

Full length article

A phytobiotic-based additive protects against the deleterious effects of fasting and refeeding challenges

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ABSTRACT

Phytobiotics are increasingly valued in functional feeds for their growth-promoting, immunostimulant, and antioxidant properties. The present study evaluates the immunomodulatory benefits of a commercially available phytobiotic-based additive (APEX) in enhancing fish performance and skin mucosa properties during fasting periods, a common challenge in aquaculture. Gilthead seabream juveniles were acclimated and fed either a commercial diet or a functional diet containing 0.3 % phytobiotic additive. Samples were collected at pre-fasting and following a 15-day fasting period and a 7-day gradual refeeding phase. The functional diet mitigated weight loss during fasting and enhanced body weight gain during refeeding, indicating improved performance. Analyses of exuded mucus, mucus metabolites, antibacterial capacity, and histological parameters provided insights into mucus properties and skin barrier status throughout the fasting and refeeding challenge. Food deprivation may affect mucus production, thereby compromising the protective condition, which was significantly reversed upon refeeding. Mucus defensive capacity, assessed via in vitro pathogenic bacteria co-cultures, showed that fasting reduced antibacterial activity against *Vibrio anguillarum*, *Pseudomonas anguilliseptica*, and *Tenacibaculum maritimum* in fish fed the commercial diet, with no recovery upon refeeding. Conversely, the functional diet preserved the antibacterial capacity of skin mucus during fasting and refeeding, meanwhile enhanced the skin barrier performance in fish fed the functional diet at pre-fasting and refeeding stages. In conclusion, short-term fasting detrimentally affects skin health and overall fish welfare, compromising the skin barrier. The use of a phytobiotic-based functional feed additive protects and prolongs the defensive properties of skin mucus during fasting, benefiting the recovery process.

1. Introduction

In their natural habitats, fish are well adapted to periods of food deprivation, possessing a remarkable capacity to mobilize metabolic reserves and structural macromolecules. This adaptation is crucial as many species experience one or more periods of rest (low activity) throughout their life cycle. In aquaculture, various species undergo extended fasting for multiple reasons. For instance, Atlantic salmon are subjected to feed withdrawal before transportation, parasite treatments, preslaughter, and depuration in recirculating systems [1]. Fasting, both voluntary and involuntary, can occur due to several factors, including strategies to prevent poor water quality by emptying the gut before major rearing operations like crowding, pumping, delousing, transport,

and slaughter [2–4]. Weather events like storms and cold drops can affect farming operations, posing a threat to both cage infrastructure and feeding regime integrity [5]. Disease can also diminish appetite [6], and winter affects many freshwater and some marine and estuarine fish species, starting a fasting period [7]. Under fasting, fish exhibit a range of physiological and metabolic regulatory responses depending on the species and the duration of stress. One of the most significant challenges in aquaculture is the reduction in growth performance and body mass, accompanied by a compromised immune system. During periods of food deprivation, fish prioritize the mobilization of internal energy reserves, which leads to diminished immune defenses and increased susceptibility to disease [8,9]. Consequently, feed intake and specific growth rates are markedly reduced [10]. Recent studies demonstrate that phytogenic

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Table 1
Morphometric parameters of gilthead sea bream juveniles submitted to a fasting and refeeding.

	Initial		Fasting		Refeeding	
Growth performance						
BW CONTROL (g)	135.5 ± 4.1	a	128.0 ± 4.0	a	149.5 ± 5.5	b
BW PHYTOBIOTIC	135.3 ± 3.2	a	131.3 ± 3.2	a	157.4 ± 4.2	b
BL CONTROL (cm)	18.1 ± 0.2		18.1 ± 0.2		18.4 ± 0.3	
BL PHYTOBIOTIC	18.0 ± 0.2	a	18.1 ± 0.1	a	18.8 ± 0.2	b
K CONTROL	2.28 ± 0.02	a	2.14 ± 0.03	b	2.37 ± 0.03	a
K PHYTOBIOTIC	2.32 ± 0.02	a	2.24 ± 0.03	b*	2.33 ± 0.02	a
DWG CONTROL	1.1 ± 0.1	a	−0.42 ± 0.06	b	1.6 ± 0.08	c
DWG PHYTOBIOTIC	1.1 ± 0.1	a	0.04 ± 0.11	b*	2.8 ± 0.32	c*
HSI CONTROL	1.47 ± 0.09	a	0.67 ± 0.05	b	1.62 ± 0.11	a
HSI PHYTOBIOTIC	1.27 ± 0.10	a	0.65 ± 0.11	b	1.66 ± 0.11	a
VSI CONTROL	5.69 ± 0.28	a	3.91 ± 0.22	b	6.31 ± 0.17	a
VSI PHYTOBIOTIC	5.12 ± 0.24	a	3.77 ± 0.16	b	6.15 ± 0.38	a

Values are shown as mean ± standard error of mean (n = 25 for growth performance, n = 6 for plasma). BW: body weight; BL: body length; K: Fulton's condition factor; DWG: daily weight gain; HIS: hepatosomatic index; VSI: viscerosomatic index. Different letters (a, b, c) indicate statistically significant differences among sampling moments, as determined by one-way ANOVA followed by Tukey's post-hoc test (p < 0.05). Asterisks (*) denote significant differences between the control diet and the phytobiotic-supplemented functional diet, assessed using Student's t-test (p < 0.05).

feed additives are promising strategies to improve fish production by enhancing fish growth performance, health, and protection against infections [11,12]. Nevertheless, upon refeeding, fish experience compensatory growth, characterized by accelerated growth following a period of fasting [13]. Short periods of fasting and refeeding can reduce feeding and labour costs [14] and prevent excessive lipid accumulation, enhancing fish quality [15]. However, compensatory growth studies in various fish species have yielded mixed results [16]. The gilthead sea bream, a significant species in Mediterranean marine aquaculture, suffers from "winter syndrome", causing chronic mortality during cold months and acute mortality when temperatures rise in early spring [17, 18].

The blend of herbs, essential oils and yeast extracts, have emerged as promising additives in aquaculture to enhance fish health and growth performance [19–22]. Besides its growth promoting effects, these compounds exhibit antimicrobial, antioxidant, and immunostimulant properties, making them effective in improving fish resilience during stressful conditions such as fasting and refeeding periods. Based on the mode of action, these products contain bioactive compounds such as β-Glucan, alkaloids or essential amino acids, in addition to vitamins and polysaccharides, which promote growth and immune system [19]. Studies have shown that phytobiotics can enhance the gut health of fish, promoting better nutrient absorption and overall growth [20]. In addition, the inclusion of phytobiotics in the diet can help maintain the fish's immune function and reduce the negative impacts of stress [21]. Upon refeeding, phytobiotics have been shown to accelerate recovery and support compensatory growth by enhancing metabolic efficiency and reducing oxidative stress [22]. The integration of these compounds in aquaculture practices not only supports fish health but also aligns with sustainable and eco-friendly farming practices, reducing the reliance on synthetic chemicals and antibiotics [23,24].

Thus, our study aimed to mitigate the deleterious impacts of fasting in gilthead seabream juveniles through the administration of a phytobiotic-based diet. Specifically, we sought to evaluate the efficacy of this dietary intervention in preserving and enhancing the fish's overall health and growth performance during periods of food deprivation and subsequent refeeding. Additionally, we focused on assessing the defensive capacity of the fish under these conditions by examining the properties of the skin mucosa. As the first line of defence against pathogens and environmental stressors, skin mucus serves as a critical

indicator of fish health [25]. It provides a valuable matrix for studying physiological responses and the effects of dietary interventions [26]. By analysing changes in skin mucus composition, exudation rates, and antibacterial properties, we aimed to gain insights into the protective mechanisms activated by the phytobiotic diet using a minimally invasive experimental approach. This comprehensive assay not only enhances our understanding of the physiological adaptations to fasting and refeeding but also underscores the potential of phytobiotics as a sustainable and effective strategy in aquaculture to improve fish welfare and resilience.

2. Material and methods

2.1. Animal maintenance and diets

Gilthead seabream juveniles were purchased from local provider (Piscimar) and kept at the University of Barcelona facilities for one month as acclimation period. The feed was formulated (45 % protein, 17 % lipid) by the Unit of Experimental Diets from Universidad de Almería (Almería, Spain). Fish (BW = 88.3 ± 1.8 g, mean ± standard error of the media, SEM) were distributed in six 200 L tanks (15 fish per tank) in a recirculating aquaculture system, and were fed two diets (three replicates per diet) for 42 days (27 days excluding the fasting period), in constant temperature, pH, dissolved oxygen, and following a natural photoperiod (December–January). Both diets, commercial (CONTROL diet) or a functional diet (including a recommended 0.3 % of a commercial phytobiotic, APEX from Adisseo, France) were offered twice a day manually until apparent satiation (*ad libitum*). APEX is mainly a mixture of herbal extracts and essential oils, organic acids, inactivated yeast and yeast extracts, on a mineral carrier. The additive is specifically formulated for use in aquafeed and is in compliance with EU feed regulation.

This study was conducted in accordance with the Guiding Principles for Biomedical Research involving Animals (EU2010/63), the guidelines of Spanish legislation (Law 32/2007 and RD 53/2013) and approved by the Ethics Committee of the University of Barcelona for the Use of Laboratory Animals and the Generalitat de Catalunya (DAAM 9383).

Table 2

Skin mucus exudation parameters of gilthead sea bream juveniles submitted to a fasting and refeeding.

	Initial		Fasting		Refeeding	
C- Exuded mucus (mg)	472 ± 39	a	330 ± 44	b	587 ± 30	c
PHYTOB- Exuded mucus (mg)	381 ± 46	a	301 ± 43	a	497 ± 8	b*
C- Exuded mucus per fish (mg/g fish)	1.65 ± 0.1	a	1.17 ± 0.3	b	2.16 ± 0.2	a
PHYTOB- Exuded mucus per fish (mg/g fish)	1.30 ± 0.1	a*	0.91 ± 0.1	b	1.90 ± 0.3	a
C- soluble fraction (%)	78.2 ± 3.3	a	80.6 ± 1.9	a	53.5 ± 6.0	b
PHYTOB- soluble fraction (%)	79.7 ± 11	a	77.5 ± 3.0	a	55.5 ± 3.7	b
C- mucus solubility ratio ¹	4.56 ± 0.77	a	4.51 ± 0.61	a	1.35 ± 0.23	b
PHYTOB- mucus solubility ratio ¹	6.19 ± 1.6	a	4.00 ± 0.65	a	1.37 ± 0.22	b
C- Protein (mg/g mucus)	4.52 ± 0.5	a	6.70 ± 1.3	ab	9.54 ± 1.48	b
PHYTOB- Protein (mg/g mucus)	4.81 ± 1.3	a	7.63 ± 1.6	b	14.5 ± 0.41	c*
C- total exuded protein (mg)	2.34 ± 0.30	a	2.32 ± 0.60	a	5.70 ± 1.01	b
PHYTOB- total exuded protein (mg)	2.23 ± 0.69	a	2.61 ± 0.77	a	7.35 ± 1.45	b
C- Glucose (µg/g mucus)	22.3 ± 3.2	a	18.7 ± 3.4	a	52.7 ± 5.0	b
PHYTOB- Glucose (µg/g mucus)	21.5 ± 3.9	a	36.7 ± 5.0	b	61.3 ± 0.6	c*
C- Total exuded glucose (µg)	1.12 ± 0.14	a	0.67 ± 0.19	a	3.09 ± 0.34	b
PHYTOB- Total exuded glucose (µg)	0.99 ± 0.19	a	1.18 ± 0.24	a	3.11 ± 0.46	b

Values are shown as mean ± standard error of mean of ten individual samples. C: control group; PHITOB: Phytobiotic group. ¹ mucus solubility ratio is calculated as soluble/insoluble ratio (arbitrary units). Different letters (a, b, c) indicate statistically significant differences among sampling moments, as determined by one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05$). Asterisks (*) denote significant differences between the control diet and the phytobiotic-supplemented functional diet, assessed using Student's t-test ($p < 0.05$).

2.2. Experimental trials and animal sampling

After the inclusion of the experimental diets (Initial period, 20 days), fish were fasted for 15 days (Fasting period) and later refed gradually for 7 days (Refeeding period). At the end of each period, fish were netted and anesthetized with tricaine methane sulfonate (MS-222, 150 mg/L), and sampled to monitor the following key performance indicators.

- Body weight (BW, g)
- Body length (BL, cm)
- Fulton's condition factor (K); $K = (BW_f / BL_f^3) \times 100$
- Daily weight gain (DWG); $DWG = (BW_f - BW_i) / \text{number days}$

BW_f and BW_i were the final and initial BW in grams, and BL_f the final BL in cm.

At each final point, blood and the skin mucus were collected from all fish for analysis. Additionally, five animals from each sampling were randomly selected and sacrificed to determine the effects on body indices, the hepatosomatic index (HSI) and the viscerosomatic index (VSI), and the skin mucus barrier.

2.3. Skin mucus collection

After anaesthetising the fish, the skin mucus was immediately collected in accordance with the methodology described by Ref. [26]. In order to cause the least stress and harm to the animals, the mucus collection process was completed in a rapid manner. The skin mucus was collected on sterile glass slides, swiped above the lateral line from the anterior to the caudal region. A sterile slide was gently wiped along both sides of the animal, and the epidermal mucus was carefully pushed and collected in a pre-weighed sterile tube (2 mL). The process was conducted in a manner that avoided contamination with blood and/or urinogenital and intestinal excretions. The collected mucus samples were homogenised using a sterile Teflon implement to desegregate the mucus mesh prior to centrifugation at 14,000g. The resulting mucus fractions were collected and weighed, and divided into separate portions, which were then stored at -80°C .

2.4. Biochemical analysis

Protein levels (µg/mL of skin mucus supernatant) were determined by the Bradford assay kit (Sigma Aldrich, Spain) using bovine serum albumin as standard (BSA; Sigma Aldrich, Madrid, Spain). Glucose and lactate levels (both µg/mL of skin mucus supernatant) were determined by colorimetric assay tests following the manufacturer protocols for glucose (LO-POD glucose, SPINREACT®, Spain) and lactate (LO-POD lactate, SPINREACT®, Spain), and adapted for 96 well plates. Glucose and lactate were also referred to soluble protein as ratios to avoid the possible dilution derived from the mucus collection. We also analysed the glucose/lactate ratio as a metabolic indicator as described in Fernandez-Alacid et al. [26].

2.5. Skin mucus antibacterial defence

To study the potential skin mucus defensive capacity against pathogenic bacteria, a selection of pathogenic bacterial strains was examined: *Vibrio anguillarum* (CECT522T), *Pseudomonas anguilliseptica* (CECT899T), and *Tenacibaculum maritimum* (CECT 4276). All bacterial strains were cultivated in Marine Broth (MB, BD Difco™). The antibacterial properties of skin mucus, derived from the sampling points (initial, fasting and refeeding), were assessed in relation to the viability of the three bacteria. This was achieved by monitoring the absorbance of co-culture grown at 400 nm (per triplicate) over a period of 14 h in 96-well plates, following the methodology adapted from Sanahuja et al. [27].

2.6. Skin morphology and barrier assessment

After 24 h of fixation with Bouin's solution at room temperature, the skin samples were dehydrated in a graded series of ethanol and stored at 4°C . The samples were cleaned with xylene, embedded in paraffin, and cut into serial sections (3-µm thick). After dewaxing and rehydration, the sections were mounted on glass slides. The slides were stained using a periodic acid-Schiff (PAS) and Alcian blue (AB) staining protocol. The morphometric slides were digitalized (Hamamatsu NanoZoomer 2.0-HT, Hamamatsu Photonics K.K., Hamamatsu City, Japan). Digital images (600 dpi) were processed and analysed using an image analysis software (ImageJ 1.52, National Institutes of Health, USA). Measurements of epidermis thickness as well as mucous cell measurements were

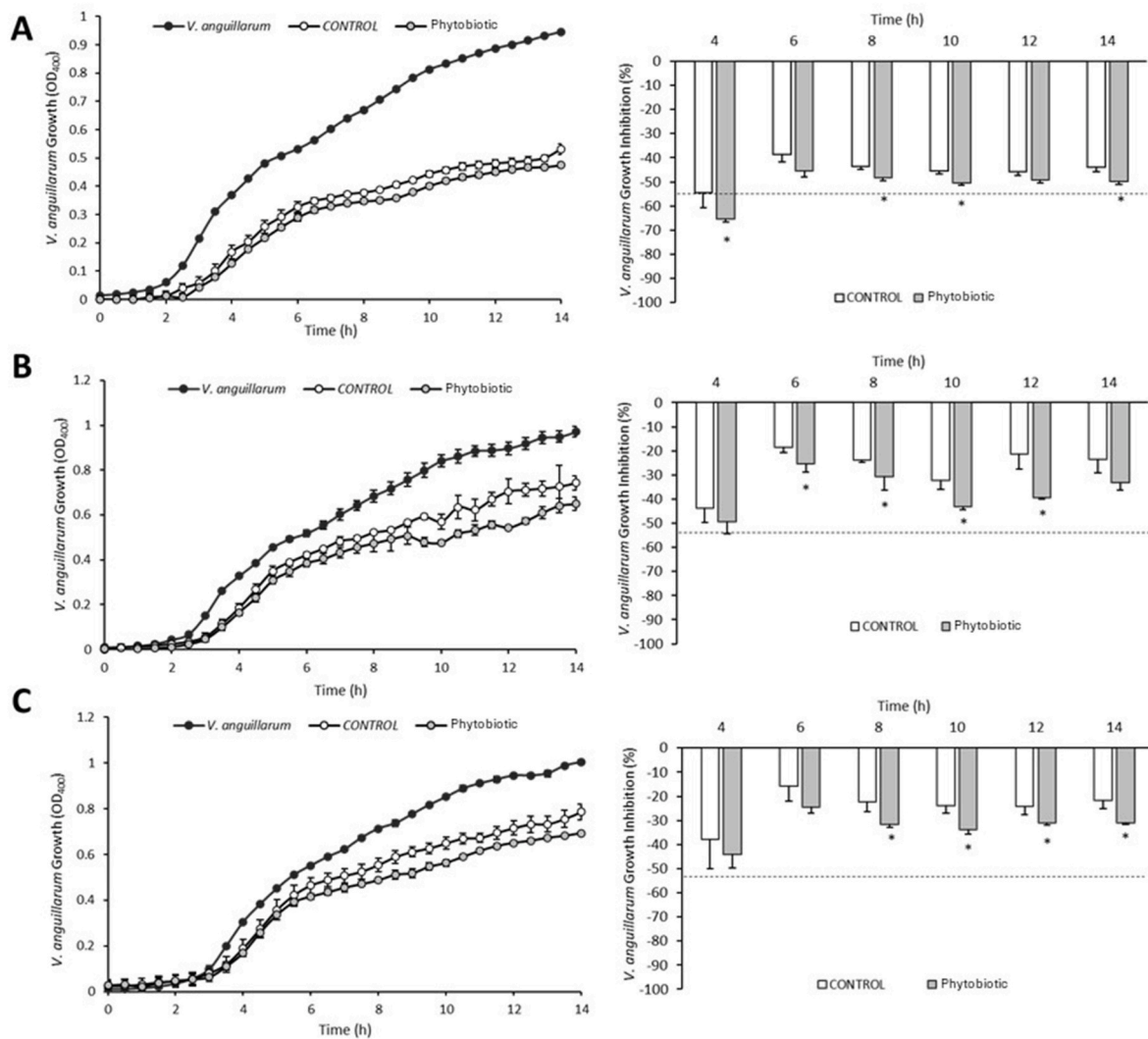


Fig. 1. Bacterial growth in the presence of skin mucus of juvenile seabream fed control diet and diet supplemented with phytobiotic. Mucus samples were obtained from 3 pools of 6 individual fish. Data corresponds to the mean $\pm$ SEM of triplicate bacterial growth curves of *V. anguillarum*. Black circles correspond to bacteria growth in medium devoid of mucus, white circles correspond to bacteria growth in the mucus of fish fed the control diet and grey circles correspond to bacteria growth in the mucus of fish fed the phytobiotic-supplemented diet. The dotted line corresponds to maximum bacterial growth inhibition for control diet as reference point. Details on the experimental groups are provided by initial period (A), fasting period (B) and refeeding period (C). Asterisks indicate significant differences in bacterial growth between dietary groups (Student's t-Test, $p < 0.05$).

based on the analysis of randomly chosen fields from each skin sample.

The skin barrier status was assessed as described in Dang et al. [28] using 3 different indices.

(a) Mean mucous cell (MC) area (μm^2)

(b) Mean MC volumetric density (%) = $\frac{\text{MC area MC number}}{\text{Epithelial area}} \times 100$

(c) Barrier status = $\frac{1}{\text{MC area MC density}} \times 100$

2.7. Statistical analysis

Normality and equality of variances were checked by the Levene's test. Differences among periods for biochemical analyses and bacterial growth were determined through one-way ANOVA test, following the post-hoc Bonferroni's or Dunnett's tests depending on the equality of the variances, with a 95 % of significance ($p < 0.05$). Differences among diets were compared by means of a student's t-test ($p < 0.05$). Statistical

analysis was performed using SPSS Statistics for Windows, version 22.0 (IBM Corp.; Armonk, NY, USA).

3. Results

3.1. Performance indicators

The outcomes of fish growth performance and organ indices under initial, fasting, and refeeding conditions are detailed in Table 1. Both dietary groups exhibited comparable responses to the dietary regimes, maintaining similar body weight levels during the fasting period and demonstrating increased weight levels during refeeding compared to initial values. In terms of body length, fish maintained initial levels during fasting. However, only those fed the experimental diet showed an increase during refeeding. The Fulton's condition index (K) revealed differences between dietary groups during the fasting period, with lower values observed compared to the initial and refeeding conditions. In contrast, the fish exhibited a cessation in daily weight gain (DWG), accompanied by a decline in weight, during the fasting period. A more pronounced negative trend was observed in the fish fed the control diet

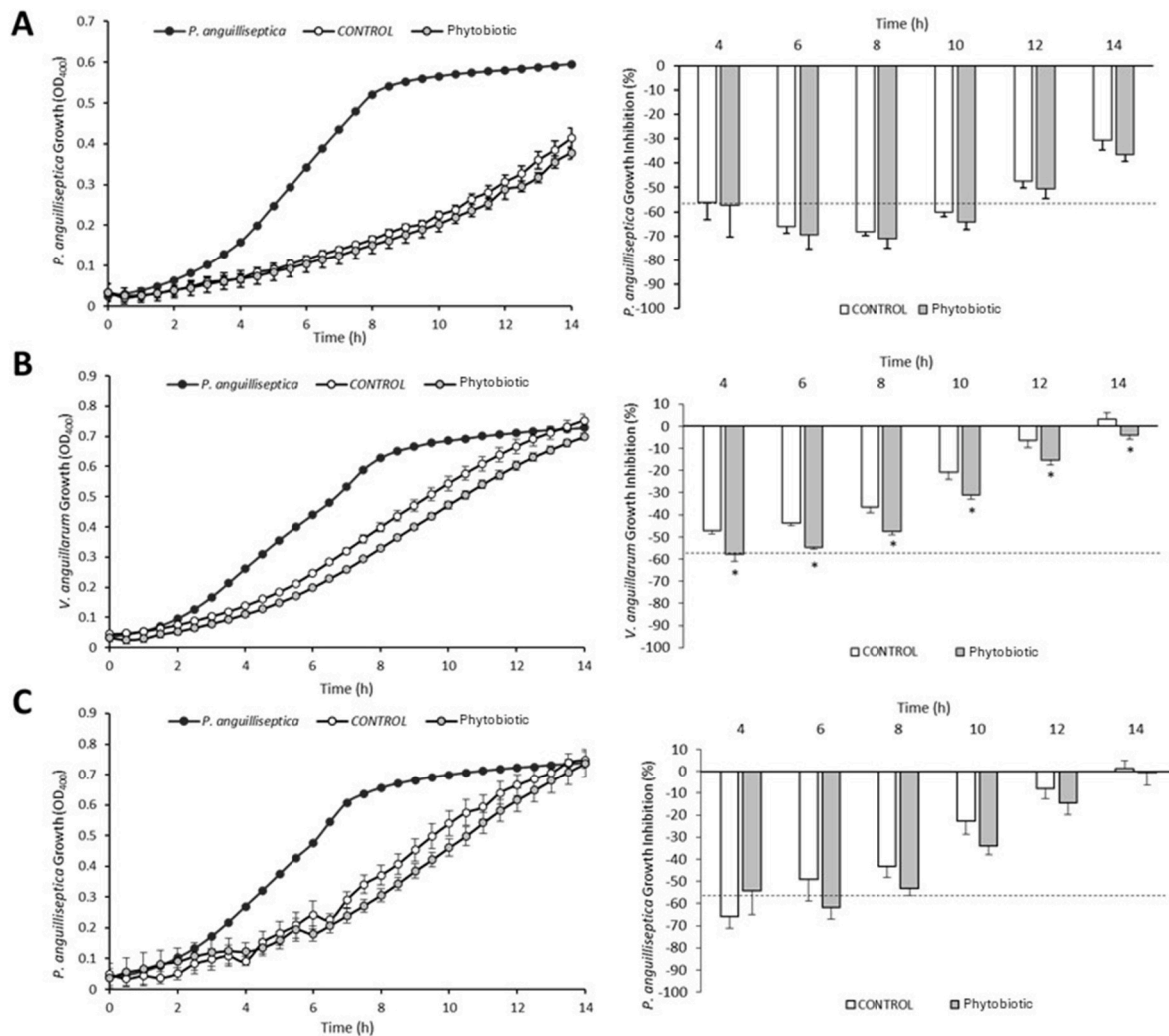


Fig. 2. Bacterial growth in the presence of skin mucus of juvenile seabream fed control diet and diet supplemented with phytobiotic. Mucus samples were obtained from 3 pools of 6 individual fish. Data corresponds to the mean $\pm$ SEM of triplicate bacterial growth curves of *P. anguilliseptica*. Black circles correspond to bacteria growth in medium devoid of mucus, white circles correspond to bacteria growth in the mucus of fish fed the control diet and grey circles correspond to bacteria growth in the mucus of fish fed the phytobiotic-supplemented diet. Dotted line corresponds to maximum bacterial growth inhibition for control diet as reference point. Details on the experimental groups are provided by initial period (A), fasting period (B) and refeeding period (C). Asterisks indicate significant differences in bacterial growth between dietary groups (Student's *t*-Test, $p < 0.05$).

compared to the fish fed the functional diet. Upon refeeding, both dietary groups reversed the fasting trends, ultimately surpassing the initial DWG values. Notably, fish fed the phytobiotics demonstrated superior results compared to those on the control diet. Regarding organ indices, both the hepatosomatic index (HSI) and the viscerosomatic index (VSI) exhibited decreased values in fish during the fasting period but returned to initial levels following refeeding. No statistical differences were observed between the dietary groups at any period.

3.2. Skin mucus associated biomarkers

Physicochemical characteristics and composition of skin mucus across dietary groups under initial, fasting, and refeeding conditions are summarized in Table 2. During the fasting period, fish fed the control diet exhibited a reduction in skin mucus quantity. Conversely, refeeding led to increased mucus production in both dietary groups, with a more pronounced effect observed in fish fed the control diet. When exudation was normalized by fish weight, the experimental diet induced a restrained mucus exudation effect only under initial conditions. Both dietary groups exhibited decreased values during fasting, with no significant differences observed during refeeding compared to initial

values. Mucus soluble fraction and solubility ratio of the skin mucus remained unchanged during the fasting period but surprisingly, during refeeding, soluble fraction exhibited reduced values resulting in a drastic reduction of solubility ratio for 3–4 folds. Regarding protein and glucose content, both metabolites followed similar trends throughout the experiment. Protein and glucose levels increased during the fasting period in the group fed the experimental diet. This trend continued during refeeding for both dietary groups, with a more pronounced increase in fish fed the experimental diet. Similarly, total protein and glucose levels showed an increase during the refeeding period, with no significant differences observed between dietary groups.

3.3. Skin mucus defence capacity against pathogenic bacteria

Figs. 1–3 illustrates the antibacterial properties of skin mucus against three pathogenic bacteria: *Vibrio anguillarum* (Fig. 1), *Pseudomonas anguilliseptica* (Fig. 2), and *Tenacibaculum maritimum* (Fig. 3). Initially, all tested bacteria showed reduced growth when co-cultured with the skin mucus. Notably, the skin mucus from fish fed the phytobiotics exhibited slightly enhanced defensive capacity against *V. anguillarum* (Fig. 1A) and *T. maritimum* (Fig. 3A) compared to those fed the control

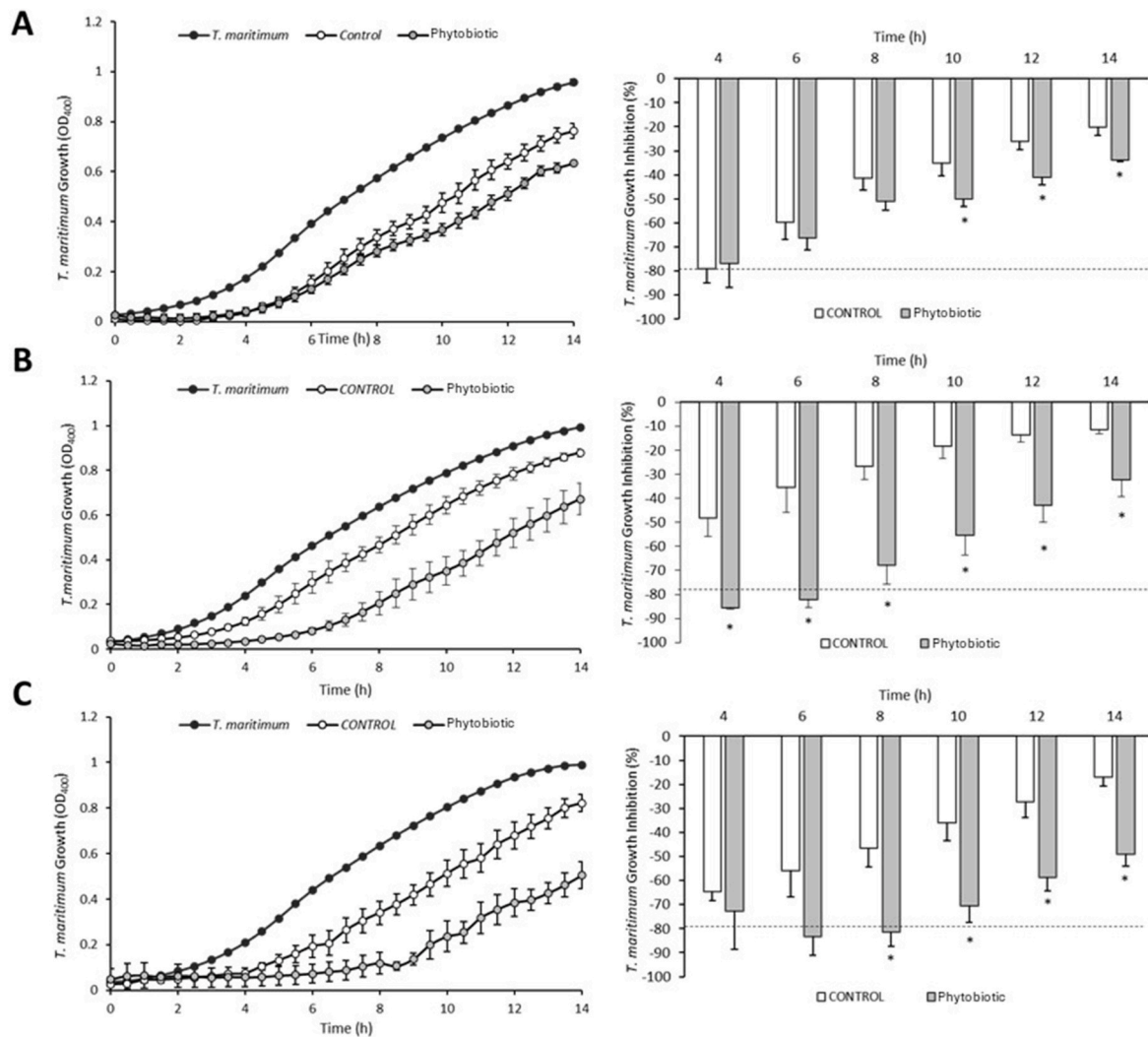


Fig. 3. Bacterial growth in the presence of skin mucus of juvenile seabream fed control diet and diet supplemented with phytobiotic. Mucus samples were obtained from 3 pools of 6 individual fish. Data corresponds to the mean $\pm$ SEM of triplicate bacterial growth curves of *T. maritimum*. Black circles correspond to bacteria growth in medium devoid of mucus, white circles correspond to bacteria growth in the mucus of fish fed the control diet and grey circles correspond to bacteria growth in the mucus of fish fed the phytobiotic-supplemented diet. Dotted line corresponds to maximum bacterial growth inhibition for control diet as reference point. Details on the experimental groups are provided by initial period (A), fasting period (B) and refeeding period (C). Asterisks indicate significant differences in bacterial growth between dietary groups (Student's *t*-Test, *p* < 0.05).

diet. After a fasting period, there was a reduction in antibacterial defence across all dietary groups relative to the initial values (Figs. 1B, 2B and 3B). However, fish fed phytobiotics demonstrated a significantly enhanced defensive capacity of the skin mucus against all tested bacteria, with a particularly strong effect against *T. maritimum*. Following the refeeding period, the defensive capacity of the skin mucus did not return to initial values. Nonetheless, fish fed phytobiotics showed improved defence against *V. anguillarum* (Fig. 1C) and especially against *T. maritimum* (Fig. 3C).

3.4. Skin mucus barrier defence

In order to evaluate the consequences of the fasting trial on skin anatomy, a histological analysis of the mucosal tissue was conducted. The epidermis from all analysed fish exhibited a normal appearance, with no visible lymphocytes or macrophages scattered across the stratified squamous epithelium, in addition to scattered mucous cells. This finding was irrespective of dietary regime, fasting or refeeding timing (see Fig. 4). However, notable changes in the mucus-related cells were detected. Under fasting conditions, the epidermal layer of fish on the

phytobiotic diet appeared significantly thickened. Conversely, post-refeeding, both dietary groups exhibited a reduced epidermal thickness compared to their initial values.

With regard to the mucus cell area the group fed the Phytobiotic diet, initially exhibited a larger mucosal cell area (Fig. 5A), with higher number of cells greater than $150 \mu\text{m}^2$ (Fig. 6B). Under fasting conditions, the control group increased its mucus cell area (Fig. 5A) and the number of cells greater than $150 \mu\text{m}^2$ (Fig. 6A), surpassing that of the phytobiotic group. Post-refeeding, the cell areas of both groups were equivalent but lower than their initial values (Figs. 5A, 6A and 6B). Regarding mucus cell density (number of mucous cells per area), both diets had increased values on refeeding (Fig. 5B). Overall, the barrier status varied across dietary regimes, with fish fed the phytobiotic diet exhibiting improved barrier status initially. However, the fasting period equalized the barrier status among all fish. Notably, fish that were fed the phytobiotic diet demonstrated enhanced barrier function following the refeeding period (Fig. 5C).

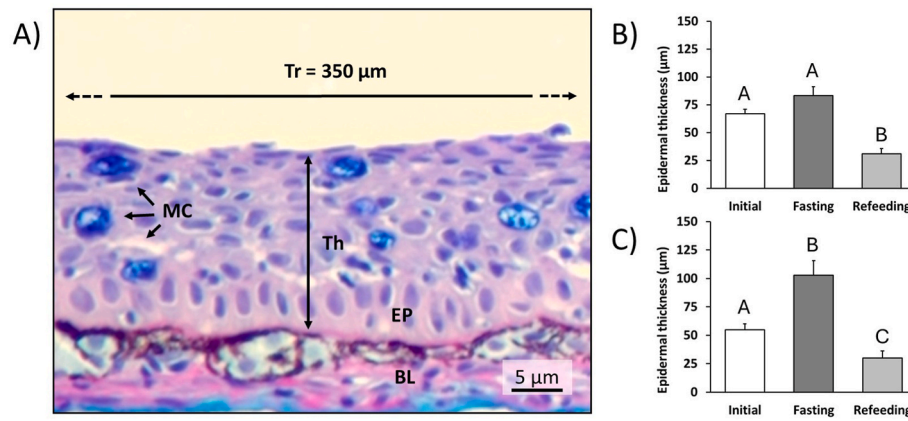


Fig. 4. (A) Representative image for the histological analysis in the skin of gilthead sea bream fed control and phytobiotic-supplemented diet. Histological organization and histological measurements for epidermis thickness, mucus cell area, and mucous cells density (Fig. 5). Each transect was of 350 μm and thickness were measured as the media of three measures per transect. Ep = epidermis BL = basal lamina, Tr = transect, Th = thickness, MC = mucus cells. Epidermal thickness values (mean $\pm$ SEM) were calculated from 4 $\times$ Transects in 6 animals per conditions for fish fed control (B) and phytobiotic (C) diets. Different capital letters within each diet indicate the presence of statistically significant differences (ANOVA). No differences between diets are reported.

4. Discussion

Fasting periods occurs in aquaculture industry, with food intake influenced by external factors such as weather changes, handling procedures, as well as internal factors including life cycle stages [29]. Phytobiotics, plant-derived additives, have been extensively used in aquaculture feeds due to their observed benefits in enhancing growth and modulating the immune system, the antioxidant defense, and the metabolism [22]. Additionally, their effects have been demonstrated in mucosal tissues, potentially improving fish mucosal barrier function and integrity [30,31]. In this study, we aimed to evaluate the effect of a phytobiotic based-additive (APEX) on gilthead seabream (*Sparus aurata*) subjected to fasting and refeeding periods, focusing on its impact on skin mucosa and the modulation of its protective capacity.

Fasting in fish halts growth and can lead to health overall deterioration. In our study, a 15-day fasting period resulted in a non-significant decrease in body weight, aligning with previous findings [32]. This weight reduction is attributed to the mobilization of energy reserves [33]. Fasting induces significant metabolic adaptations to maintain energy homeostasis, including a change in the carbohydrate metabolism and the preference for the lipid catabolism [34]. This shift is evidenced by the depletion of muscle and liver glycogen stores and the increased mobilization of lipids [35]. Although we did not directly measure fat content, the approximately 50 % reduction in both hepatosomatic and viscerosomatic indexes reflect mobilization of energy reserves, including lipid stores [36]. In gilthead seabream, lipid reserves in the liver and adipose tissues are mobilized to provide energy, resulting in alterations in the fatty acid composition of these tissues [37,38]. Furthermore, studies have demonstrated that fasting leads to a change in muscle protein turnover and an increase of serum concentrations of urea cycle-related compounds [39]. However, a phytobiotic-supplemented diet may mitigate the adverse effects of fasting in these fish, as indicated by the improved Fulton's index and reduced weight loss compared to the control group. Phytobiotics have been reported to stimulate appetite and promote weight gain due to the presence of bioactive molecules such as alkaloids, terpenoids, saponins, and flavonoids [20]. The modulation of nutrient absorption and antioxidant properties of phytobiotic diets can significantly influence lipid metabolism and gut microbiota in fish. This dietary intervention may enhance metabolic resilience during fasting by protecting against lipid oxidation [22]. Furthermore, recent studies have shown that dietary lipid content can reorganize gut microbiota and influence metabolic pathways in fish [40, 41]. Thus, hypothetically, the interaction between the phytobiotic-supplemented diet and gut microbiota could play a crucial

role in moderating metabolic health and mitigating the deleterious effects of fasting. Further studies, particularly regarding microbiota composition, are needed to fully elucidate these potential benefits.

On the other hand, the reduction in nutrient intake and the mobilization of body reserves can compromise the immune response of fish, making them more susceptible to infections and diseases due to increased stress sensitivity during fasting [42,43]. Phytobiotics have been demonstrated to exhibit a broad spectrum of biological activities, with some studies indicating their capacity to modulate the health and immune system of farmed animals [44–46]. Our results demonstrate that a phytobiotic-based diet can enhance the skin mucosa, providing protection against all tested pathogenic bacteria, particularly *T. maritimum*. Recent studies have indicated that phytobiotic additives can improve the immune condition and reduce stress levels in farmed fish following vaccination by bath immersion [21] or with 0 % water change [47]. Both studies indirectly are related with skin mucosa, the primary portal of pathogen entry, which is an active organ responsive to various dietary and environmental conditions. Firmino et al. [30] revealed that dietary administration of phytobiotics induces a multi-factorial response, including the transcriptional regulation of skin innate immunity factors and activation of the mucosal immune response. It is plausible that components of the phytobiotic diet continue to provide protection during fasting, extending the defense against pathogens. Additionally, fasting typically reduces mucus glucose content [26]. However, in fish fed a phytobiotic diet, this reduction is not observed, suggesting an improved metabolic response to fasting and, consequently, unaltered immune activity.

After a fasting period, fish exhibit a physiological process known as compensatory growth. In gilthead seabream, this adaptive response to food deprivation is characterized by accelerated growth rates upon refeeding [10]. Extensive studies in aquaculture have shown that while gilthead seabream can achieve significant weight gain during compensatory phases, the increase in length often lags behind [35,48]. In our study, both groups demonstrated increased body weight following refeeding, indicative of compensatory growth. However, only the fish fed a phytobiotic diet exhibited increased body length. This disparity is attributed to a rapid increase in condition factor rather than proportional skeletal growth in the control group, suggesting a dietary differential complex interplay between metabolic and physiological processes [49]. Notably, phytobiotics have been shown to improve feed efficiency and growth rates in fish by stimulating appetite. Dietary supplementation with phytobiotics has been observed to enhance weight gain and feed conversion ratios (FCR) in farmed animals, including fish [50]. The observed differences in daily weight gain (DWG) between groups

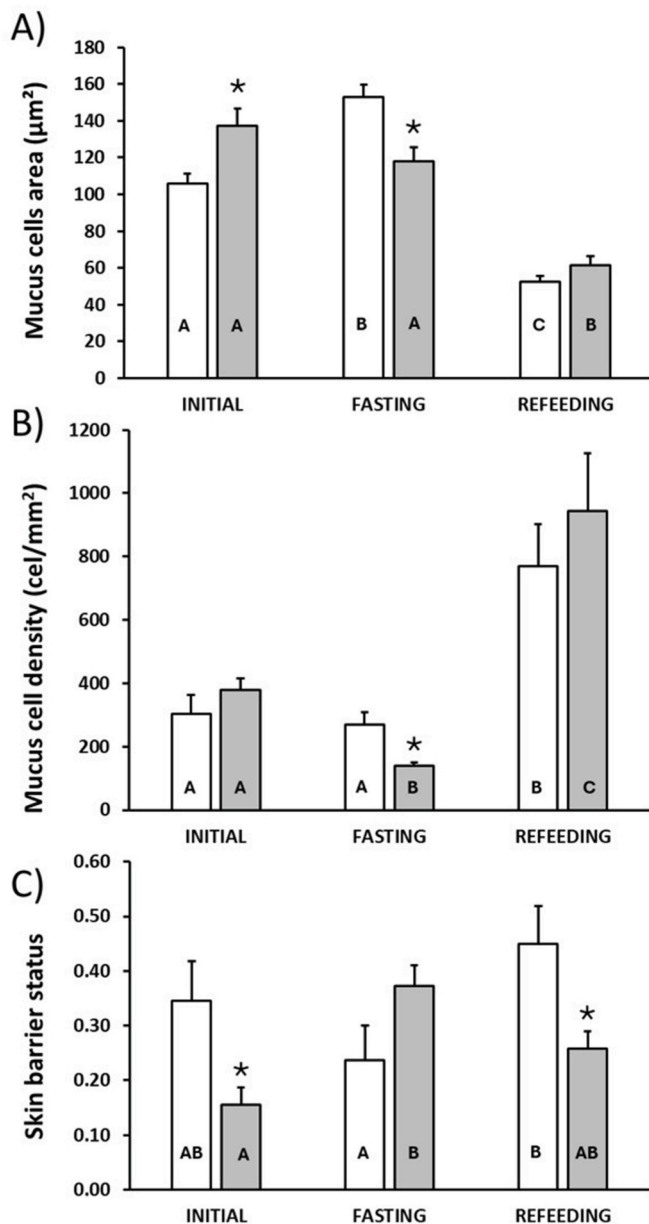


Fig. 5. Mucus cell area (A), density (B) and calculated skin barrier status (C) of gilthead sea bream skin fed control and phytobiotic-based diet. White bars correspond to the control diet; and grey bars correspond to fish fed the phytobiotic-supplemented diet. Values are mean $\pm$ standard errors of the media (SEM) from individual fish (N = 6). Capital letters indicate differences among periods for each dietary group (one-way ANOVA). Asterisks indicate significant differences between dietary groups (Student's *t*-Test; $p < 0.05$).

underscore the growth benefits of phytobiotic additives, both under normal conditions and following a fasting period.

Phytobiotics can facilitate these transitions by modulating metabolic pathways and supporting energy homeostasis. Research indicates that phytobiotic feed additives can reduce oxidative stress and improve antioxidant status in fish, which is crucial during the refeeding phase when metabolic rates are high [22]. Thus, the enhanced antioxidant activity helps in maintaining cellular integrity and overall health, maintaining the immune system and the defensive capacity. This is supported in our study by the increase in the antibacterial defense showed in bacterial growth inhibition in the mucus of fish fed the phytobiotic diet. In addition, the mucus of the phytobiotic group showed an enhanced protective property compared with the control group. This

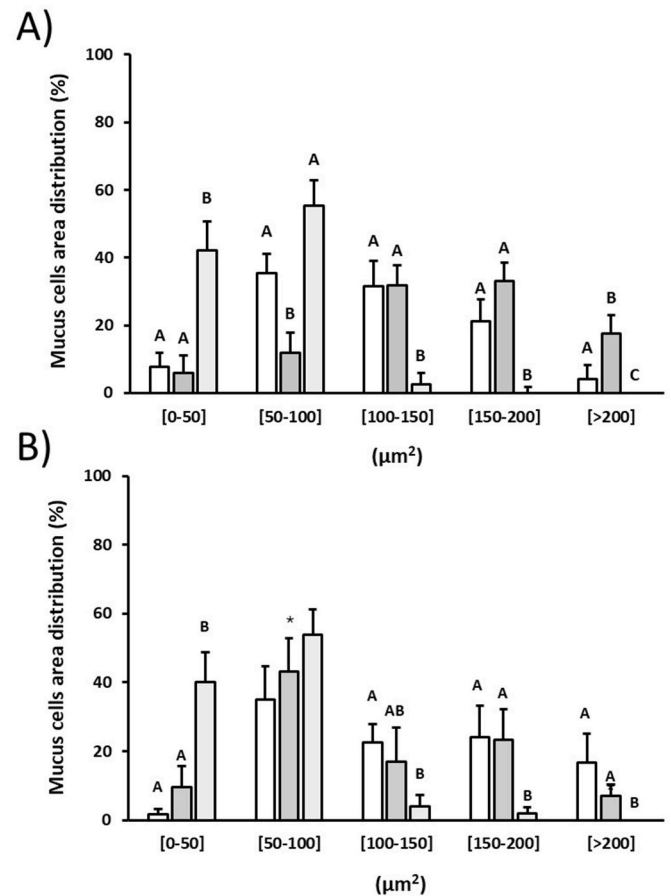


Fig. 6. Mucus cells aggruppation by area. Data correspond to the percentage of cell area of the skin mucus cells of fish fed a control diet (A) or the phytobiotic diet (B). White bars correspond to initial condition, dark grey bars correspond to the fasting period, and light grey bars correspond to refeeding period. Capital letters indicate significant differences among periods (one-way ANOVA) and asterisks denotes significant differences between diets (Student's *t*-test; $p < 0.05$).

hypothesis could be explained by different factors. Despite the inferior mucus exudation exhibited, the phytobiotic dietary group had superior protein and glucose levels. Variations in mucus composition, including glycoproteins such as mucins, reflect the fish's ability to adapt to different environmental stressors and maintain homeostasis [51]. This is one of the defensive mechanisms against bacterial or parasitic challenge, where fish responds by increasing mucus production to 'drag' those pathogens [26]. However, the biochemical defensive capacity of the mucus decrease [52]. On the other hand, high-quality mucus is characterized by its viscosity, thickness, and the presence of antimicrobial peptides, lysozymes, immunoglobulins, and lectins, which collectively enhance the fish's immune response [53]. Exuding less mucus but increasing its protective contents molecules could explain the increased antimicrobial potential of the mucus of fish fed the phytobiotic diet, in addition to the incorporation of antimicrobial components described in the phytobiotic additives [22,30]. Studies in this field show an interesting relationship between mucous cell density and its area, which is also described as the "barrier status", indicating the reactive capacity of the epithelium [28]. The barrier status can be altered by several factors such as nutrition and contaminants [54,55], denoting the feasibility of its use [52]. Here we demonstrated that fasting and refeeding conditions alter the skin performance. Moreover, the phytobiotic addition in fed would enhance the skin barrier, mainly during the critical refeeding period. However, further studies are needed to explain how phytobiotics interacts with mucus formation and how is involved in its composition.

However, our first results show that phytobiotics has potential as an alternative to antibiotics, reinforcing the skin mucosa as showed by the increasing of the barrier function during occasional fasting challenges. In addition to the known beneficial effects on antioxidant defence and immunostimulant activity [22], it has a beneficial effect on tissues, particularly the skin, which serves as the primary defence barrier against potentially harmful agents to fish health, as observed in this study.

5. Conclusions

The administration of a phytobiotic-based diet effectively mitigates the negative impacts of fasting in gilthead seabream juveniles, enhancing their growth performance and overall health during periods of food deprivation and subsequent refeeding. The functional diet not only reduced weight loss during fasting but also improved body weight gain during refeeding, indicating better performance. Additionally, the phytobiotic diet preserved the antibacterial capacity of skin mucus, maintaining its defensive properties against pathogens or increasing it. These findings highlight the potential of phytobiotics as a sustainable and effective strategy in aquaculture to improve fish welfare and resilience. APEX, a commercially available phytobiotic, has been shown to be suitable for use in farm animals. Its relevance for aquaculture is attributable to its demonstrated capacity to modulate the skin mucosa, which constitutes the main immunological barrier in fish.

Author contributions

IS, LF-A, WGN-O and AI designed the experiments. IS, MT and LF-A carried out the experiments analyses. Biological samplings and skin mucus analyses were performed by IS, MT and LF-A. IS, CB, CM, and LF-A reviewed and validated the methodology used in the study. The study was supervised by IS, LF-A, WGN-O and AI. IS wrote the original draft. All the authors provided the critical feedback, read and agreed to the published version of the manuscript.

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Data availability

Data will be made available on request.

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