longa* L.): A review. International Journal of Chemical Studies, 6, 1354-1357.

Vessey, D. A., Benfatto, A. M., Zerweck, E., and Vestweber, C. (1990). Purification and characterization of the enzymes of bile acid conjugation from fish liver. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 95, 647-652.

Viver, T., Ruiz, A., Bertomeu, E., Martorell-Barceló, M., Urdiain, M., Grau, A., et al. (2023). Food determines ephemerous and non-stable gut microbiome communities in juvenile wild and farmed Mediterranean fish. Science of The Total Environment, 889, 164080.

Wang, L., Yu, A., Yu, C., Ibrahim, U. B., Chen, J., and Wang, Y. (2023). Curcumin supplementation enhances the feeding and growth of largemouth bass (*Micropterus salmoides*) fed the diet containing 80 g/kg fish meal. Aquaculture Research, 2023, 5454248.

Wang, S., Zhang, W., Cao, A., Pan, Z., and Liu, T. (2022). Dietary supplementation of bile acids in tongue sole (*Cynoglossus semilaevis*): a promising strategy to improve hepatic health status. Frontiers in Marine Science, 9, 899768.

Wang, Y. X., Lee, C. H., Tiep, S., Ruth, T. Y., Ham, J., Kang, H., et al. (2003). Peroxisome-proliferator-activated receptor δ activates fat metabolism to prevent obesity. Cell, 113, 159-170.

Wang, Y., Wang, Q., Xing, K., Jiang, P., and Wang, J. (2021a). Dietary cinnamaldehyde and *Bacillus subtilis* improve growth performance, digestive enzyme activity, and antioxidant capability and shape intestinal microbiota in tongue sole, *Cynoglossus semilaevis*. Aquaculture, 531, 735798.

Wang, M., Wang, K., Liao, X., Hu, H., Chen, L., Meng, L., et al. (2021b). Carnitine palmitoyltransferase system: a new target for anti-inflammatory and anticancer therapy?. Frontiers in Pharmacology, 12, 760581.

Wassef, E. A., Chatzifotis, S., Sakr, E. M., and Saleh, N. E. (2010). Effect of two natural carotenoid sources in diets for gilthead seabream, *Sparus aurata*, on growth and skin coloration. Journal of Applied Aquaculture, 22, 216-229.

Wassef, E. A., Shalaby, S. H., and Saleh, N. E. (2015). Comparative evaluation of sunflower oil and linseed oil as dietary ingredient for gilthead seabream (*Sparus aurata*) fingerlings. OCL, 22, A201.

Weil, C., Lefèvre, F., and Bugeon, J. (2013). Characteristics and metabolism of different adipose tissues in fish. Reviews in Fish Biology and Fisheries, 23, 157-173.

Weisman, J. L., and Miller, D. L. (2006). Lipoid liver disease and steatitis in a captive sapphire damsel, *Pomacentrus pavo*. Acta Ichthyologica et Piscatoria, 36, 99-104.

Wen, J., Mercado, G. P., Volland, A., Doden, H. L., Lickwar, C. R., Crooks, T., et al. (2021). Fxr signaling and microbial metabolism of bile salts in the zebrafish intestine. Science Advances, 7, eabg1371.

Westerterp-Plantenga, M., Diepvens, K., Joosen, A. M., Bérubé-Parent, S., and Tremblay, A. (2006). Metabolic effects of spices, teas, and caffeine. Physiology & Behavior, 89, 85-91.

Wiertsema, S. P., van Bergenhenegouwen, J., Garssen, J., and Knippels, L. M. (2021). The interplay between the gut microbiome and the immune system in the context of infectious diseases throughout life and the role of nutrition in optimizing treatment strategies. Nutrients, 13, 886.

Williams, I. R. (2006). CCR6 and CCL20: partners in intestinal immunity and lymphorganogenesis. Annals of the New York Academy of Sciences, 1072, 52-61.

Wojno, M., Mandas, A., Kwasek, K., and Dabrowski, K. (2021). The effect of dietary supplements of black pepper *Piper nigrum* and turmeric *Curcuma longa* extracts on dietary amino acid utilization and growth performance in common carp. North American Journal of Aquaculture, 83, 155-164.

Wu, D., Wang, H., Xie, L., and Hu, F. (2022). Cross-talk between gut microbiota and adipose tissues in obesity and related metabolic diseases. Frontiers in Endocrinology, 13, 908868.

Wu, S., Pan, M., Zan, Z., Jakovlić, I., Zhao, W., Zou, H., et al. (2024). Regulation of lipid metabolism by gut microbiota in aquatic animals. Reviews in Aquaculture, 16, 34-46.

Xiao, Z., Mescher, M. F., and Jameson, S. C. (2007). Detuning CD8 T cells: down-regulation of CD8 expression, tetramer binding, and response during CTL activation. The Journal of Experimental Medicine, 204, 2667-2677.

Xie, D., Chen, C., Dong, Y., You, C., Wang, S., Monroig, O., et al. (2021). Regulation of long-chain polyunsaturated fatty acid biosynthesis in teleost fish. Progress in Lipid Research, 82, 101095.

Xiong, F., Wu, S. G., Zhang, J., Jakovlić, I., Li, W. X., Zou, H., et al. (2018). Dietary bile salt types influence the composition of biliary bile acids and gut microbiota in grass carp. Frontiers in Microbiology, 9, 2209.

Xu, D., Xiang, X., Li, X., Xu, N., Zhang, W., Mai, K., et al. (2022a). Effects of dietary vegetable oils replacing fish oil on fatty acid composition, lipid metabolism and inflammatory response in adipose tissue of large yellow croaker (*Larimichthys crocea*). Journal of Marine Science and Engineering, 10, 1760.

Xu, J., Li, X., Yao, X., Xie, S., Chi, S., Zhang, S., et al. (2022b). Protective effects of bile acids against hepatic lipid accumulation in hybrid grouper fed a high-lipid diet. Frontiers in Nutrition, 9, 813249.

Xu, R., Li, M., Wang, T., Zhao, Y. W., Shan, C. J., Qiao, F., et al. (2022c). *Bacillus amyloliquefaciens* ameliorates high-carbohydrate diet-induced metabolic phenotypes by restoration of intestinal acetate-producing bacteria in Nile Tilapia. British Journal of Nutrition, 127, 653-665.

Xue, M., Luo, L., Wu, X., Ren, Z., Gao, P., Yu, Y., et al. (2006). Effects of six alternative lipid sources on growth and tissue fatty acid composition in Japanese sea bass (*Lateolabrax japonicus*). Aquaculture, 260, 206-214.

Xun, P., Zhou, C., Huang, X., Huang, Z., Yu, W., Yang, Y., et al. (2023). Effects of dietary sodium acetate on intestinal health of juvenile *Trachinotus ovatus* based on multi-omics approach. Aquaculture, 562, 738776.

Yaakob, Z., Ali, E., Zainal, A., Mohamad, M., and Takriff, M. S. (2014). An overview: biomolecules from microalgae for animal feed and aquaculture. Journal of Biological Research-Thessaloniki, 21, 6.

Yamamoto, T., Suzuki, N., Furuita, H., Sugita, T., Tanaka, N., and Goto, T. (2007). Supplemental effect of bile salts to soybean meal-based diet on growth and feed utilization of rainbow trout *Oncorhynchus mykiss*. Fisheries Science, 73, 123-131.

Yan, H., Ren, J., and Liu, G. H. (2023). Fecal microbiota transplantation: a new strategy to delay aging. Hlife, 1, 8-11.

Yanar, M., Büyükçapar, H. M., and Yanar, Y. (2016). Effects of hot and sweet red peppers (*Capsicum annuum*) as feed supplements on pigmentation, sensory properties and weight gain of rainbow trout (*Oncorhynchus mykiss*). Annals of Animal Science, 16, 825-834.

Yang, B., Huang, S., Zhao, G., and Ma, Q. (2022). Dietary supplementation of porcine bile acids improves laying performance, serum lipid metabolism and cecal microbiota in late-phase laying hens. Animal Nutrition, 11, 283-292.

Yang, C., Diarra, M. S., Choi, J., Rodas-Gonzalez, A., Lepp, D., Liu, S., et al. (2021). Effects of encapsulated cinnamaldehyde on growth performance, intestinal digestive and absorptive functions, meat quality and gut microbiota in broiler chickens. Translational Animal Science, 5, txab099.

Yang, C., Kennes, Y. M., Lepp, D., Yin, X., Wang, Q., Yu, H., et al. (2020). Effects of encapsulated cinnamaldehyde and citral on the performance and cecal microbiota of broilers vaccinated or not vaccinated against coccidiosis. Poultry Science, 99, 936-948.

Yang, H., Xiang, Y., Robinson, K., Wang, J., Zhang, G., Zhao, J., et al. (2018). Gut microbiota is a major contributor to adiposity in pigs. Frontiers in Microbiology, 9, 3045.

Yao, S., Ren, S., Cai, C., Cao, X., Shi, Y., Wu, P., et al. (2022). Glycocholic acid supplementation improved growth performance and alleviated tissue damage in the liver and intestine in *Pelteobagrus fulvidraco* fed a high-pectin diet. Fish Physiology and Biochemistry, 50, 41-57.

Yeh, M. M., Bosch, D. E., and Daoud, S. S. (2019). Role of hepatocyte nuclear factor 4-alpha in gastrointestinal and liver diseases. World Journal of Gastroenterology, 25, 4074-4091.

Yigit, N. O., Ozdal, A. M., Bahadir Koca, S., and Ozmen, O. (2021). Effect on pigmentation, growth and liver histopathology of red pepper (*Capsicum annuum*) added in different ratios to jewel cichlid (*Hemichromis guttatus*) diet. Aquaculture Research, 52, 5579-5584.

Yılmaz, S., and Ergün, S. (2011). Effect of red pepper (*Capsicum annuum*) on pigmentation of blue streak hap (*Labidochromis caeruleus*). The Israeli Journal of Aquaculture - Bamidgeh, 63, IIC:63.2011.633.

Yılmaz, S., Çelik, E. Ş., Ergün, S., Gürkan, M., Kesbic, F. I., and Abdel-Latif, H. M. (2024). The effects of *Capsicum annuum* oleoresin, as a dietary carotenoid, on growth, gut microbiome, intestinal histomorphometry, and sensory characteristics of *Oncorhynchus mykiss*. Journal of the World Aquaculture Society, 55, 149-168.

Yılmaz, S., Ergün, S., and Soytas, N. (2013a). Enhancement of growth performance and pigmentation in red *Oreochromis mossambicus* associated with dietary intake of astaxanthin, paprika, or capsicum. The Israeli Journal of Aquaculture - Bamidgeh, 65, 825-831.

Yılmaz, S., Ergün, S., and Çelik, E. Ş. (2013b). Effect of dietary herbal supplements on some physiological conditions of sea bass *Dicentrarchus labrax*. Journal of Aquatic Animal Health, 25, 98-103.

Yin, P., Xie, S., Zhuang, Z., He, X., Tang, X., Tian, L., et al. (2021). Dietary supplementation of bile acid attenuate adverse effects of high-fat diet on growth performance, antioxidant ability, lipid accumulation and intestinal health in juvenile largemouth bass (*Micropterus salmoides*). Aquaculture, 531, 735864.

Yin, Q., Yu, J., Li, J., Zhang, T., Wang, T., Zhu, Y., et al. (2024). Enhancing milk quality and modulating rectal microbiota of dairy goats in starch-rich diet: the role of bile acid supplementation. Journal of Animal Science and Biotechnology, 15, 7.

Yonar, M. E., Yonar, S. M., İspir, Ü., and Ural, M. Ş. (2019). Effects of curcumin on haematological values, immunity, antioxidant status and resistance of rainbow trout (*Oncorhynchus mykiss*) against *Aeromonas salmonicida* subsp. *achromogenes*. Fish & Shellfish Immunology, 89, 83-90.

Yousefi, M., Hoseini, S. M., Kulikov, E. V., Kharlitskaya, E. V., Petukhov, N. V., and Khomenets, N. G. (2024). Dietary propionate administration improves growth performance, hepatic lipid deposition, and intestinal activity of digestive enzymes, inflammation, bacterial population, and antioxidant capacity in rainbow trout, *Oncorhynchus mykiss*. Aquaculture, 578, 740099.

Yu, C., Zhang, M., Liu, J., Zhang, J., Xu, J., and Xu, W. (2022). Effects of sodium acetate on lipid metabolism, antioxidant capability and cell apoptosis of blunt snout bream (*Megalobrama amblycephala*) hepatocytes treated by sodium palmitate. Aquaculture Research, 53, 1098-1109.

Yu, H., Zhang, L., Chen, P., Liang, X., Cao, A., Han, J., et al. (2019). Dietary bile acids enhance growth, and alleviate hepatic fibrosis induced by a high starch diet via AKT/FOXO1 and cAMP/AMPK/SREBP1 pathway in *Micropterus salmoides*. Frontiers in Physiology, 10, 1430.

Yu, Y. Y., Ding, L. G., Huang, Z. Y., Xu, H. Y., and Xu, Z. (2021). Commensal bacteria-immunity crosstalk shapes mucosal homeostasis in teleost fish. Reviews in Aquaculture, 13, 2322-2343.

Yu, Y., Wang, Q., Huang, Z., Ding, L., and Xu, Z. (2020). Immunoglobulins, mucosal immunity and vaccination in teleost fish. Frontiers in Immunology, 11, 567941.

Yun, B., Xue, M., Wang, J., Fan, Z. Y., Wu, X. F., Zheng, Y. H., et al. (2013). Effects of lipid sources and lipid peroxidation on feed intake, growth, and tissue fatty acid compositions of largemouth bass (*Micropterus salmoides*). Aquaculture International, 21, 97-110.

Zachariah, T. J., and Gobinath, P. (2008). Paprika and chilli. In: Parthasarathy, V. A., Chempakam, B., and Zachariah, J. T. (Eds.), Chemistry of Spices (pp. 260-286), CAB International, Wallingford, UK.

Zhang, J., Kong, X., Zhou, C., Li, L., Nie, G., and Li, X. (2014). Toll-like receptor recognition of bacteria in fish: ligand specificity and signal pathways. Fish & Shellfish Immunology, 41, 380-388.

Zhang, J., Wang, Y., Liu, J., Xu, W., Yin, Z., Liu, Y., et al. (2023). Effects of fecal bacteria on growth, digestive capacity, antioxidant capacity, intestinal health of large yellow croaker (*Larimichthys crocea*) larvae. Aquaculture, 562, 738796.

Zhang, L., Zhang, J., Yan, E., He, J., Zhong, X., Zhang, L., et al. (2020). Dietary supplemented curcumin improves meat quality and antioxidant status of intrauterine growth retardation growing pigs via Nrf2 signal pathway. Animals, 10, 539.

Zhang, Y., Feng, H., Liang, X. F., He, S., Lan, J., and Li, L. (2022a). Dietary bile acids reduce liver lipid deposition via activating farnesoid X receptor, and improve gut health by regulating gut microbiota in Chinese perch (*Siniperca chuatsi*). Fish & Shellfish Immunology, 121, 265-275.

Zhang, X., Hu, Y., Ansari, A. R., Akhtar, M., Chen, Y., Cheng, R., et al. (2022b). Caecal microbiota could effectively increase chicken growth performance by regulating fat metabolism. Microbial Biotechnology, 15, 844-861.

Zhang, Z., Li, Q., Huang, Y., Jiang, B., Li, X., Huang, M., et al. (2022c). Molecular characterization of a novel C-type lectin receptors (CD302) in Nile tilapia (*Oreochromis niloticus*) and its functional analysis in host defense against bacterial infection. Aquaculture Reports, 27, 101405.

Zhao, A., Yu, J., Lew, J. L., Huang, L., Wright, S. D., and Cui, J. (2004). Polyunsaturated fatty acids are FXR ligands and differentially regulate expression of FXR targets. DNA and Cell Biology, 23, 519-526.

Zhao, L., Cheng, L., Hu, Y., Li, X., Yang, Y., Mu, J., et al. (2024). Dietary sodium acetate and sodium butyrate attenuate intestinal damage and improve lipid metabolism in juvenile largemouth bass (*Micropterus salmoides*) fed a high carbohydrate diet by reducing endoplasmic reticulum stress. Animal Nutrition, 16, 443-456.

Zheng, T., Kang, J. H., Sim, J. S., Kim, J. W., Koh, J. T., Shin, C. S., et al. (2017). The farnesoid X receptor negatively regulates osteoclastogenesis in bone remodeling and pathological bone loss. Oncotarget, 8, 76558.

Zhou, H., and Hylemon, P. B. (2014). Bile acids are nutrient signaling hormones. Steroids, 86, 62-68.

Zhou, J. S., Chen, H. J., Ji, H., Shi, X. C., Li, X. X., Chen, L. Q., et al. (2018a). Effect of dietary bile acids on growth, body composition, lipid metabolism and microbiota in grass carp (*Ctenopharyngodon idella*). Aquaculture Nutrition, 24, 802-813.

Zhou, M., Wan, Q., Babu, V. S., Qiu, Q., Kou, H., Lin, C., et al. (2018b). Bacterial features in tilapia (*Oreochromis niloticus*) and environments in a goose-tilapia polyculture model. Aquaculture, 497, 313-319.

Zhou, J. S., Guo, P., Yu, H. B., Ji, H., Lai, Z. W., and Chen, Y. A. (2019). Growth performance, lipid metabolism, and health status of grass carp (*Ctenopharyngodon idella*) fed three different forms of sodium butyrate. Fish Physiology and Biochemistry, 45, 287-298.

Zhou, W. H., Limbu, S. M., Li, R. X., Luo, Y., Ren, J., Qiao, F., et al. (2023). Dietary sodium acetate improves high-fat diet utilization through promoting differential nutrients metabolism between liver and muscle in Nile tilapia (*Oreochromis niloticus*). Aquaculture, 565, 739142.

Zhou, Y., Jiang, W. D., Zhang, J. X., Feng, L., Wu, P., Liu, Y., et al. (2020). Cinnamaldehyde improves the growth performance and digestion and absorption capacity in grass carp (*Ctenopharyngodon idella*). Fish Physiology and Biochemistry, 46, 1589-1601.

Zhu, T., Corraze, G., Plagnes-Juan, E., and Skiba-Cassy, S. (2020). Cholesterol metabolism regulation mediated by SREBP-2, LXRα and miR-33a in rainbow trout (*Oncorhynchus mykiss*) both *in vivo* and *in vitro*. PloS One, 15, e0223813.

Zhu, T., Corraze, G., Plagnes-Juan, E., Quillet, E., Dupont-Nivet, M., and Skiba-Cassy, S. (2018). Regulation of genes related to cholesterol metabolism in rainbow trout (*Oncorhynchus mykiss*) fed a plant-based diet. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 314, R58-R70.

Zou, J., and Secombes, C. J. (2016). The function of fish cytokines. Biology, 5, 23.

APPENDICES

Appendix 1. Extended summary of studies compiling the effect of bile acid/salt dietary supplementation on the growth and feed performance of different fish species.

Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Dietary inclusion level (%)	Growth performance	Feed performance	References
GIFT tilapia (*Oreochromis niloticus*)	8.2 ± 0.2 g	9 weeks	Wet weight (10.3% moisture): 32% CP + 6% CL	Vegetal protein-based diets	69.9% HDCA, 18.9% CDCA, 7.8% HCA (Shangdong Longchang Animal Health Product Co., China)	0.005	=BW_f ↑WGR	=FE	Jiang et al. (2018)
						0.015	↑BW_f ↑WGR	↑FE	
						0.045	=BW_f =WGR	=FE	
						0.135	=BW_f ↓WGR	↓FE	
	2.5 ± 0.01 g	60 days	38% CP + 8% CL	N/I	CA (Himedia Laboratories India Pvt. Ltd.)	0.05	↑WGR ↑SGR	=FCR =PER	Bhusare et al. (2023)
						0.10	↑WGR ↑SGR	=FCR =PER	
			35% CP + 11% CL	N/I	CA (Himedia Laboratories India Pvt. Ltd.)	0.05	↑WGR ↑SGR	=FCR =PER	
						0.10	=WGR =SGR	=FCR =PER	
			32% CP + 14% CL	N/I	CA (Himedia Laboratories India Pvt. Ltd.)	0.05	=WGR =SGR	↓FCR ↑PER	
						0.10	=WGR =SGR	↓FCR =PER	
Hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂)	7.8 ± 0.01 g	8 weeks	48% CP + 15% CL	High-lipid diet	Na T-CA (>95%; Sigma-Aldrich)	0.03	=WGR =SGR	=FI =FCR	Xu et al. (2022b)
						0.06	=WGR =SGR	=FI =FCR	
						0.09	↑WGR ↑SGR	↓FI ↓FCR	
						0.12	=WGR =SGR	=FI =FCR	
						0.15	=WGR =SGR	=FI =FCR	

Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Dietary inclusion level (%)	Growth performance	Feed performance	References
Chinese perch (*Siniperca chuatsi*)	171.3 ± 0.77g	56 days	47% CP + 7% CL	N/I	70.9% HDCA, 20.2% CDCA, 8.0% HCA	0.090	↑BW_f ↑WGR	↓FCR	Zhang et al. (2022a)
Leopard coral grouper (*Plectropomus leopardus*)	13.1 ± 0.14 g	10 weeks	52% CP + 10% CL	N/I	68% HDCA, 17% CDCA, 9% HCA (99%; porcine BS; Shandong Longchang Animal Health Product Co. Ltd., China)	0.15	↑BW_f ↑WGR ↑SGR	=FI =FCR	Gao et al. (2023)
						0.30	↑BW_f ↑WGR ↑SGR	=FI =FCR	
						0.45	=BW_f =WGR ↑SGR	=FI =FCR	
						0.60	=BW_f =WGR =SGR	=FI =FCR	
Large yellow croaker (*Larimichthys crocea*)	12.0 ± 0.20 g	10 weeks	45% CP + 18% CL	N/I	N/I (Bovine BS)	0.015	=BW_f =WGR =SGR	↓FCR ↑PER	Ding et al. (2020)
						0.030	↑BW_f ↑WGR ↑SGR	↓FCR ↑PER	
						0.045	=BW_f =WGR =SGR	↓FCR ↑PER	
	13.1 ± 0.18 g	10 weeks	42% CP + 12% CL	Soybean oil as the main lipid source	HDCA + HCA (≥77%), CDCA (≥17%) (Shandong Longchang Animal Health Product Co. Ltd., China)	0.03	=BW_f =SGR	=FI =FCR	Li et al. (2023)
						0.06	=BW_f =SGR	=FI ↓FCR	
						0.12	=BW_f =SGR	=FI =FCR	
Black seabream (*Acanthopagrus schlegelii*)	2.2 ± 0.00 g	8 weeks	42% CP + 17% CL	Increase of fish oil and decrease of cellulose levels	N/I	0.020	=BW_f =WGR =SGR	=FE	Jin et al. (2019)

Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Dietary inclusion level (%)	Growth performance	Feed performance	References
Largemouth bass (*Micropterus salmoides*)	6.2 ± 0.03 g	70 days	51% CP + 14% CL	N/I	70.9% HDCA, 20.2% CDCA, 8.0% HCA (Shandong Longchang Animal Health Care Co. Ltd., China)	0.008	=BW_f =WGR =SGR	↑FIR =FCR =PPV =PLV	Yu et al. (2019)
						0.016	=BW_f =WGR =SGR	=FIR =FCR =PPV ↑PLV	
						0.024	=BW_f =WGR =SGR	↑FIR =FCR =PPV ↑PLV	
						0.030	↑BW_f ↑WGR ↑SGR	↑FIR =FCR =PPV ↑PLV	
						0.060	=BW_f =WGR =SGR	↑FIR =FCR =PPV =PLV	
	18.4 ± 0.05 g	9 weeks	49% CP + 18% CL	High fat diet	CDCA (≥96%, Sigma-Aldrich)	0.030	=BW_f =WG =SGR	=FI =FCR	Yin et al. (2021)
						0.060	=BW_f =WG =SGR	=FI =FCR	
						0.090	=BW_f =WG =SGR	=FI =FCR	
Grass carp (*Ctenopharyngodon idella*)	69.9 ± 6.24 g	8 weeks	Wet weight (8.5% moisture): 35% CP + 7% CL	Basal diet supplemented with 2% soybean oil	N/I (Longchang Animal Health Products Co. Ltd., China)	0.006	↑BW_f ↑SGR	=FE =PER ↑PPV	Zhou et al. (2018a)

Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Dietary inclusion level (%)	Growth performance	Feed performance	References
Turbot (*Scophthalmus maximus*)	8.5 g	56 days	48% CP + 12% CL	Plant protein meal-based diet	Na T-CA (95%; Aladdin Co., China)	0.5	↑BW_f ↑SGR ↑WGR	=FI ↑FER	Gu et al. (2017)
Tongue sole (*Cynoglossus semiliaevis*)	10.9 ± 0.32 g	8 weeks	53% CP + 8% CL	N/I	69.9% HDCA, 18.9% CDCA, 7.8% HCA (Porcine bile, 99%; Longchang Group, China)	0.030	↑BW_f ↑WGR ↑SGR	N/I	Li et al. (2021b)
						0.090	↑BW_f ↑WGR ↑SGR	N/I	
	13.1 ± 2.4 g	10 weeks	53% CP + 8% CL	N/I	69.9% HDCA, 18.9% CDCA, 7.8% HCA (99%; BS from livestock and poultry; Longchang Group, China)	0.030	↑BW_f ↑WGR ↑SGR	N/I	Wang et al. (2022)
						0.090	↑BW_f ↑WGR ↑SGR	N/I	
Rainbow trout (*Oncorhynchus mykiss*)	13.3 ± 1.1 g	10 weeks	43% CP + 14% CL	Total replacement of fish meal by defatted soybean meal and corn gluten meal	N/I (Bovine BS; Wako Pure Chemicals, Japan)	1.5	↑BW_f ↑SGR	=FI ↑FER	Yamamoto et al. (2007)
	11.3 ± 1.3 g	10 weeks	43% CP + 15% CL	Total replacement of fish meal by defatted soybean meal and corn gluten meal	N/I (Bovine gall powder, with 70% of BS content; Wako Pure Chemicals, Japan)	1.5	↑BWf ↑SGR	=FI ↑FER	Iwashita et al. (2008)
					Na T-CA (> 95%; Sigma Aldrich, USA)	1.0	↑BWf ↑SGR	=FI ↑FER	
	10 g	6 weeks	44% CP + 15% CL	Defatted soybean meal-based diet supplemented with 0.38% soya saponin	Na T-CA (> 95%; Sigma-Aldrich, USA)	1.0	=BW_f	=FI	Iwashita et al. (2009)
				Defatted soybean meal-based diet supplemented with 0.38% soya saponin and 0.0075% soya lectin	Na T-CA (> 95%; Sigma-Aldrich, USA)	1.0	=BW_f	=FI	

Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Dietary inclusion level (%)	Growth performance	Feed performance	References
Atlantic salmon (*Salmo salar*)	362 ± 95 g	77 days	41% CP + 30% CL	Partial replacement of fish meal by soy protein concentrate and pea protein concentrate	Na T-CA (>95%; HC Handelscenter, Denmark)	1.8	=BW_f =TGC =SGR	N/I	Kortner et al. (2016)
					N/I (Bovine BS; HC Handelscenter, Denmark)	1.8	=BW_f ↓TGC ↓SGR	N/I	
				Partial replacement of fish meal by soya protein	N/I (Bovine BS; HC Handelscenter, Denmark)	1.8	=BW_f =TGC =SGR	N/I	
Striped catfish (*Pangasianodon hypophthalmus*)	10.3 ± 0.20 g	70 days	32% CP + 5% CL	N/I	6.2% HCA, 13.8% CDCA, 53.1% HDCA (Lachance, RUNEON, China)	0.025	↑BW_f ↑WG ↑ADG ↑SGR	=FI ↓FCR ↑PER ↑PPV	Adam et al. (2023)
						0.050	↑BW_f ↑WG ↑ADG ↑SGR	↑FI ↓FCR ↑PER ↑PPV	
						0.075	↑BW_f ↑WG ↑ADG ↑SGR	↑FI ↓FCR ↑PER ↑PPV	
						0.100	↑BW_f ↑WG ↑ADG ↑SGR	↑FI ↓FCR ↑PER ↑PPV	
						0.125	↑BW_f ↑WG ↑ADG ↑SGR	↑FI ↓FCR ↑PER ↑PPV	
						0.150	↑BW_f ↑WG ↑ADG ↑SGR	↑FI ↓FCR =PER =PPV	

Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Dietary inclusion level (%)	Growth performance	Feed performance	References
Yellow catfish (*Pelteobagrus fulvidraco*)	19 ± 5 g	56 days	37% CP + 5% CL	High-pectin diet	G-CA (≥97%; Suzhou Ketong Biomedical Technology Co. Ltd., China)	0.06	↑BW_f ↑SGR	=FIR ↓FCR	Yao et al. (2022)

Abbreviations:

- CP, crude protein
- CL, crude lipid
- BS, bile salts
- CA, cholic acid
- CDCA, chenodeoxycholic acid
- HCA, hyocholic acid
- HDCA, hyodeoxycholic acid
- T-CA, taurocholic acid
- G-CA, glycocholic acid
- Na T-CA, sodium taurocholate
- BW_i, Initial Body Weight
- BW_f, Final Body Weight
- WG, Weight Gain (WG (g) = BW_f - BW_i)
- WGR, Weight Gain Rate (WGR (%) = 100 x WG / BW_i)
- ADG, Average Daily Gain (g/day)
- SGR, Specific Growth Rate
- FI, Feed Intake
- TGC, Thermal Growth Coefficient (TGC = 1000 x ($BW_f^{1/3}$ - $BW_i^{1/3}$) / (average temperature in ^oC x days))
- FCR, Feed Conversion Ratio
- FER, Feed Efficiency Ratio (FER = WG / feed intake)
- FE, Feed Efficiency (FE (%) = 100 x FER)
- FIR, Feed Intake Ratio, Voluntary Feed Intake, or Feeding Rate (FIR (%/day) = 100 x feed intake / [(BW_f + BW_i) / 2] / days
- PER, Protein Efficiency Ratio (PER = biomass increase / protein intake)
- PPV, Productive Protein Value (PPV (%) = 100 x protein gain / protein intake)
- PLV, Productive Lipid Value (PLV (%) = 100 x lipid gain / lipid intake)
- GIFT, genetically improved farmed tilapia
- N/I, not identified or not assessed

Appendix 2. Extended summary of studies compiling the effect of capsicum (*Capsicum* spp.), black pepper (*Piper nigrum*), ginger (*Zingiber officinale*), turmeric (*Curcuma longa*), and cinnamaldehyde on the growth and feed performance of different fish species.

Spice/active principle	Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Additive format	Dietary inclusion level (%)	Growth perf.	Feed perf.	References
Capsicum (*Capsicum* spp.)	Blue streak hap (*Labidochromis caeruleus*)	1.1 ± 0.02 g	45 days	34% CP + 13% CL	N/I	Capsicum	Powder (meal)	2	$=BW_f$ =WGR =SGR	=FCR	Yılmaz and Ergün (2011)
								5	$=BW_f$ =WGR =SGR	=FCR	
	Jewel cichlid (*Hemichromis guttatus*)	3.4 ± 0.03 g	90 days	37% CP + 11% CL	N/I	Capsicum (from a local market in Turkey)	Powder (flour)	3	$=BW_f$ =WG =SGR	=FCR	Yigit et al. (2021)
								7	$=BW_f$ =WG =SGR	=FCR	
								11	$=BW_f$ =WG =SGR	=FCR	
								15	$=BW_f$ =WG =SGR	=FCR	
	Gilthead seabream (*Sparus aurata*)	94.9 ± 0.3 g	6 weeks	48% CP + 13% CL	N/I	Edible portion of capsicum devoid of stem ends, seeds, and core (from a local market in Alexandria, Egypt, harvested on 2007 season)	Powder (meal)	0.3	$=BW_f$ =WG =SGR	=FI =FCR	Wassef et al. (2010)
	Mozambique tilapia (*Oreochromis mossambicus*)	5 g	45 days	37% CP + 10% CL	N/I	Capsicum	Oleoresin (from Kutluer, Turkey)	0.7	$=BW_f$ =WG =SGR	=FCR	Yılmaz et al. (2013a)
								1.4	$=BW_f$ =WG =SGR	=FCR	

Spice/active principle	Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Additive format	Dietary inclusion level (%)	Growth perf.	Feed perf.	References
Capsicum (*Capsicum* spp.)	Nile tilapia (*Oreochromis niloticus*)	22.3 ± 0.19 g	60 days	37% CP + 10% CL	N/I	Capsicum (from a local market in Zagazig, Egypt)	Powder	0.04	↑BW_f ↑WG =SGR	=FI ↓FCR ↑PER	Ibrahim et al. (2024)
								0.08	↑BW_f ↑WG =SGR	=FI ↓FCR ↑PER	
								0.16	↑BW_f ↑WG ↑SGR	=FI ↓FCR ↑PER	
	Rainbow trout (*Oncorhynchus mykiss*)	93 ± 0.64 g	20 days	40% CP + 12% CL	N/I	Capsicum (from local producers from Iran)	Powder	0.0033	=BW ↑TL	N/I	Talebi et al. (2013)
								0.0044	↑BW ↑TL	N/I	
								0.0055	↑BW ↑TL	N/I	
			40 days	40% CP + 12% CL	N/I	Capsicum (from local producers from Iran)	Powder	0.0033	↑BW ↑TL	N/I	
								0.0044	↑BW ↑TL	N/I	
								0.0055	↑BW ↑TL	N/I	
			60 days	40% CP + 12% CL	N/I	Capsicum (from local producers from Iran)	Powder	0.0033	↑BW_f ↑TL =SGR =WGR	=FCR	
								0.0044	↑BW_f ↑TL =WGR =SGR	=FCR	
								0.0055	↑BW_f ↑TL =WGR =SGR	=FCR	

Spice/active principle	Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Additive format	Dietary inclusion level (%)	Growth perf.	Feed perf.	References
Capsicum (*Capsicum* spp.)	Rainbow trout (*Oncorhynchus mykiss*)	58–60 g	20 days	45% CP + 20% CL	N/I	Leaves of capsicum (Kahramanmaraş, Turkey, harvested in September)	Meal (extracted with acetone)	0.5	=BW	N/I	Yanar et al. (2016)
								2	=BW	N/I	
								4.4	=BW	N/I	
			40 days	45% CP + 20% CL	N/I	Leaves of capsicum (Kahramanmaraş, Turkey, harvested in September)	Meal (extracted with acetone)	0.5	=BW	N/I	
								2	=BW	N/I	
								4.4	=BW	N/I	
			60 days	45% CP + 20% CL	N/I	Leaves of capsicum (Kahramanmaraş, Turkey, harvested in September)	Meal (extracted with acetone)	0.5	=BW	N/I	
								2	=BW	N/I	
								4.4	=BW	N/I	
			80 days	45% CP + 20% CL	N/I	Leaves of capsicum (Kahramanmaraş, Turkey, harvested in September)	Meal (extracted with acetone)	0.5	$=BW_f$ =TL =SGR	=FCR	
								2	$=BW_f$ =TL =SGR	=FCR	
								4.4	$=BW_f$ =TL =SGR	=FCR	
		195.1 ± 1.55 g	30 days	14% CP + 8% CL	N/I	Capsicum	Oleoresin (from Smart Kimya Tic. ve Danışmanlık Ltd., Turkey)	0.7	$\uparrow BW_f$ $\uparrow$WGR $\uparrow$SGR	$\downarrow$FCR	Yılmaz et al. (2024)
								1.4	$\uparrow BW_f$ $\uparrow$WGR $\uparrow$SGR	$\downarrow$FCR	
								2.1	$=BW_f$ =WGR =SGR	=FCR	
								2.8	$=BW_f$ =WGR =SGR	=FCR	

Spice/active principle	Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Additive format	Dietary inclusion level (%)	Growth perf.	Feed perf.	References
Black pepper (*Piper nigrum*) / piperine	African catfish (*Clarias gariepinus*)	60.3 ± 0.44 g	30 days	31% CP + 7% CL	Basal diet supplemented with 0.5% turmeric	Black pepper	Powder (from a local market in Zagazig, Egypt)	0.1	$=BW_f$ =WG =SGR	N/I	El-Houseiny et al. (2019)
					Basal diet supplemented with 0.5% turmeric and fish exposed to cadmium at 0.8 mg/L in the water	Black pepper	Powder (from a local market in Zagazig, Egypt)	0.1	$=BW_f$ =WG =SGR	N/I	
	Rohu (*Labeo rohita*)	22.1 ± 0.98g	12 weeks	34% CP (%CL N/I)	N/I	Leaves of black pepper (collected during October-December 2019 from nursery farms in District Kasur, Pakistan)	Powder (extracted with methanol)	1	=TL ↓FL ↑WG	↑FI ↑FCR	Ullah et al. (2023)
								2	↑TL ↑FL ↑WG	↑FI ↑FCR	
								3	↓TL ↓FL =WG	↑FI ↑FCR	
	Common carp (*Cyprinus carpio*)	3.8 ± 0.12 g	40 days	53% CP + 7% CL	Diet based on casein, gelatin, and free amino acids supplemented with 0.4% methionine	Piperine	Powder (>97%; Sigma-Aldrich)	0.02	=WG	↑FCR	Wojno et al. (2021)
						Fruits of black pepper (from a local vendor from Ohio)	Powder (extracted with hexane and filtered)	0.02	↓WG	=FCR	

Spice/active principle	Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Additive format	Dietary inclusion level (%)	Growth perf.	Feed perf.	References
Black pepper (*Piper nigrum*) / piperine	Common carp (*Cyprinus carpio*)	12.3 ± 0.31 g	8 weeks	33% CP + 9% CL	N/I	Piperine	Powder (98%; from Senran-Shengwu, China)	0.05	=BW_f =WGR =SGR	=FCR	Giri et al. (2023)
								0.1	=BW_f =WGR ↑SGR	↓FCR	
								0.2	↑BW_f ↑WGR ↑SGR	↓FCR	
								0.3	=BW_f ↑WGR ↑SGR	=FCR	
								0.4	=BW_f ↑WGR =SGR	=FCR	
	Rainbow trout (*Oncorhynchus mykiss*)	41 ± 8 g	30 days	45% CP + 16% CL	N/I	Black pepper	Powder	0.1	=BW	N/I	Stoev and Zhelyazkov (2021)
			60 days	45% CP + 16% CL	N/I	Black pepper	Powder	0.1	=BW_f =WG	↓FCR	
	Olive flounder (*Paralichthys olivaceus*)	27.6 ± 0.4 g	8 weeks	50% CP + 11% CL	N/I	Piperine (from Synergen, South Korea)	N/I	0.025	=BW_f =WG =SGR	=FCR =PER	Malintha et al. (2023)
								0.050	↑BW_f ↑WG ↑SGR	=FCR ↑PER	
								0.075	=BW_f =WG ↑SGR	=FCR ↑PER	
								0.1	=BW_f =WG =SGR	=FCR =PER	
								0.2	=BW_f =WG =SGR	=FCR =PER	

Spice/active principle	Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Additive format	Dietary inclusion level (%)	Growth perf.	Feed perf.	References
Ginger (*Zingiber officinale)*	Asian sea bass (*Lates calcarifer*)	18 ± 1 g	15 days	Wet weight (9.7% moisture): 42% CP + 17% CL	N/I	Peeled rhizomes of ginger (from the local market in Kuala Terengganu, Malaysia)	Powder (extracted with methanol)	0.1	↑WGR ↑SGR	↓FCR	Talpur et al. (2013)
								0.2	↑WGR ↑SGR	↓FCR	
								0.3	↑WGR ↑SGR	↓FCR	
								0.5	↑WGR ↑SGR	↓FCR	
								1	↑WGR ↑SGR	↓FCR	
	Rohu (*Labeo rohita*)	12.3 ± 0.11 g	30 days	28% CP + 7% CL	N/I	Peeled rhizomes of ginger (from a local market in Thanjavur, India)	Powder	0.2	=WG =SGR	=FI =FCR	Sukumaran et al. (2016)
								0.4	=WG =SGR	=FI =FCR	
								0.6	=WG =SGR	↑FI =FCR	
								0.8	↑WG ↑SGR	↑FI ↓FCR	
								1	=WG ↑SGR	↑FI =FCR	
			60 days	28% CP + 7% CL	N/I	Peeled rhizomes of ginger (from a local market in Thanjavur, India)	Powder	0.2	=WG =WGR =SGR	=FI =FCR	
								0.4	=WG =WGR =SGR	=FI =FCR	
								0.6	↑WG ↑WGR ↑SGR	=FI ↓FCR	
								0.8	↑WG ↑WGR ↑SGR	=FI ↓FCR	
								1	↑WG ↑WGR ↑SGR	=FI ↓FCR	

Spice/active principle	Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Additive format	Dietary inclusion level (%)	Growth perf.	Feed perf.	References
Ginger (*Zingiber officinale*)	Common carp (*Cyprinus carpio*)	16 g	60 days	41% CP + 9% CL	Fish were reared at high stocking densities (20 kg/m^3)	Ginger (from a local shop in Iran)	Powder	0.5	=BW_f ↑WGR ↑SGR	↓FCR	Fazelan et al. (2020)
								1	=BW_f ↑WGR ↑SGR	↓FCR	
		10.9 ± 0.17 g	60 days	Wet weight (5-11% moisture): 38-41% CP + 4-16% CL	N/I	Rhizomes of ginger (from Zarringiah medicinal plants company in Urmia, Iran)	Powder (extracted with ethanol)	0.1	=BW_f =WG ↑SGR	↓FCR	Mohammadi et al. (2020)
								0.2	↑BW_f ↑WG ↑SGR	↓FCR	
								0.4	↑BW_f ↑WG ↑SGR	↓FCR	
	Striped catfish (*Pangasianodon hypophthalmus*)	19.93 ± 0.29 g	90 days	25% CP + 6% CL	N/I	Rhizomes of ginger	Powder (from a local market in Egypt)	0.5	↑BW_f ↑WG ↑SGR	=FI ↓FCR ↑PER	Ashry et al. (2023)
								1	↑BW_f ↑WG ↑SGR	=FI ↓FCR ↑PER	
								1.5	↑BW_f ↑WG ↑SGR	=FI ↓FCR ↑PER	
	Rainbow trout (*Oncorhynchus mykiss*)	7.5 ± 0.1 g	8 weeks	46% CP + 13% CL	N/I	Rhizomes of ginger	Powder (extracted with ethanol, from Saha Jesa Medicinal Plants Co., Iran)	0.5	↑BW_f ↑WG =WGR =SGR	=FI =FCR ↑PER ↑LER	Aqmasjed et al. (2023)

Spice/active principle	Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Additive format	Dietary inclusion level (%)	Growth perf.	Feed perf.	References
Ginger (*Zingiber officinale)*	Nile tilapia (*Oreochromis niloticus*)	1.8 ± 0.52 g	55 days	Wet weight (12.5% moisture): 40% CP + 5% CL	N/I	Rhizomes of ginger (harvested in the State of Amazonas, Brazil)	Essential oil (extracted by hydro-distillation)	0.5	= BW_f =TL =SGR	=FCR	Brum et al. (2017)
								1	= BW_f =TL =SGR	=FCR	
								1.5	↓BW_f ↓TL ↓SGR	↑FCR	
Turmeric (*Curcuma longa*) / curcumin	Gilthead seabream (*Sparus aurata*)	20.0 ± 0.37 g	150 days	44% CP + 16% CL	N/I	Curcumin	Powder (from a local market in Egypt)	1.5	=BW_f =WG =SGR	↑FCR =PER	Ashry et al. (2021)
								2	↑BW_f ↑WG =SGR	=FCR =PER	
								2.5	↑BW_f ↑WG =SGR	↓FCR =PER	
								3	↑BW_f ↑WG ↑SGR	↓FCR ↑PER	
	Large yellow croaker (*Larimichthys crocea*)	15.9 ± 0.16 g (SEM)	10 weeks	43% CP + 18% CL	High-fat diet	Turmeric	N/I	0.02	=BW_f =WGR =SGR	N/I	Ji et al. (2021)
								0.04	↑BW_f ↑WGR ↑SGR	N/I	
								0.06	=BW_f =WGR =SGR	N/I	
	Largemouth bass (*Micropterus salmoides*)	37.8 ± 0.2 g	8 weeks	52% CP + 12% CL	80% fish meal replacement by poultry meal	Curcumin	Powder (from Dulai Biotechnology Co., China)	0.5	=BW_f =WG	=FI =FCR	Wang et al. (2023)
								1	↑BW_f ↑WG	↑FI =FCR	

Spice/active principle	Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Additive format	Dietary inclusion level (%)	Growth perf.	Feed perf.	References
Turmeric (*Curcuma longa*) / curcumin	Common carp (*Cyprinus Carpio*)	1.4 ± 0.06 g	10 weeks	9% CP + 4% CL	N/I	Turmeric	Powder (from a local market in Egypt)	0.1	↑BW_f ↑WGR =SGR	↑FI =FCR =PER	Abdel-Tawwab and Abbass (2017)
								0.2	↑BW_f ↑WGR =SGR	↑FI =FCR =PER	
								0.5	↑BW_f ↑WGR =SGR	↑FI =FCR =PER	
		3.8 ± 0.12 g	40 days	53% CP + 7% CL	Diet based on casein, gelatin, and free amino acids supplemented with 0.4% methionine	Turmeric (Verdure Sciences, Noblesville, Indiana)	N/I	0.02	=WG	=FCR	Wojno et al. (2021)
	Crucian carp (*Carassius auratus*)	76.3 ± 0.10 g	105 days	37% CP + 8% CL	N/I	Curcumin	Powder (Sigma-Aldrich Co., USA)	0.1	=BW_f =WGR	=FI ↑FER	Jiang et al. (2016)
								0.5	↑BW_f ↑WGR	=FI ↑FER	
	Grass carp (*Ctenopharyngodon idella*)	5.3 ± 0.10 g	60 days	31% CP + 5% CL	N/I	Curcumin	Powder (Sigma-Aldrich Co., USA)	0.02	=BW_f ↑WGR ↑SGR	↓FCR	Ming et al. (2020)
								0.04	↑BW_f ↑WGR ↑SGR	↓FCR	
								0.06	↑BW_f ↑WGR ↑SGR	↓FCR	
								0.08	↑BW_f ↑WGR ↑SGR	↓FCR	

Spice/active principle	Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Additive format	Dietary inclusion level (%)	Growth perf.	Feed perf.	References
Turmeric (*Curcuma longa*) / curcumin	African catfish (*Clarias gariepinus*)	60.3 ± 0.44 g	30 days	31% CP + 7% CL	N/I	Turmeric	Powder (from a local market in Zagazig, Egypt)	0.1	$=BW_f$ =WG =SGR	N/I	El-Houseiny et al. (2019)
					Fish were exposed to cadmium at 0.8 mg/L in the water	Turmeric	Powder (from a local market in Zagazig, Egypt)	0.1	$\uparrow BW_f$ =WG =SGR	N/I	
	Rainbow trout (*Oncorhynchus mykiss*)	31.3 ± 1.17 g	8 weeks	47% CP + 10% CL	N/I	Curcumin	Powder (from Merck)	1	$\uparrow BW_f$ ↑WG ↑SGR	↓FCR	Yonar et al. (2019)
								2	$\uparrow BW_f$ ↑WG ↑SGR	↓FCR	
								4	$\uparrow BW_f$ ↑WG ↑SGR	↓FCR	
		7.5 ± 0.1 g	8 weeks	46% CP + 13% CL	N/I	Curcumin	Powder (from Dineh Pharmaceutical Co., Iran)	0.5	$\uparrow BW_f$ ↑WG =WGR =SGR	=FCR ↑PER ↑LER	Aqmasjed et al. (2023)
	Nile tilapia (*Oreochromis niloticus*)	60 ± 5 g	7 weeks	32% CP (%CL N/I)	N/I	Turmeric	Powder	1	$\uparrow BW_f$ ↑WG	↓FCR	Diab et al. (2014)
								2	$\uparrow BW_f$ ↑WG	↓FCR	
Cinnamaldehyde	Tongue sole (*Cynoglossus semilaevis*)	188 ± 5 g	60 days	52% CP + 14% CL	N/I	Cinnamaldehyde, lecithin, α-tocopherol, ethanol, and potassium dihydrogen phosphate	Liposome-encapsulated product	0.1	$=BW_f$ ↑WGR ↑SGR	↓FCR ↑PER	Wang et al. (2021a)
					Diet supplemented with 10^7 *Bacillus subtilis*		Liposome-encapsulated product	0.1	$\uparrow BW_f$ ↑WGR ↑SGR	↓FCR ↑PER	

Spice/active principle	Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Additive format	Dietary inclusion level (%)	Growth perf.	Feed perf.	References
Cinnamaldehyde	Fat greenling (*Hexagrammos otakii*)	6.2 ± 0.19 g	8 weeks	51% CP + 10% CL	N/I	Cinnamaldehyde (from Improved McLin Biotech Co., Shanghai, China)	N/I	0.02	↑WGR ↑SGR	↓FCR	Gu et al. (2022)
								0.04	↑WGR ↑SGR	↓FCR	
								0.06	↑WGR ↑SGR	↓FCR	
								0.08	↑WGR ↑SGR	↓FCR	
								0.10	↑WGR ↑SGR	↓FCR	
	Grass carp (*Ctenopharyngodon idella*)	227.3 ± 0.46 g	60 days	28% CP + 4% CL	N/I	Cinnamaldehyde (>98%; from the Shanghai Menon Animal Nutrition Technology Co. Ltd., China) diluted to 18% with silicon dioxide to enhance it stability	Essential oil	0.02 (0.004% cin.)	↑BW_f ↑WGR ↑SGR	↑FI ↑FE	Zhou et al. (2020)
								0.04 (0.007% cin.)	↑BW_f ↑WGR ↑SGR	↑FI ↑FE	
								0.06 (0.011% cin.)	↑BW_f ↑WGR ↑SGR	↑FI ↑FE	
								0.08 (0.014% cin.)	↑BW_f ↑WGR ↑SGR	↑FI ↑FE	
	Nile tilapia (*Oreochromis niloticus*)	10.2 ± 0.06 g	15 days	33% CP + 9% CL	N/I	Cinnamaldehyde (from Flaka Chemical, Switzerland)	Essential oil (≥98%)	0.105	=BW =WG	=FI =FCR	Amer et al. (2018)
								0.210	=BW =WG	=FI =FCR	
			75 days	33% CP + 9% CL	N/I	Cinnamaldehyde (from Flaka Chemical, Switzerland)	Essential oil (≥98%)	0.105	=BW_f =WG =ADG =SGR	=FI =FCR =PER	
								0.210	=BW_f =WG =ADG =SGR	=FI =FCR =PER	

Spice/active principle	Fish species	Fish BW_i	Feeding period	Diet composition (dry-matter basis)	Remarks	Additive composition (origin if given)	Additive format	Dietary inclusion level (%)	Growth perf.	Feed perf.	References
Cinnamaldehyde	Nile tilapia (*Oreochromis niloticus*)	20.6 ± 0.15 g	12 weeks	32% CP + 8% CL	N/I	Cinnamaldehyde, polyoxyethylene, monooleate (Tween 80, food grade), sodium alginate (medium viscosity, A-2033) (from Sigma-Aldrich Co., St. Louis, USA)	Nanoemulsion	0.01	↑BW_f ↑WG ↑WGR ↑SGR	↓FI ↓FCR ↑PER	Abd El-Hamid et al. (2021)
								0.02	↑BW_f ↑WG ↑WGR ↑SGR	↓FI ↓FCR ↑PER	
								0.03	↑BW_f ↑WG ↑WGR ↑SGR	↓FI ↓FCR ↑PER	

Abbreviations:

- CP, crude protein
- CL, crude lipid
- BW, Body Weight
- BW_i, Initial Body Weight
- BW_f, Final Body Weight
- TL, Total Length
- FL, Fork Length
- WG, Weight Gain (WG (g) = BW_f - BW_i)
- WGR, Weight Gain Rate (WGR (%) = 100 x WG / BW_i)
- ADG, Average Daily Gain (g/day)
- SGR, Specific Growth Rate
- FI, Feed Intake
- FCR, Feed Conversion Ratio
- FER, Feed Efficiency Ratio (FER = WG / feed intake)
- FE, Feed Efficiency (FE (%) = 100 x FER)
- PER, Protein Efficiency Ratio (PER = biomass increase / protein intake)
- LER, Lipid Efficiency Ratio (PER = biomass increase / lipid intake)
- N/I, not identified or not assessed

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