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Impact of dietary porcine blood by-products in meagre (*Argyrosomus regius*) physiology, evaluated by welfare biomarkers and the antibacterial properties of the skin mucus

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ABSTRACT

Tools are required for quick and easy preliminary evaluation of functional feeds efficiency on fisheries. The analysis of skin mucus biomarkers is a recent alternative approach providing a faster feed-back from the laboratory which is characterized by being less invasive, more rapid and with reduced costs. The effect of replacing fishmeal and fish protein hydrolysates by means of two porcine by-products, the porcine spray-dried plasma (SDPP) and pig protein hydrolysate (PPH), in compound diets (50.4% crude protein, 16.2% crude protein, 22.1 MJ/kg feed) was evaluated in juvenile meagre (*Argyrosomus regius*) during a two-months period. To determine the impact of these dietary replacements, growth and food performance were measured together with digestive enzymes activities and filet proximal composition. Additionally, skin mucus was collected and characterized by determining main mucus biomarkers (protein, glucose, lactate, cortisol, and antioxidant capacity) and its antibacterial properties, measured by the quick *in vitro* co-culture challenges. In comparison to the control group, the inclusion of PPH and SDPP, in meagre diets reduced growth (7.4–8.8% in body weight), increased feed conversion ratios (9.0–10.0%), results that were attributed to a reduction in feed intake values (24.2–33.0%) ($P < 0.05$). Porcine blood by-products did not modify the activity of gastric and pancreatic digestive enzymes as well as those involved in nutrient absorption (alkaline phosphatase) nor liver oxidative stress condition ($P > 0.05$). In contrast, a reduction in fillet lipid content associated to an increase in fillet protein levels were found in fish fed SDPP and PPH diets ($P < 0.05$). As compared to the control diet, the dietary replacement did not alter the levels of the skin mucus biomarkers related to stress (cortisol and antioxidant capacity) or nutritional status (soluble protein, glucose and lactate) ($P > 0.05$). Interestingly, regardless of the worst performance in somatic growth, meagre fed diets containing both tested porcine by-products showed a significantly improved antibacterial capacity of their skin mucus. This enhancement was more prominent for fish fed with the PPH diet, which may be attributed to a higher content of immunomodulatory bioactive compounds in PPH. Further research will be necessary to provide insights on how the inclusion of SDPP and PPH, at the expense of dietary fishmeal and fish protein hydrolysates, affects feed intake and growth performance in meagre. However, the use of skin mucus biomarkers has been demonstrated to be an excellent methodology for a preliminary characterization of the functional feeds, in particular for their prophylactic properties by the study of mucus antibacterial activity.

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1. Introduction

Diet formulation is a critical issue in terms of fish performance. Several studies reported that diet promotes and regulates specific metabolic pathways and resistance to environmental stressors and pathogenic organisms; thereby, improving survival, growth, development, health, welfare and reproductive capacity in fish [1,2]. During the lasts years, the feed manufacture industry focused in developing feed formulations that promotes fish health and welfare without compromising somatic growth or product final quality [3]. Thus, the development of the so-called functional feeds is nowadays part of the business plan for all aquafeed companies. Moreover, functional feeds aim to reduce the use of antibiotics, which its use as growth promoters was banned in Europe (Regulation (EC) 1831/2003 of the European Parliament and of the Council). A long and growing list of functional additives and ingredients exists in the field of animal production, among which a lot of them have been tested and validated due to their properties in functional feeds for aquatic animals as immunomodulators [4,5]. Their effects may vary among fish species depending on the composition, route, dose and duration of administration, and association/interaction with other feed components [5]. In the current study, we focused in two by-products from the pig industry, such as the porcine spray-dried plasma (SDPP) and pig protein hydrolysate (PPH). SDPP has been reported to promote fish growth due to its high digestibility and the improvement of feed intake and feed efficiency parameters [6,7]. Moreover, SDPP has been recommended for livestock nutrition as a source of immunological support due to its content in immunoglobulins and bioactive peptides [8]. Recently, the effects of SDPP on gilthead sea bream skin mucosa and mucus composition was investigated [9]. We reported that the nutritional stimulus from SDPP supplementation favoured the stimulation of the skin mucosa cell protein turnover and the activation of the exudation machinery, resulting in putatively higher antioxidant and antimicrobial properties. The benefits of the dietary inclusion of fish protein hydrolysates have been extensively studied in several fish species. The benefits of such ingredients have been reported in terms of fish growth, survival, feed utilization, immune response and disease resistance [10]. Regarding the use of livestock by-products like plasma protein hydrolysates as functional feed additives, they have not extensively evaluated in fish [11], regardless their promising properties as functional ingredients due to their content in bioactive peptides with antioxidant and antimicrobial properties [12,13].

Studies evaluating functional feeds and their efficacy are long, complex and expensive, especially at the bench level when biological samples have to be processed and analysed. Thus, novel tools are required to preliminarily evaluate the efficiency of functional feeds, and these tools should be user-friendly (*i.e.*, quick, and easy to use). It is well known that feeding regimes and diet composition modulate plasma haematological and biochemical parameters, and these could be used as potential biomarkers for assessing the functional and nutritional status of the organism [14]. In this sense, the analysis of the skin mucus components and their properties could be an alternative reliable methodology, minimally invasive, as it was recently proposed during evaluation of the effects of stress and other physiological responses in fish [15–20]. However, there is limited information about the use of skin mucus biomarkers when evaluating functional diets [21].

Skin mucus acts as a natural, physical, biochemical, dynamic, and semipermeable barrier that accomplish a number of important functions in fish, such as osmoregulation, respiration, nutrition and locomotion [22]. However, acting as a mechanical protective barrier against abiotic and biotic aggressions, particularly blocking the entry of pathogens, is the primary essential function. Therefore, it is considered an important immunological factor in fish innate defences [18,19,23]. Furthermore, recently was shown that skin mucus layer is continuously renewed [24] preventing the stable colonization and adherence of potential infectious microorganisms [25]. Thus, skin mucus is a dynamic secretion whose composition largely depends on the physiological and health condition of the organism [26]. Both, endogenous factors, like the developmental stage or nutritional status of fish, and exogenous factors, such as stress, environmental disturbances and infections can influence its composition and properties. Classic biomarkers of fish welfare status such as glucose, lactate, cortisol, and antioxidant power are also detectable in fish skin mucus [15–18,21,27,28]. In addition to these classical biomarkers, the *in vitro* antimicrobial activity of skin mucus is one of the recent major interests in studies of mucus properties [19,21,29], which can be applied to evaluate the responses to infections, to environmental challenges or to nutritional studies. Characterization of skin mucus would also be a useful approach to easily study functional feeds in fish.

The current study aims to determine the adequacy of skin mucus analyses, as a minimally invasive and injurious sampling procedure, to study the effects of functional diets based on blood porcine by-products on meagre (*Argyrosomus regius*). Meagre is a commercial fish species, with great new importance in the Mediterranean aquaculture, characterised by its fast growth, adaptation to domestication and high tolerance to wide ranges of salinity and temperature [30]. Meagre aquaculture started in the late nineties (30–35 t), from 2008 its production remarkably increase (1712 t), reaching a maximal production in 2018 (7032 t) [31]. Regardless of this, there is still fragmented information about the nutritional requirements and diet formulation for this species and this issue becomes more evident when dealing with new diet ingredients and functional feeds. Two different functional diets containing porcine by-products were assayed, the porcine spray-dried plasma (SDPP) and pig protein hydrolysate (PPH), for a period of two months. To evaluate the impact of the above-mentioned diets, the skin mucus was collected and analysed for several specific biomarkers such as total soluble protein, glucose and lactate, as main markers of nutritional status and cortisol levels and antioxidant power, as markers of stress. The expected benefits on fish defensive systems were indirectly evaluated by the antibacterial power of the skin mucus via *in vitro* co-culture challenges. Moreover, main digestive enzymes activities, together with filet proximal composition were analysed to determine the effects on digestive function and commercial value of final product, respectively. Our data indicate that the applied skin mucus methodology by testing all those parameters is powerful, rapid and reliable to monitor fish condition and can be used as a preliminary and minimally invasive tool to study functional diets in aquaculture.

2. Material and methods

2.1. Animals, diets, and experimental procedures

Unvaccinated meagre fingerlings (body weight, BW = 7.4 ± 1.3 g, mean ± standard deviation) were obtained from a commercial hatchery (Piscimar, Andromeda Group, Burriana, Spain) and transported by road to research facilities (IRTA, Sant Carles de la Ràpita, Spain), where they were acclimated in 2 x 2000-L tanks for two weeks. After acclimation, all fish were anaesthetized (tricaine methanesulfonate, MS-222, 150 mg L^{-1}) and individually weighted for initial BW (BWi) and measured for standard length (SLi) to the nearest 0.1 g and 1 mm, respectively. Then, fish were distributed into twelve 500-L cyclindroconical tanks at a density of 50 fish per tank. Experimental tanks were connected to an IRTAmar® water recirculation unit to guarantee adequate water quality through ultraviolet, biological and mechanical filtration.

Functional diets consisted of replacing 2% of fishmeal (FM) and 3.4% of fish protein hydrolysate (CPSP 90; Sopropêche, Willime, France) from the control diet (CD) by 5% of spray-dried porcine plasma (SDPP-diet, Appetein™ GS, APC Europe SA, Granollers, Spain) or 5% hydrolysed pig protein (PPH-diet, Pepteiva, APC Europe SA). The level of SDPP and PPH inclusion in experimental diets was chosen according to previous results on gilthead seabream [6]. The main ingredients and proximate composition of experimental diets are shown in Table 1. Diets were manufactured by Sparos Lda. (Olhão, Portugal) as described in Gisbert et al. (2015) [6]. Each diet was assayed in four replicate-tanks during a

Table 1
Diet formulation and proximate biochemical composition of experimental diets evaluated in meagre (*A. regius*) juveniles.

	Experimental diets		
Ingredients (%)	CD	SDPP	PPH
Fishmeal LT70 (Norvik)	35.00	33.20	33.65
CPSP90	3.40		
Appetein GS (APC) [a]		5.00	
Pepteiva (APC) [b]			5.00
Soy protein concentrate (Soycomil)	10.00	10.00	10.00
Wheat gluten	6.60	6.60	6.60
Corn gluten	8.00	8.00	8.00
Soybean meal 48	5.00	5.00	5.00
Rapeseed meal	4.50	4.50	4.50
Wheat meal	13.63	13.48	13.08
Sardine oil – Sopropeche	12.35	12.70	12.65
Vitamin & Mineral Premix INVIVO 1%	1.00	1.00	1.00
Antioxidant powder (VERDILOX)	0.20	0.20	0.20
Sodium propionate	0.10	0.10	0.10
L-Taurine	0.20	0.20	0.20
Total	**100.00**	**100.00**	**100.00**
Proximate composition	**CD**	**SDPP**	**PPH**
Crude protein, % feed	50.42	50.37	50.35
Crude fat, % feed	16.24	16.18	16.17
Ash, % feed	8.00	7.90	8.20
Gross Energy, MJ/kg feed	22.10	22.10	22.10

The biochemical and amino acid composition of the tested functional porcine blood by-products (SDPP and PPH) is supplied in Supplementary File 1.
[a] Spray-dried plasma (Appetein, APC Europe SL, Granollers, Spain).
[b] Pig protein hydrolysed (Pepteiva, APC Europe SL).

period of 68 days. Fish were fed four times per day (08:00, 11:00, 14:00 and 17:00 h) over the experimental period with automated feeders (ARVO-TEC T Drum 2000™, Arvotec, Finland) at a 4.5% feeding rate of the stocked biomass, which approached apparent satiation. During this time, water quality parameters were: 23.1 ± 1.1 °C, 7.5 ± 0.2 ppm of dissolved oxygen (OXI330, Crison Instruments, Barcelona, Spain) and pH values of 7.5 ± 0.1 (pH meter 507, Crison Instruments). Water flow rate in the experimental tanks was maintained at approximately 9.0 L min^{-1} via recirculation that maintained adequate water quality (total ammonia and nitrite were ≤ 0.10 and 0.4 mg L^{-1}, respectively). No signs of bacterial infections were found along the trial, and the overall health condition of animals was considered as good.

The IRTA facilities are certified and have the required authorisation for the breeding and husbandry of animals for scientific purposes. All procedures involving the handling and treatment of the fish were approved as far as the care and use of experimental animals are concerned, by the European Union (86/609/EU), the Spanish Government (RD 1201/2005) and the University of Barcelona (Spain).

2.2. Sample collection

At the end of the experimental period, all animals in each tank were measured in BW and SL, and condition factor were calculated as well as the daily weight gain, specific growth rate (SGR) and Fulton's condition factor (K). The former parameters were measured using the following formulae: Specific growth rate (SGR; % BW day^{-1}) = (ln BWf− ln BWi) x 100/days; Fulton's condition factor (K) = (BWf/SL^3f) x 100, where BWf and BWi were the final and initial BW in grams, and SLf the final SL in cm. Fish survival was also recorded. Twenty animals were randomly sampled for each dietary condition (5 per tank) and slightly anaesthetized with tricaine methane sulfonate (MS-222, 150 mg L^{-1}). Skin mucus was individually and minimally-invasively collected as described in Fernandez-Alacid et al. (2018) [15]. Briefly, skin mucus was collected in a very fast process (less than 2 min) using sterile glass slides from the over-lateral line in a front to caudal direction and the epidermal mucus was carefully pushed and collected in a sterile tube (2 mL), avoiding the contamination with blood and/or urine-genital and intestinal excretions. Mucus samples were homogenized using a sterile Teflon implement to desegregate mucus mesh before centrifugation at 14, 000×*g* for 15 min at 4 °C. The resultant skin mucus supernatants were collected, avoiding the surface lipid layer, aliquoted and stored at −80 °C for further analyses. Sampled fish were subsequently sacrificed with an overdose of MS-222. Muscle and liver samples and their digestive tract were individually collected, immediately frozen in liquid nitrogen and stored at −80 °C until further analyses.

2.3. Mucus biomarkers

The soluble protein concentration of homogenized skin mucus was determined using the Bradford assay [32] with bovine serum albumin (BSA; Sigma-Aldrich, Madrid, Spain) as a standard. The optical density (OD) was determined at λ = 596 nm. Protein values were expressed as mg mL^{-1} of skin mucus. Glucose and lactate concentrations of homogenized skin mucus were determined by the respective enzymatic colorimetric tests (SPINREACT®, Barcelona, Spain) following the manufacturer's instructions for plasma determinations, but with slight modifications [15]. The OD was determined at λ = 505 nm. The glucose and lactate values were expressed as μg mL^{-1} of skin mucus and as μg mg^{-1} of mucus protein.

Skin mucus cortisol levels were measured using an ELISA kit RE52611 (IBL International, Germany) with lower cross-reactivity with other corticosteroid compounds in fish. The methodology of the kit was previously adapted for fish skin mucus samples [15]. Briefly, 50 μL of mucus extract or standard solutions was mixed with enzyme conjugate (100 μL) and incubated for 2 h at room temperature. After rinsing the wells with a wash solution, the substrate solution (100 μL) was added and incubated for 30 min. The reaction was stopped by adding 100 μL of stop solution and the OD was determined at λ = 450 nm. The cortisol values were expressed as ng mL^{-1} of skin mucus and ng · g^{-1} of mucus protein.

Ferric reducing antioxidant power (FRAP) detection is a measure of antioxidant status, by gauging the ability of antioxidants to convert ferric ions to ferrous ions. FRAP concentration was determined by an enzymatic colorimetric test (Ferric antioxidant status detection kit, Invitrogen, Thermo Fisher Scientific, Spain). An aliquot of either 20 μL of mucus extract or standard solutions (from 0 to 1000 μM μL^{-1} of $FeCl_2$) in triplicate was mixed with 75 μL of FRAP colour solution and incubated for 30 min at room temperature. The OD was determined at λ = 560 nm. Antioxidant values were expressed as nmol FRAP mL^{-1} of skin mucus, and nmol mg^{-1} of mucus protein. All OD measurements were done with a microplate spectrophotometer reader (Infinity 171 Pro200 spectrophotometer, Tecan, Spain).

2.4. Evaluation of skin mucus antibacterial activity by co-culture challenges

The study of mucus antibacterial activity was performed using three different bacteria: a non-pathogenic bacterium for fish, *Escherichia coli* (DSMZ423), and two pathogenic bacteria for fish marine species, *Vibrio anguillarum* (CECT522T) and *Pseudomonas anguilliseptica* (CECT899T). *E. coli* were grown in Tryptic Soy Broth culture media (TSB, Conda, Spain), while *V. anguillarum* and *P. anguilliseptica* were grown in Marine Broth culture media (MB, Difco Laboratories, Detroit). The effect of skin mucus on bacterial viability was determined by monitoring the absorbance of bacterial cultures grown in flat-bottomed 96-well plates. For the co-culture challenge, each well was loaded with 50 μl of bacterial suspension (optical density, OD = 0.2) in the appropriate culture media (1X) plus 100 μL of skin mucus (2 μg μL^{-1} of mucus protein) and 50 μL of culture media (3X) to obtain a 200 μL final volume. Controls without bacterial suspension were prepared by adding 100 μL of culture media (2X) and 100 μL of skin mucus (2 μg μL^{-1} of mucus protein). Bacterial growth without mucus (control) were prepared by adding 50 μL of bacterial suspension (OD = 0.2) in culture media (1X) and 150 μL of

culture media (1X). Blanks (control bacterial growth without bacteria and mucus) were prepared by adding 200 μL culture media (1X). The absorbance of the bacteria was measured at λ = 400 nm every 30 min for 14 h at 25 °C in flat-bottomed 96-well plates. Average absorbance of controls without bacteria [skin mucus at 2 μg μL^{-1} of protein (100 μL) plus 100 μL of medium] was subtracted from the absorbance from co-culture (bacteria plus skin mucus) samples. All assays were done in triplicate (methodological replicates). Data are presented as growth curves (increased absorbance at λ = 400 nm per unit of time) and as percentage of inhibition with respect to bacterial growth for each 2 h of co-culture.

2.5. Proximal composition of the muscle

Pools of five individual meagre muscle samples per tank were homogenized, and small aliquots were dried (120 °C for 24 h) to estimate their water content. The total fat content from feed and fish tissues was gravimetrically quantified after fat extraction in a chloroform-methanol solution (2:1) and evaporation of the solvent under a stream of nitrogen followed by vacuum desiccation overnight [33]. Protein and carbohydrate contents were determined according to Lowry et al. (1951) [34] and Dubois et al. (n.d) [35], respectively. Ash contents were determined by keeping the sample at 500–600 °C for 6 h in a muffle furnace [36]. All chemical analyses were performed in triplicate per pooled fish and feed samples.

2.6. Activity of digestive and antioxidative stress enzymes

Stomach and pyloric caeca were dissected for measuring the activity of gastric (pepsin) and pancreatic proteases (trypsin and total alkaline protease activities), bile salt-activated lipase, and α-amylase, whereas the anterior and posterior regions of the intestine were obtained for measuring the activity of alkaline phosphatase. Enzyme extracts were prepared, and spectrophotometric analyses performed as recommended by Solovyev and Gisbert (2016) [37] in order to prevent sample deterioration. Stomach and pyloric caeca samples were homogenized in 5 vol (wet weight; ww/v) of distilled water at 4 °C for 1 min, followed by a sonication process of 30 s. Intestinal samples were homogenized in 30 vol (w/v) of ice-cold mannitol (50 mM), Tris-HCl buffer (2 mM) pH 7.0 as described in Gisbert et al. (2009) [38].

Total alkaline protease activity was measured using azocasein (0.5%) as substrate in Tris-HCl 50 nmol L^{-1} (pH = 9). One unit (U) of activity was defined as the nmoles of azo dye released per minute and per mL of tissue homogenate (λ = 366 nm) [39] Trypsin activity was assayed using BAPNA (N-benzoyl-DL-arginine p-nitroanilide) as substrate; one unit of trypsin per mL (U) was defined as 1 μmol BAPNA hydrolysed min^{-1} mL^{-1} of enzyme extract (λ = 407 nm) [40]. Alpha-amylase activity was determined using 0.3% soluble starch as substrate, and its activity (U) was defined as the amount of starch (mg) hydrolysed during 30 min per mL of homogenate (λ = 580 nm) [41]. Bile salt-activated lipase activity was assayed for 30 min using p-nitrophenyl myristate as substrate; and its activity (U) was defined as the amount (nmol) of substrate hydrolysed per min per mL of enzyme extract (λ = 405 nm) [42]. Pepsin was quantified using 2% hemoglobin as substrate in 1 N HCl buffer as substrate, and its activity (U) defined as the nmol of tyrosine liberated per min per mL of tissue homogenate (λ = 280 nm) [43]. Alkaline phosphatase was quantified using 4-nitrophenyl phosphate (PNPP) as substrate. One unit (U) was defined as 1 μmol of pNP released min^{-1} mL^{-1} of brush border homogenate at λ = 407 nm [44]. All digestive enzyme activities were measured at 23 °C and expressed as specific activity defined as units per mg of protein (U mg $protein^{-1}$).

Quantification of lipid peroxidation in the intestine and liver was conducted using the thiobarbituric acid reactive substances method described by Solé et al. (2004) [45]. In brief, lipid peroxidation was measured using 200 μL of the homogenate mixed with 650 μL of methanol and 1-methyl-2-phenylindole (10.3 mM) in acetonitrile: methanol (1:3; vol/vol) and 150 μL of 37% HCl. This mixture was incubated for 40 min at 45 °C, cooled on ice for 10 min, and centrifuged at 21,000×*g* for 10 min at 4 °C to remove protein precipitates. Absorbance was read at λ = 586 nm, and the amount of peroxidized lipids (in nmol malondialdehyde/100 g tissue; wt/wt) was evaluated by means of a calibration curve made of a standard solution of 1,1,3,3-tetramethoxypropane (10 mM). Homogenized samples, prepared for the determination of the levels of lipid peroxidation, were also used to measure antioxidant enzyme activities. Catalase activity was measured in sampled tissues by the decrease in absorbance at λ = 240 nm (extinction coefficient, e = 40 M^{-1} cm^{-1}) using 50 mM H_2O_2 as substrate [46]. Glutathione S-transferase (GST) activity was assayed by the formation of glutathione chlorodinitrobenzene adduct at λ = 340 nm (e = 9.6 40 M^{-1} cm^{-1}), using 1 mM 1-chloro-2,4-dinitrobenzene, and 1 mM glutathione as substrates [47]. Glutathione reductase (GR) activity was determined by measuring the oxidation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) at λ = 340 nm (e = 6.22 mM^{-1} cm^{-1}), using 20 mM glutathione disulphide and 2 mM NADPH as substrates [48]. Total glutathione peroxidase (GPX) was determined by measuring the consumption of NADPH at λ = 340 nm (e = 6.22 mM^{-1} cm^{-1}), using 75 mM glutathione and 8.75 mM NADPH as substrates [49]. Oxidative stress enzyme activities were expressed as specific enzyme activities (nmol min^{-1} mg $protein^{-1}$). Soluble protein of crude enzyme extracts was quantified by means of the Bradford's method [32] using bovine serum albumin as standard. All the assays for evaluating the activity of digestive and antioxidative stress enzymes were made in triplicate (methodological replicates) for each tank and the absorbance was read using a spectrophotometer (Tecan™ Infinite M200, Männedorf, Switzerland).

2.7. Statistical analysis

Data are presented as mean values ± standard error of mean (SEM) and the differences through the dietary condition were analysed by one-way ANOVA (Bonferroni's post-hoc test). Normality and homoscedasticity of all data sets were checked through the Kolmogorov-Smirnov and Levene tests, respectively. Data expressed as percentage were arcsine square root transformed before analysed. Differences were considered statistically significant at $P < 0.05$. All statistical analysis were performed using SPSS Statistics for Windows, Version 22.0 (IBM Corp.; Armonk, NY, USA).

3. Results

Diets formulated for meagre containing 5% of SDPP or 5% PPH showed lower BWf when compared to the control group (−8.8% and −7.4%, respectively). Similarly, lower daily weight gain and TEC values were found in meagre fed SDPP- and PPH-diets (Table 2; $P < 0.05$). However, no differences in SL or Fulton's condition factor were found among dietary groups (Table 2; $P > 0.05$).

Table 2
Growth parameters in meagre fed two functional diets, containing 5% porcine spray-dried plasma (SDPP) and pig protein hydrolysate (PPH), over 9 weeks.

	Experimental diets		
	CD	SDPP	PPH
Final body weight (BWf, g)	70.6 ± 1.6 a	64.4 ± 0.7 b	65.4 ± 0.7 b
Final standard length (SLf, cm)	15.8 ± 0.1	15.6 ± 0.1	15.7 ± 0.1
Δ daily body weight (g)	1.05 ± 0.05 a	0.95 ± 0.02 b	0.97 ± 0.02 b
SGR (%)	3.77 ± 0.06 a	3.62 ± 0.04 b	3.64 ± 0.04 b
K	1.74 ± 0.04	1.69 ± 0.02	1.68 ± 0.05
FCR	1.31 ± 0.05 b	1.51 ± 0.07 a	1.46 ± 0.05 a
FI (g $fish^{-1}$ 60 $days^{-1}$)	16.5 ± 1.7 a	11.1 ± 2.1 b	12.5 ± 1.9 b

Values are mean ± standard error (n = 4). Different letters in the same row indicates the existence of statistically significant differences among dietary groups (ANOVA, $P < 0.05$).

Furthermore, the inclusion of SDPP or PPH in meagre diets slightly increased the protein content (1.5% and 1.7%, respectively) and reduced the lipid levels (2.4 and 2.2%, respectively) in muscle (Table 3; $P < 0.05$).

Experimental diets did not alter the specific activity of gastric (pepsin) and pancreatic (trypsin, total alkaline proteases, bile salt-activated lipase and α-amylase) digestive enzymes, nor that of alkaline phosphatase from the intestine (Table 4; $P > 0.05$).

No statistically significant differences were found regarding the levels of lipid peroxidation (TBARS) in the liver nor in the activity of selected (SOD, CAT, GR, GST and GPX) oxidative stress enzymes (Table 5; $P > 0.05$).

Results from the main skin mucus biomarkers and ratios as well as the mucus antioxidant power values are shown in Table 6. The replacement of 5% of FM and fish product hydrolysed by porcine by-products SDPP or PPH did not modify skin mucus composition in terms of glucose (ranging 15–20 μg mL^{-1}), lactate (6–8 μg mL^{-1}), soluble protein (2–4 mg mL^{-1}), cortisol (0.1–0.3 ng mL^{-1}) and antioxidant power (190–230 μmol mL^{-1}) contents ($P > 0.05$). Furthermore, the calculated ratios of these parameters with respect to protein values are also provided to avoid possible mucus dilution or concentration effects due to mucus collection. As no significant changes in the amount of soluble protein content was detected between treatments, different ratios of glucose/protein, lactate/protein or cortisol/protein led to the same results than the respective levels of these biomarkers ($P > 0.05$). Finally, the metabolic aerobic response measured in mucus as glucose/lactate ratio was not modified regardless of the dietary condition ($P > 0.05$).

To evaluate the effect of experimental diets on the antibacterial activity of skin mucus, bacterial growth curve studies were performed using a non-pathogenic *E. coli* strain and two sea fish pathogen bacterial species, *V. anguillarum* and *P. anguilliseptica*. Fig. 1 describes the meagre skin mucus antibacterial activity against *E. coli*, showing the bacterial growth curve (Fig. 1A) and the growth inhibition percentage of the bacteria when cultured with the skin mucus (Fig. 1B). Skin mucus from meagre fed with the control diet are able to limit *E. coli* growth for a maximum of 25% at 4 h, followed by an extended period of 8 h (from 6 to 14 h) when the bacterial growth inhibition was reduced below 10%. Meagre fed the diet containing SDPP showed the same inhibitory capacity than the control diet with regard to *E. coli* growth during the first hours of the assay (4–6 h). However, a slight but significant improvement in comparison to the control diet between the following hours (from 8 to 14 h) was observed ($P < 0.05$). Interestingly, skin mucus from meagre fed the experimental diet containing PPH showed an increased inhibitory capacity against *E. coli* during all the co-culture assay, being the maximum inhibition values recorded at 4 h (60% of growth inhibition, over two-fold higher than the other diets) ($P < 0.05$).

The antibacterial activity of skin mucus against marine pathogenic bacteria *V. anguillarum* and *P. anguilliseptica* is presented in Figs. 2 and 3, respectively. Contrary to *E. coli* co-culture, meagre skin mucus co-cultured with *V. anguillarum* showed the minimum inhibition capacity the first 6 h and maximum inhibitory growth values between the 8–12 h period (from 15% at 4 h to 25% at 10 h). The antibacterial activity of skin mucus from meagre juveniles fed the 5% SDPP experimental diet did not significantly differ from that of the control diet (Fig. 2A and B, $P > 0.05$ for all time values). However, the inclusion of PPH in the diet significantly enhanced the inhibitory activity of skin mucus against *V. anguillarum* (Fig. 2A and B). The inhibitory dynamics of this co-culture evidenced a two-fold higher inhibitory capacity than both control and SDPP diets through all the time-course. The maximal inhibitory capacity of meagre skin mucus fed the PPH diet against *V. anguillarum* was

Table 3
Proximal composition of the meagre muscle (wet basis) of fish fed two functional diets, containing 5% porcine spray-dried plasma (SDPP) and pig protein hydrolysate (PPH), over nine weeks.

	Experimental diets		
	CD	SDPP	PPH
Humidity (%)	76.66 ± 0.23	77.75 ± 0.59	77.21 ± 0.70
Protein (%)	12.85 ± 0.51 a	14.31 ± 0.31 b	14.55 ± 0.48 b
Lipids (%)	8.59 ± 1.16 a	6.22 ± 0.51 b	6.41 ± 0.93 b
Carbohydrates (%)	0.30 ± 0.06	0.26 ± 0.07	0.28 ± 0.05
Ashes (%)	1.63 ± 0.09	1.46 ± 0.06	1.53 ± 0.18

Values are mean ± standard error (n = 4). Different letters in the same row indicates the existence of statistically significant differences among dietary groups (ANOVA, $P < 0.05$).

Table 4
Specific activity of selected pancreatic and intestinal enzymes in meagre juveniles fed different experimental diets.

	Experimental diets		
Activity	CD	SDPP	PPH
Trypsin (mU mg $prot^{-1}$)	0.85 ± 0.13	0.74 ± 0.10	0.83 ± 0.22
Total alkaline proteases (mU mg $prot^{-1}$)	17.05 ± 2.00	10.10 ± 2.50	8.04 ± 2.10
Amylase (U mg $prot^{-1}$)	0.33 ± 0.07	0.27 ± 0.07	0.34 ± 0.07
Bile salt-activated lipase (mU mg $prot^{-1}$)	4.00 ± 1.10	4.00 ± 1.30	4.70 ± 0.80
Pepsin (U mg $prot^{-1}$)	0.47 ± 0.07	0.47 ± 0.06	0.44 ± 0.02
Alkaline phosphatase (mU mg $prot^{-1}$)	35.10 ± 10.08	47.10 ± 10.21	41.06 ± 11.12

Values are mean ± standard error of mean.

Table 5
Lipid peroxidation values (TBARS), and specific activity levels of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GP) measured in the liver of meagre juveniles fed different experimental diets.

	Experimental diets		
	CD	SDPP	PPH
TBARS (mmol mg $protein^{-1}$)	0.051 ± 0.011	0.081 ± 0.024	0.074 ± 0.019
SOD (% inhibition)	8.79 ± 0.95	8.22 ± 0.79	8.45 ± 0.66
CAT (nmol min^{-1} mg $protein^{-1}$)	151.08 ± 7.68	143.15 ± 6.22	149.9 ± 3.14
GR (nmol min^{-1}mg $protein^{-1}$)	10.07 ± 5.71	12.01 ± 3.78	11.96 ± 2.22
GST (nmol min^{-1} mg $protein^{-1}$)	155.57 ± 7.58	147.59 ± 6.22	160.33 ± 4.87
GPX (nmol min mg $protein^{-1}$)	57.26 ± 5.97	50.44 ± 3.21	49.21 ± 7.23

Values are mean ± standard error of mean.

Table 6
Main biomarkers and ratios of skin mucus of meagre fed two functional diets, containing 5% porcine spray-dried plasma (SDPP) and pig protein hydrolysate (PPH), over nine weeks.

	Experimental diets		
	CD	SDPP	PPH
Mucus biomarkers			
Glucose (μg mL^{-1})	17.15 ± 2.44	19.46 ± 2.60	15.17 ± 1.97
Lactate (μg mL^{-1})	6.49 ± 0.82	6.18 ± 0.48	7.59 ± 1.23
Protein (mg mL^{-1})	3.29 ± 0.49	3.40 ± 0.31	2.36 ± 0.34
Cortisol (ng mL^{-1})	0.26 ± 0.03	0.20 ± 0.04	0.19 ± 0.05
FRAP (μmol mL^{-1})	196 ± 26	221 ± 19	192 ± 19
Mucus ratios			
Glucose/Protein (μg mg^{-1})	5.36 ± 0.33	5.44 ± 0.27	5.88 ± 0.66
Lactate/Protein (μg mg^{-1})	2.07 ± 0.10	1.89 ± 0.13	3.31 ± 0.56
Cortisol/Protein (ng g^{-1})	78.3 ± 10.7	86.0 ± 19.1	93.5 ± 17.2
FRAP/Protein (μmol mg^{-1})	71.3 ± 1.6	68.4 ± 2.5	68.4 ± 3.3
Glucose/Lactate (mg mg^{-1})	2.68 ± 0.04	2.80 ± 0.26	2.14 ± 0.18

Values are mean ± standard error of mean.

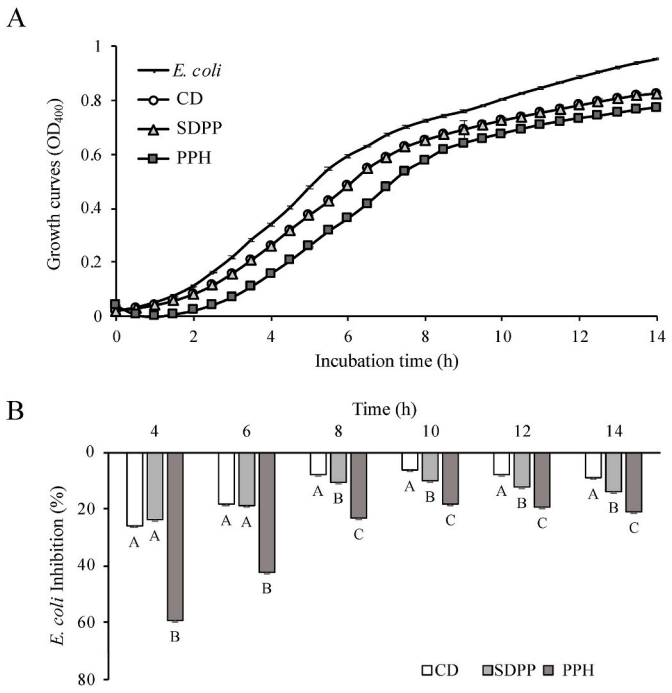

Fig. 1. Bacterial growth (A) and inhibition rate (B) against *Escherichia coli* of meagre skin mucus fed with the indicated diets. Different letters indicate significant differences among diets within each time ($P < 0.05$, one-way ANOVA).

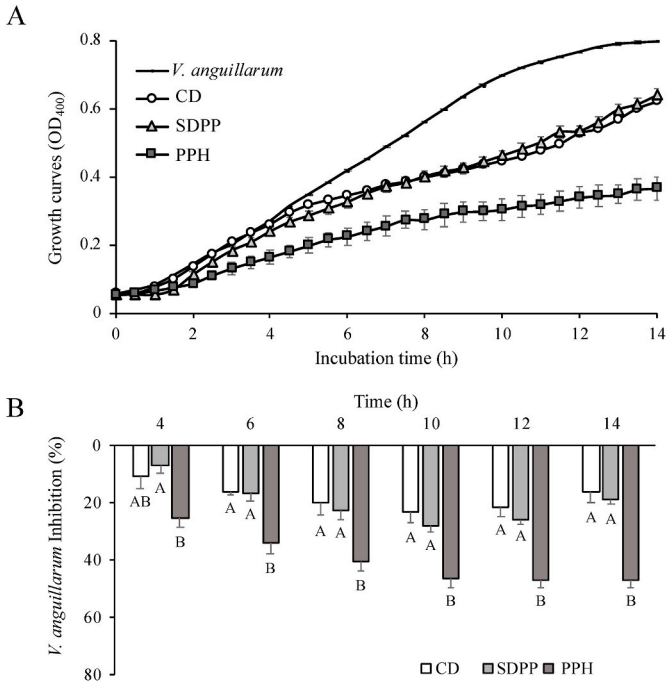

Fig. 2. Bacterial growth (A) and inhibition rate (B) against *Vibrio anguillarum* of meagre skin mucus fed with the indicated diets. Different letters indicate significant differences among diets within each time ($P < 0.05$, one-way ANOVA).

recorded during the 10–14 h interval.

With regard to *P. anguilliseptica,* the co-cultures with skin mucus from the different experimental diets showed a great capacity to inhibit bacterial growth, already at initial period (over 30%) and increased during the rest of co-culture (Fig. 3A and B). Even considering the great inhibitory potential of the fish mucus against *P. anguilliseptica*, PPH diet exhibited slight but significant higher inhibitory capacity than the other

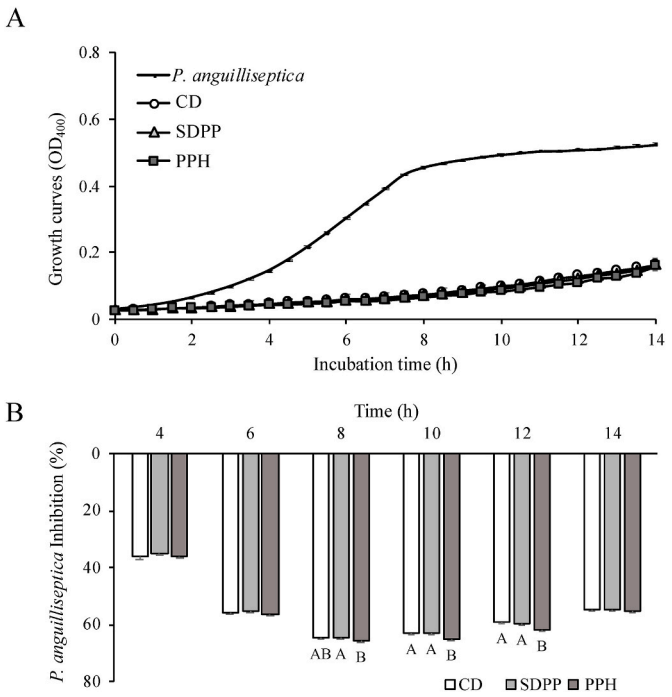

Fig. 3. Bacterial growth (A) and inhibition rate (B) against *Pseudomanas anguillseptica* of meagre skin mucus fed with the indicated diets. Different letters indicate significant differences among diets within each time ($P < 0.05$, one-way ANOVA).

two diets for the interval of 10–12 h ($P < 0.05$).

4. Discussion

The validation of functional diets in aquaculture is a common trend within this industry; to corroborate if they incorporate different growth and health-promoting bioactive compounds that may have positive effects on the fish immune system and welfare (reviewed in Tacchi et al. (2011) [50]). The effectiveness of these diets in terms of growth and welfare are classically measured by several key performance indicators like growth performance and feed conversion parameters, whereas other variables like plasma biomarkers, digestive enzymes and microbiota or immune response biomarkers may be also considered to provide insights into their mode of action (reviewed in Guerreiro et al. (2018) [51]). Most of these necessary diagnoses involve animal sacrifice and require relatively long-term laboratory analyses, delaying feedback information from the academy to the industry (i.e., feed manufacturers and fish farmers). Herein, we aim to evaluate a new, rapid, easy and affordable approach to evaluate functional feeds (diets containing SDPP and PPH) in juvenile meagre by measuring welfare biomarkers and the antibacterial properties of skin mucus.

In the current experiment, functional diets were formulated by replacing 5% of FM and fish product hydrolysed by two functional ingredients obtained from the rendering industry: the porcine spray-dried plasma, SDPP, and the pig protein hydrolysed, PPH. These hemoderivates are abattoir by-products obtained from animal blood, which have been reported to promote feed intake, somatic growth, improve FCR and/or reduce stress in several livestock [8,52] and aquaculture species [6,7,9]. Surprisingly, under present experimental conditions the replacement of low levels of FM (2%) and the absence of fish protein hydrolysate (CPSP90) in meagre experimental diets resulted in the reduction of 8.8 and 7.1% in somatic growth in fish fed SDPP and PPH diets, respectively. This reduction in BW was also coupled with a slight reduction in daily weight gain, a slight increase in FCR and decrease in feed intake values. Additionally, the above-mentioned changes in

growth and feed efficiency parameters were accompanied by changes in muscle composition with increased protein content and decreased lipid levels. The results from the current study in meagre were not in agreement with previous studies which reported that SDPP inclusion in diet enhanced growth performance in several fish species, such as rainbow trout [53], gilthead sea bream [6,9] and Nile tilapia [7]. Such findings in terms of somatic growth and muscle proximate composition may be attributed to a reduction in the content of FM and CPSP90 in isoproteic experimental diets, which has traditionally been used as the main protein source in aquafeeds. In particular, FM is not only nutritionally reputed by its excellent amino acid profile, palatability and digestibility, but also because it is a source of nucleotides, essential fatty acids, phospholipids, minerals and lipid- and water-soluble vitamins [54]. Furthermore, reductions in FI may also be attributed to changes in diet palatability and changes in dietary amino acids known as feed attractants (alanine, glycine, isoleucine, histidine, leucine, and proline) [55]. Considering these unexpected results further research is needed to provide insights on how a small reduction in FM resulted in a significant reduction of the performance indicators. A limited knowledge on the nutritional requirements of the meagre exists [56] and further studies should be focused on growth parameters, for instance studying amino acid profile requirements or the key growth signalling pathways.

Pancreatic and gastric digestive enzymes are widely considered as indicators of the digestive capacity in fish, whereas brush border intestinal enzymes are biomarkers of intestinal absorptive capacities and enterocytes' integrity [57]. In the present study, FM and CPSP90 replacement by porcine by-products did not modify the activity of selected pancreatic (trypsin, total alkaline proteases, α-amylase and bile salt-activated lipase), gastric (pepsin) and intestinal brush border (alkaline phosphatase) enzymes, which indicated that these ingredients did not impair the digestive performance of meagre fed SDPP or PPH diets. Thus, the reduction of growth and feed efficiency observed in meagre fed the above-mentioned diets may not be attributed to a worsened digestive capacity, but probably to the other dietary factors previously hypothesized.

In order to evaluate the tested functional diets, we proposed to study meagre's skin mucus biomarkers and its antibacterial activity as a tool to monitor in a preliminary and minimally invasive way the welfare of fish. Recently, different plasma metabolites considered as biomarkers of the fish physiological condition (i.e., glucose, lactate, cortisol, and total protein levels) have been correlated with their contents in the skin mucus levels in different freshwater [27] and marine species [16,17,20,29]. These classic biomarkers are rapid and easy to be measured, especially in skin mucus, providing valuable information on the fish response to different biotic and abiotic stressors [15]. Under present experimental conditions, meagre skin mucus content on glucose, lactate or soluble protein from different dietary groups were within the range of values previously reported for this species [15,16,21] and it was not affected by the porcine-derived ingredient included in the diet. Although data describing the effects of the nutritional status on mucus metabolites are scarce, the maintenance of these soluble components in the mucus seems to be important for the adequate functionality of the skin mucus layer [15,18,20,26]. Beside the main mucus metabolites, stress markers like lactate and cortisol levels are also of major interest in cultured fishes. Cortisol is secreted and released in responses to stress, although the levels strongly vary within species according to the duration or severity of the stressor [58]. Skin mucus cortisol have been detected in several fish species [29,59], and the correlation between mucus cortisol and plasma cortisol has been recently confirmed in meagre [16]. However, the mechanisms involved in cortisol exudation in skin mucus are still unknown in fish. From our best knowledge, only one study evaluated mucus cortisol levels in meagre under a sustained dietary modified condition [21]. The former authors related lower levels of mucus cortisol to stress-attenuated role of tryptophan addition to diet. As it could be expected, in the current study, experimental diets assayed did not modify the skin mucus cortisol levels or the lactate levels, indicating that the dietary modifications did not impair the stress condition of animals. Skin mucus antioxidant capacity was also measured using FRAP analysis [19,27,60]. In fish plasma and tissues, FRAP levels have been considered as a key mechanism in the epidermis response to oxidative stress [61,62]. The FRAP levels obtained were within the range of values reported for this species [19], although no changes of the mucus antioxidant power were observed for both tested experimental diets. Dietary plasma porcine products were reported to enhance the antioxidant capacity in the intestine of gilthead sea bream [6]. However, this characteristic was not evidenced from our mucus FRAP's analyses in the mucus, similarly to other studies testing phytogenic in this species [60]. In addition, under current experimental conditions no differences in the levels of lipid peroxidation and activity of antioxidative stress enzymes in the liver were found between experimental diets. The divergence between current results in meagre and those previously reported from the gut of gilthead seabream [6] need to be further explored to get insights into the potentially different mode of action of the tested functional ingredients on different species as well as in different mucosal tissues that may differently respond to dietary ingredients.

Predicting fish response to functional diets is very difficult due to complex interactions between diet ingredients and the host condition [63]. Plasma porcine contains bioactive compounds like immunoglobulins, albumins, growth factors and biologically active peptides, which may mediate anti-inflammatory and immunomodulatory effects [64,65]. Although the classical models for evaluating functional feeds with immunomodulatory properties are conducted using bacterial challenges [66,67], the use of mucus co-culture *in vitro* is a recent novel approach for evaluating the antibacterial activity of the skin mucus [19]. Considering that fish skin mucus provides a stable physical, biological, and chemical barrier against invading pathogens, knowledge of its antibacterial capacity when exposed to a pathogenic organism is of relevance. Following the criteria of dynamic co-culture of skin mucus, we performed three co-cultures to evaluate the antibacterial activity potential benefits of both SDPP and PPH inclusion on meagre diet. The pathogenic bacteria selected, *V. anguillarum* and *P. anguilliseptica* are well characterised causing different fish diseases in this marine species [68,69]. The non-pathogenic bacterium for fish, *E. coli*, has been used as indicative of the potential antibacterial capacity of skin mucus without considering previous putative host contact with *V. anguillarum* and *P. anguilliseptica,* which could generate specific acquired defences.

Depending on the ingredient considered, the inclusion of SDPP or PPH in meagre diets resulted in different results in terms of the antibacterial activity of skin mucus. Interestingly, the antibacterial activity of the meagre skin mucus also differed depending on the pathogenic bacteria considered. In particular, skin mucus from PPH-fed fish caused a greater inhibition of *V. anguillarum* in comparison to control and SDPP dietary groups. Vibriosis generally affects cultured fish recurrently, and *V. anguillarum* has been demonstrated to be a responsible agent for chronic pathogenic outbreaks in meagre among other farmed species. Moreover, high incidences of vibriosis can occur in hatchery and pre-on-growing facilities, as juveniles are more sensitive to this infectious disease; thus, the benefits of the application of a preventive nutritional strategy targeting for the improvement of the mucus antibacterial activity may be advisable and alienated with the strategy of reducing antimicrobial agents use. Our results seemed to indicate that the hydrolysed form of porcine hemoderivates seemed to be more effective than the atomized form of plasma, which may be attributed to their different composition of bioactive compounds. In particular, SDPP is an ingredient with highly digestible proteins and amino acids, and significant concentrations of functional bioactive components including immunoglobulins, transferrin, growth factors, peptides, and other biologically active components [65]. Protein hydrolysates also includes biologically active peptides with immune-stimulating and antibacterial properties produced during the hydrolysing procedure, regardless of the aquatic [10] or terrestrial [12] origin considered. In this sense, it has been reported that the hydrophobicity of peptides has been related to

their antimicrobial activity, as higher hydrophobicity is useful in the binding of lipopolysaccharides on the outer cell membrane of the bacteria [12]. Furthermore, the characterization of the antibacterial activity of meagre skin mucus against *P. anguilliseptica* was previously described in Sanahuja et al. (2019) [19]. In the present study, we showed that meagre skin mucus was able to strongly inhibit the bacterial growth of *P. anguilliseptica* regardless of the experimental group considered, although this antibacterial activity was slightly enhanced by the administration of the PPH diet. *P. anguilliseptica* is considered an opportunistic pathogen whose infections occur when fish immune system is depressed, mainly at low water temperatures [70]. Different results on the antibacterial capacity of meagre skin mucus against *V. anguillarum* and *P. anguilliseptica* may be also attributed to different bacteria virulence, differences in chemotaxis to skin mucus and their adherence capacity [26]. As fish condition during the current experiment was optimal, the real benefits of both porcine by-products against *P. anguilliseptica* could be masked by the unaltered mucus composition from external or internal stressors. Finally, the non-pathogenic fish bacterium *E. coli* was also used as an indicator of the potential antibacterial capacity of the skin mucus, neglecting a potentially acquired immunization against fish-specific pathogens [19,23]. Similarly, to *V. anguillarum*, the PPH diet enhanced the inhibitory capacity of meagre skin mucus against *E. coli* growth when compared to SDPP. It is also interestingly to mention that the evaluation of mucus antibacterial activity using growth curves was time-dependent [19]. Thus, the maximum inhibitory activity was detected at different intervals depending on the bacteria considered. In particular, this maximum inhibitory activity was found between 4 and 8h of co-culture for *E. coli*, at the 10–14h interval for *V. anguillarum* and at the 8–12h interval for *P. anguilliseptica*, irrespective of the diet administered. These results revealed the relevance of skin mucus renewal process and the importance of the mucus components exudation processes to guarantee the antibacterial mucus capacity over time [24]. Regarding the observed differences in the antibacterial capacities of skin mucus between meagre fed diets containing PPH and SDPP, it could be speculated that the tested hemoderivates have a different way of acting on the skin-associated lymphoid tissue, as they have a different content in immunoglobulins (SDPP = 270 g/kg; data provided by the manufacturer). Further studies are necessary to elucidate how the antibacterial capacity is improved beyond the study of the current mucus biomarkers such as cortisol, glucose, lactate or soluble proteins. Skin mucus is biochemically complex [71,72], integrating a wide variety of biological processes of relevance to fish health. Thus, the activities of these pig hemo-compounds as innate humoral parameters inductors in skin mucus should be deeper studied to better understand the bactericidal enhancement of the skin mucosa and even its extrapolation to other fish mucosas.

5. Conclusions

The effectiveness of functional diets or their components in cultured fish species are classically measured by several key performance indicators like growth and other physiological parameters that require fish sacrifice relatively long-lasting laboratory analyses and expensive costs. The use of skin mucus analyses represents an alternative approach for the study of functional diets, characterized as being less invasive, more rapid and with reduced costs, providing a faster feedback from the laboratory. Here, the replacement of FM and fish protein hydrolysate (CPSP90) by the inclusion of two porcine by-products, PPH and SDPP, in meagre diets impaired growth performance and feed efficiency, results that may be explained due to the great dependence of this species on FM at juvenile stages. However, the inclusion of the two tested porcine by-products did not modify the activity of gastric and pancreatic digestive enzymes as well as those involved in nutrient absorption (alkaline phosphatase), liver oxidative stress condition, nor the classical stress markers from skin mucus. On the other hand, the inclusion of the two tested porcine hemoderivates enhanced the mucus antibacterial activity, even though this enhancement was more notorious in mucus from fish fed the PPH diet, which may be attributed to its content in immunomodulatory bioactive compounds of this ingredient. Although further research is needed to understand the decrease in meagre performance in terms of growth and feed efficiency parameters when SDPP and PPH were included in diets at the expense of FM and fish protein hydrolysates, results from this study regarding the enhancement of the antibacterial activity in mucus from fish fed SDPP and PPH diets are promising as prophylactic strategies to enhance fish protection from pathogenic bacteria in the water.

CRediT authorship contribution statement

Laura Fernández-Alacid: Conceptualization, Methodology, sampling, procedure related to skin mucus, co-culture challenges including processing and data analysis, Conceptualization, and design of figures and tables, Formal analysis, wrote the original draft. **Joana P. Firmino:** Conceptualization, feeding trial, sampling, growth parameters, activity of digestive, and antioxidative stress enzymes, Formal analysis, wrote the original draft. **Ignasi Sanahuja:** Sampling, procedure related to skin mucus, Conceptualization, and design of figures and tables, Formal analysis. **Cristina Madrid:** Co-culture challenges including processing and data analysis. **Javier Polo:** Methodology, diets formulation and chemical composition. **Maude R. de Borba:** Feeding trial, Formal analysis. **Carlos Balsalobre:** Co-culture challenges including processing and data analysis. **Enric Gisbert:** Conceptualization, Methodology, feeding trial, growth parameters, activity of digestive, and antioxidative stress enzymes, Conceptualization, and design of figures and tables, Formal analysis, wrote the original draft, Funding acquisition. **Antoni Ibarz:** Conceptualization, Methodology, Conceptualization, and design of figures and tables, Formal analysis, wrote the original draft, Funding acquisition, All the authors corrected, read, and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2021.09.011.

Author statement

The conceptualization of the experiment was developed by LFA, JPF, EG, and AI. The methodology was originally proposed by LFA, JP, AI, and EG. The feeding trial was performed by JPF, MRB and EG, while sampling was conducted by LFA, JPF, IS and MRB. The diets formulation and chemical composition was formulated by JP. Growth parameters, activity of digestive, and antioxidative stress enzymes, including processing and data analysis was done by JPF and EG. The procedure related to skin mucus, including processing and data analysis was done by LFA, IS and AI. The co-culture challenges including processing and data analysis was done by LFA, CM and CB. The conceptualization and design of figures and tables were in charge of LFA, JPF, IS, EG, and AI. All the authors contributed to the data analysis. LFA, JPF, EG, and AI wrote the original draft. Funding acquisition was charge of EG and AI. All the authors corrected, read, and approved the final manuscript.

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