SHRIMP HYDROLYSATE-BASED PALATABILITY ENHANCER: A CIRCULAR ECONOMY AND COST-EFFECTIVE STRATEGY TO REDUCE FISH IN FISH OUT RATIO IN MARINE FISH SPECIES

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Abstract

Reducing the reliance of the aquaculture industry on wild fish resources remains a key challenge. Generally obtained from food coproducts, protein hydrolysates have emerged as promising functional and sustainable protein sources that can help compensate for the limitations of alternatives to marine proteins. In this study, we evaluated their use as a palatability enhancer (PE) by top-coating them directly on the feed, as a liquid and at low dose. We tested a PE made primarily from a shrimp liquid hydrolysate, as a cost-effective dietary solution to replace significant amounts of fish meal (FM) with plant proteins in juvenile red sea bream diets. The experimental feeds consisted in a high FM diet (HFM, 30% FM), a low FM diet (LFM, 15% FM) and two additional diets with 2% PE top-coated (HFM+PE and LFM+PE). Six replicate tanks, each containing fish weighing 27.2 ± 0.2 g, were provided with one of the test diets twice daily until apparent satiety for a duration of 15 weeks. Half of the

tanks were exposed to an intermittent stress consisting in a 1 min net-chasing, 1 hour before their first meal. After the nutritional trial, individuals were subjected to an *Edwarselia tarda* challenge through injections. The basal diets, LFM and HFM, achieved comparable growth and feed efficiency. The use of LFM, therefore, mechanically reduced wild fish use by 25% if referring to the eFIFO index (Kok et al., 2020). PE supplementation in both diets resulted in enhanced fish growth and health performance, and improved feed use. Consequently, we estimated that PE use can significantly, and additionally, reduce by 6% the use of wild fish biomass in these conditions. The beneficial effects of PE on the growth performance were stable despite the stress challenge, which led however to overall strong reduction in growth performances in all groups. Survival was significantly increased by PE addition in response to the bacterial challenge compared to the LFM diet. All these results were supported by underlying physiological results. We concluded that liquid hydrolysate PE represent a great potential for feed formulators to cost-effectively reduce pressure on wild resources, while maintaining high performance of their feed.

Keywords: Fish meal replacement, eFIFO, Repeated stress, Disease challenge, Red seabream.

1. Introduction

The production of key aquaculture-fed species grew by 10.5% annually, on average, from 2000 to 2020, whereas fish meal (FM) use in aquaculture feeds rose by 2.46% per year over this interval (FAO, 2022). In response to resource limitations and sustainability challenges, the aquafeed industry has investigated and adopted novel protein ingredients, enabling substantial FM replacement. Consequently, FM inclusion in marine fish diets dropped from 50 % down to 14 % between 1997 and 2017 (Naylor et al., 2021), resulting in an economic Fish In Fish Out

ratio (eFIFO) of 0.94 (Kok et al. 2020) making, based on this calculation, marine fish farming a net fish producer overall. During this period, however, aquaculture's reliance on FM also rose, making it the primary consumer of FM worldwide – its share of global FM volumes increased from 42% in 2000 to 83% in 2020 (FAO, 2022).

Soybean meal has emerged as the primary replacement for fishmeal, thanks to its advantageous nutritional properties, high production capacity, worldwide accessibility, and cost-effectiveness (Gatlin et al., 2007; Boyd and McNevin, 2022). As a premium alternative to soybean meal due to its enhanced digestibility and lower levels of anti-nutritional compounds, soy protein concentrate (SPC) is often included in carnivorous fish feeds (Refstie et al., 2001; Gyan et al. 2019). Substituting FM with SPC has demonstrated satisfactory zootechnical outcomes in multiple fish species, provided that essential dietary nutrients are adequately supplied and a certain level of palatability is retained (Bureau et al., 1998; Gunathilaka et al., 2021). Nevertheless, underlying negative effects on fish welfare and immunity were highlighted, when investigated, resulting in a higher susceptibility to bacterial diseases (Khosravi et al., 2015b, Herault et al., 2023).

The global production of sparids exceeded 485,000 metric tons in 2020 (Fishstat, 2023), with the largest share through species like the Gilthead seabream (*Sparus aurata*), which alone accounted for approximately 282,100 metric tons (FAO, 2022). Sparidae production is driven by the demand for carnivorous species that require high-quality diets, often rich in FM. Among these, red seabream, a member of the Sparidae family, is an important species within East Asia. It ranked as the third most commonly farmed fish during the 2000s in South Korea and, in Japan, it continues to hold the position of second most farmed fish (Tabata and Taniguchi, 2000; FAO, 2022).

Earlier studies on red seabream showed that dietary FM could be partially substituted with SPC at levels ranging from 25 to 50% without compromising health performance. This was achieved by 5% Aquatic Product Hydrolysates-based supplementation into low FM diets (Khosravi et al., 2015b; Gunathilaka et al., 2021; Herault et al., 2023). Despite these promising results, the main limit to a generalized use of APH in aquafeed industry is the cost and availability.

One potential strategy to enhance the cost-effectiveness of APH and broaden their adoption within the industry would be to use them in liquid form at reduced dosages. Top-coating is also the preferred application mode to increase diet palatability, making palatable compounds more readily available to fish taste receptors (Kolkovski, 2006; Fournier, 2013; Tola et al., 2019; Rigos et al., 2001). Nevertheless, they are usually added to lower extent when top-coated as a liquid (~2-3%) compared to powder inclusion (~2-5%); consequently, lower fish zootechnical and health performance are expected because of reduced addition of functional components. This is corroborated by recent studies, which demonstrated a positive correlation between the dietary concentrations of bioactive peptides, or APH, and the final fish zootechnical or health performance (Gunathilaka et al., 2020; Wang et al., 2023). To our knowledge, however, there is a limited number of studies investigating the real impact of hydrolysate-based products top-coated on fish meal substituted diets, and at low doses (Tola et al., 2022).

We therefore propose to explore the potential of a formulated palatability enhancer (PE) made from a shrimp liquid hydrolysate as a cost-effective dietary solution to replace half of the FM with plant proteins in the diet of red seabream juveniles in controlled or unstressed conditions. Resulting FM consumption and eFIFO will be assessed for each experimental group while a repeated daily stress will be implemented at regular intervals to mimic husbandry stress found in fish farming operations. The performance indicators and physiological biomarkers used were

identical to those in Herault et al. (2023, Table 4) to ensure that the proposed dietary optimization did not negatively impact the fish physiological processes and to make easier the interpretation of any changes observed in the performance indicators.

2. Materials and Methods

All the procedures used during this nutritional and health trial adhered to the guidelines established by the Institutional Animal Care and Use Committee of Jeju National University (approval number: 2021-0041) and utilized a methodology adapted from Herault et al. (2023).

2.1. Experimental diets

Four test diets were prepared to have equivalent nitrogen and energy contents on dry matter basis (46.8% crude protein and 20.7 $kJ.g^{-1}$, respectively). The reference diet included 30% FM of fair average quality (brown FM, 67.7% crude protein) and was designated as the high FM (HFM) diet (Table 1). For the low FM (LFM) formulation, half of the FM in the HFM diet was replaced by SPC, wheat, and corn gluten meals while essential nutrients – such as amino acids, lipids, omega-3 fatty acids, phosphorus, and calcium – were adjusted accordingly. The dry ingredients were mixed thoroughly, followed by the addition of fish oil, soybean oil, and 15-20% double-distilled water. The resulting dough was pelletized using a pelletizer (SP-50, Gum Gang Engineering, Daegu, Korea), yielding pellets with diameters of 2-3mm and lengths of 3-4mm. These pellets were freeze-dried at −40 °C for 24 h before being coated with a 50% mixture fish and soybean oils (Table 1). Two experimental diets were prepared in addition by top-coating HFM and LFM at 2% with Extrapal Shrimp, a palatability enhancer provided by Symrise Aqua Feed (part of Taste, Nutrition & Health segment of Symrise group, Elven, France). Extrapal Shrimp (Table 3) is made of a shrimp hydrolysate derived from

enzymatically treated cephalothorax co-products of farmed white shrimp (*Litopenaeus vannamei*), combined with sensory flavors and stabilized with phosphoric acid to achieve a pH of 3.5. This 2% dietary supplementation of Extrapal Shrimp results in a nutritional addition of 0.45% dry matter and 0.25% crude protein. Supplemented diets can therefore be considered iso nutrients compared to their control. Diets were stored at -20 °C until used. Details on the proximate composition and amino acid profiles of each diet are provided in Tables 1 and 2.

2.2. Fish and feeding trial

Juveniles of red seabream were obtained from a local hatchery and subsequently reared at the Marine Sciences Institute of Jeju National University, South Korea. Upon arrival, the fish underwent a 30-minute oxytetracycline treatment (100 ppm bath). For an acclimation period of two weeks, the fish were fed a commercial diet (VOGUELUCK, Jeju, South Korea; 52% crude protein, 8% crude lipid).

A total of 960 fish (mean initial body weight: 27.2 ± 0.11 g) were distributed randomly among 24 circular tanks (40 fish per 240 L tank), each receiving a continuous flow of sand-filtered seawater at 3 L/min. Aeration in each tank ensured dissolved oxygen levels of 7.5 ± 0.6 ppm. During the summer season, water temperatures ranged from 20.5°C to 29.7°C, reflecting natural coastal conditions. Six tanks were assigned randomly to each dietary treatment. Fish were fed the test diets to visual satiety twice daily (at 09:30 and 18:30) for 15 weeks. Uneaten feed was collected 20-30 minutes after feeding, dried at 125°C for 4 hours, and weighed. Feed intake was calculated by adjusting for the initial moisture content and subtracting the uneaten feed weight from the total feed offered. For the stress protocol, fish in three tanks per diet (n=3) were exposed to daily stress, consisting of one minute of net chasing at 08:30 every morning. This procedure was repeated for 7 days every two weeks, mirroring intermittent farm-related

stressors. Compared to a daily stress model used in a previous study (Herault et al., 2023), this approach imposed less frequent stress. Fish were weighed in bulk before and after each 7-day stress period, occurring weekly except during weeks 1, 4, 7, 10, and 13.

2.3. Calculations of zootechnical & environmental parameters

The zootechnical performance indicators were calculated as follows:

Specific growth rate (%/d) = [Ln (final body weight (g)) - Ln (initial body weight(g))] / duration of feeding trial (d)] × 100

Feed intake (g) = total feed provided (g) – uneaten feed (including moisture content) (g)

Economic feed conversion ratio (eFCR) = crude feed distributed / (final biomass (g) – initial biomass (g))

Condition factor = (Fish weight (g) / Fish length(cm) ^3) × 100

Fish Meal Consumption (kg of FM / kg of produced fish) = eFCR × dietary FM content (%)

In addition to FM consumption (FMC), we propose calculating the economic Fish in Fish Out (eFIFO) ratio to place greater emphasis on the limiting feed ingredient, whether FM or FO, that contributes the most to FIFO ratio (Kok et al., 2020).

Economic fish in fish out ratio (eFIFO) = eFCR x (% dietary FM content x Pm) + (% dietary FO content x Po) where Pm and Po are economic embodiments of FM and FO respectively, calculated based on the following formula:

Pm or Po = EVm or EVo / (EVm x FmY + Evo x FoY) where EVm or EVo are economic values of FM, or FO (cost / mT). FmY and FoY are embodiments of FM and FO, which are usually 22.5% and 5%, respectively (Tacon and Metian, 2008). When writing this manuscript, FAQ65

FM and pelagic FO prices were 1,800 and 3,700 US$/mT respectively resulting in Pm and Po at 1,95 and 4,01 respectively.

2.4. Sample collection and analysis

At the end of the feeding trial, following a 24-hour fasting period, each fish was individually weighted. 9 fish were then randomly selected, and identified from 1 to 9 (Table 4), for tissue sampling. These selected fish were euthanized immediately using a 500-ppm solution of 2-phenoxyethanol.

All the procedures used in this study to collect and analyze samples have been well described in our previous research (Herault et al., 2023), with the controlled physiological biomarkers listed in Table 4. We propose here to adapt this table to summarize sampled fish allocation and tissues, and methods or commercial kits used for biomarker analysis (Table 4).

2.5. Bacterial challenge

Following the nutritional study, 15 individuals in each tank (total of 45 per statistical group) were randomly selected and intraperitoneally injected with 0.1mL of a suspension of *Edwardsiella tarda* (ATCC 15947, American type culture collection) at a concentration of 1×10^6 CFU mL^{-1}. The *E. tarda* broth was prepared at the required concentration according to the method outlined by Bui et al. (2014). The fish were then placed into twenty-four 120L fiberglass aquarium. No mortality or abnormal behavior was observed during the first five days. Therefore, fish were re-injected with 0.1 mL of 1×10^8 CFU mL^{-1} *E.tarda* suspension from the same strain on day six of the trial. After the second injection, fish were kept in the same tanks and mortality was recorded for 11 days, when the survival rates remain steady. The injected fish were not fed, and the water temperature was maintained at 25°C throughout the bacterial challenge trial. Tanks were siphoned daily, with 75% of the water replaced.

2.6. Estimation of apparent digestibility coefficients

Apparent digestibility coefficients (ADCs) were estimated in specific dedicated trials, each of them lasting 10 days and repeated twice (n = 3).

The full procedure was previously described in Herault et al. (2023). In brief, modified Guelph system (Cho et al., 1982) was used to collect fish feces while 1% chromic oxide (Cr_2O_3) (Sigma-Aldrich, St. Louis, USA) was added to experimental feeds as an inert indicator of digestibility. Eighty red seabreams of 100g BW (bulk mean weight of 8kg) were distributed in each of the 4 feacal collection tank (300L). Chromic oxide levels were then analyzed following the procedure described by Divakaran et al. (2002) and applying following formula:

ADC of nutrients or dry matter (%) = 100 - 100 × (% Cr_2O_3 in diet / % Cr_2O_3 in faeces) × (% nutrients in faeces /% nutrients or dry matter in diet)

2.7. Statistical analysis

All measurements were analyzed at the tank level, using the average value of all individuals within the tank. All statistical methods used in this study were previously described in Herault et al. (2023).

3. Results

3.1. Effects of dietary FM level and PE application on growth performance in control and stressed conditions

Red seabream responded well to the tested diets, including LFM, leading to consistent growth across all groups (Figure 1) and high survival rates exceeding 95%. The substitution of FM did not result in significant differences in growth and feed efficiency, whatever the stress

conditions ($p > 0.05$, Figures 2A and 2B). However, PE supplementation showed improved growth and feed performance for the two basal diets ($p < 0.001$), with increased effects for LFM dietary groups ($p < 0.05$ for the PExFM interaction). These effects were observed in stressed and controlled conditions ($p > 0.05$ for the SxPE interaction). PE supplementation also resulted in higher individual feed intake (Figure 2C; $p < 0.05$) especially for LFM dietary groups ($p < 0.001$ for PExFM interactions). The chasing stress significantly reduced the survival and growth performance of all groups ($p < 0.01$). Stress induction and PE supplementation showed effects on fish growth after 3 weeks of feeding trial, with each 1-week period of daily net chasing increasing the amplitude of these differences (Figure 1). FM replacement and chasing stress negatively impacted fish IGF-I mRNA expression ($p \leq 0.001$) while PE dietary supplementation positively impacted it ($p < 0.05$ and non-significant interactions, Figure 2D).

No significant change was observed in the proximate compositions of the fish whole-body, or carcass, regardless of the PE supplementation or chasing stress (Tables S1 and S2). Similar to growth performance, fish condition factor was negatively impacted by chasing stress while it was improved by PE dietary coating ($p < 0.001$, Table S3). Repeated periods of chasing stress significantly lowered fish somatic indexes and liver fat content ($p < 0.01$). Dietary changes did not impact fish somatic indices nor viscera or liver fat contents (Table S3). Fish exposed to chasing stress conditions or receiving the FM substituted diets showed lower water retention in muscle tissue ($p < 0.05$, Table S3).

3.2. LFM and PE on the reduction of wild fish use

Because of little growth differences, 50% FM replacement with plant proteins led to a FM consumption (FMC) divided by around 2 (Figure 3A). PE supplementation significantly

reduced FMC ($p < 0.01$), while stress increased FMC ($p < 0.001$), but both to a much lower extend. Similarly, 50% FM replacement resulted in a 30% reduction of eFIFO approximately (Figure 3B, $p < 0.001$). Stress and PE supplementation impacted eFIFO in the same direction as for FMC ($p \leq 0.001$).

3.3. Underlying digestive and stress physiology

Dietary FM replacement significantly decreased ADC of proteins and amino acids but not of dry matter (Table 5). Dietary PE supplementation resulted in enhanced ADCs, regardless of FM levels. Interestingly, the LFM diet top-coated with 2% PE resulted in significantly higher ADC than the ones observed for HFM dietary group. In most cases, the availability of individual amino acids was enhanced by the dietary PE supplementation compared to the levels of the non-supplemented LFM and HFM diets (Table S4).

While FM reduction only affected fish gut EH ($p < 0.05$, Table 6), repeated periods of chasing stress resulted in compromised status of fish gut morphometry, with significant impairment in EH and GC counts, and numerical decrease in VL and ID ($p \leq 0.065$). Whatever the basal diet or stress conditions, PE dietary coating resulted in significantly improved values of all fish gut morphometric parameters ($p < 0.01$, Table 6). The Spearman correlation coefficient analysis revealed a significant negative correlation ($p < 0.05$, Table S7) between fish FCR values and their gut morphometrics, with the exception of EH ($p > 0.05$). It also indicated that all fish gut morphometric parameters analyzed in this study were strongly correlated among themselves ($p \leq 0.001$).

Morphologies of fish intestine and liver tissues are illustrated with Figure S1 and S2, respectively. Higher liver vacuolation was observed in LFM non coated groups (Figure S2, b

and f) while the lowest vacuolation was shown in the fish groups fed PE supplemented diets, regardless of stress conditions (Figure S2, c, d, g and h).

Both plasma and muscle cortisol were strongly affected by repeated stress periods ($p < 0.001$, Table 7). However, fish tissues were not affected the same ways with 6-10 times higher cortisol levels in muscle of the stressed groups, while differences observed for circulating plasma cortisol were limited to 2-3 times the observed basal values. FM reduction also significantly increased cortisol levels in fish muscle ($p < 0.05$) but not in fish plasma. Fish receiving the PE supplement in the LFM diet exhibited muscular cortisol levels ($p < 0.05$), similar to those observed in groups receiving HFM, irrespective of stress conditions.

Plasma levels of metabolic indicators were not influenced by feed changes, except for slightly lower circulating TG values observed in LFM groups ($p < 0.05$, Table S5). Repeated periods of stress showed a significant reduction of all fish circulating fat components (TG, cholesterol, LDL, $p < 0.01$, Table S5) except for HDL which increased. No effects on glucose and protein levels were observed.

Haematocrit and haemoglobin levels were not impacted by the stress nor PE coating or FM reduction ($p > 0.05$, Table S6). However significant interactions ($p < 0.01$, Table S6) were shown for Ht between PE coating and FM reduction, indicating a differential trend of Ht values, following PE coating when associated to LFM groups.

3.4. Survival to the bacterial challenge

The survival kinetics and log-rank analysis clearly highlighted 3 distinct statistical groups ($p < 0.001$, Figure 4). In the first group, highest survival rates were observed for the non-stressed fish fed HFM basal diets or with PE dietary supplementations. Oppositely, the lowest survival

rate, i.e. almost 7 times lower than the average one observed for the first statistical group, was seen for the fish fed LFM control diet in stress conditions. Between these two groups, survival rates at the end of the bacterial challenge were approximately 30% for LFM, and stressed fish receiving either HFM basal diet or PE supplementation.

3.5. Underlying immune physiology

Except for lysozyme activity, all the measures of non-specific immune response were reduced in repeated stress conditions (Table 8). The dietary top coating with PE resulted in a substantial enhancement of all innate immune responses whatever the stress conditions (Table 8, SxPE interactions > 0.05), with the exception of myeloperoxidase levels. Dietary FM reduction resulted in significantly lower levels of lysozyme activities and circulating Ig. Significant interactions, between PE and FM were also observed for these two parameters, when fish groups received the LFM diet top-coated with PE.

All markers associated with antioxidative capacities were substantially impacted by periodical repeated stress, resulting in a simultaneous decrease in TAC and an elevation in malondialdehyde muscle concentrations (Table 9). While FM reduction mostly impacted SOD, TAC and MDA ($p < 0.05$), dietary PE coating resulted in improved values of all parameters reflecting fish antioxidative status, with significant differences compared to LFM fish groups ($p < 0.05$) but not for HFM ones (Table 9).

4. Discussion

4.1. FM reduction preserves fish zootechnical performance but compromises stress levels and resistance to bacterial challenges.

Consistent with previous findings (Herault et al., 2023), the reduction of dietary FM from 30 to 15% did not impair fish growth or FCR, despite a reduction in FI and IGF-I levels. However, the digestibility trial revealed a significantly lower ADC of protein ($p < 0.05$) in the LFM group, after 10 days of feeding, compared to the HFM group. This suggests that fish fed the LFM diet may exhibit an adaptive capacity, enabling them to compensate during the 15 weeks of feeding. This compensation likely reflects the ability of the fish to adjust their digestive processes or metabolism over time, allowing for improved nutrient utilization despite the initial reduction in protein digestibility. This phenomenon was also observed in gilthead seabream in pilot scale trials implemented by Benedito-Palos et al. (2016) and Simo-Mirabet et al. (2018). However, Kokou et al. (2016) did not observe this trend, maybe because SPC was the only source of plant protein for FM replacement.

Similarly to Herault et al. (2023), 50% FM replacement with plant proteins resulted in noticeable immunodeficiencies and reduced antioxidative defenses. Similarly to previous studies, FM reduction had no significant effects on plasmatic cortisol values (Ribeiro et al., 2015; Sadoul et al., 2016). However, low FM diets showed, in our current study, elevated levels of muscle cortisol, supposed to be more integrative. This suggests that replacing FM with plant proteins can sometimes be perceived by fish as a long-term nutritional stressor (Bonaldo et al., 2015), even though fish zootechnical parameters remain unaffected. Long term exposures to cortisol are known to be deleterious (Yada and Tort, 2016) and resulting immunodeficiencies are likely to make fish less resistant to any infection process from an opportunistic pathogen as illustrated in this study by the increased mortality kinetics observed in LFM fish group.

318 This study confirmed that acceptable zootechnical performance can be achieved with red
319 seabream sub-adults fed a diet containing only 15% fishmeal (FM). However, this comes at the
320 cost of silently compromised fish welfare and health, possibly leading, like any significant
321 stressor, to severe losses if a pathogen infection occurs.

322 *4.2. Intermittent repeated stress periods result in decreased fish performance and health.*

323 Compared to a previous study (Herault et al., 2023) where a 1 min net chasing stress was
324 repeated daily for 15 weeks, the impact of the same stressor on fish zootechnical and health
325 performance remained very severe in the current study despite of 2-weeks resting periods
326 following the 1-week daily repeated stress.

327 Deleterious impact of repeated stress on fish welfare, feeding motivation and resulting
328 compromised growth and health performance have been well described in the literature
329 (Bernier et al. 2004; Sadoul and Vijayan, 2016; Yada and Tort, 2016).

330 In this research, the declines in zootechnical and health performance caused by intermittent
331 repeated stress were similar for all diets, illustrated by very few interactions between FM
332 replacement and stress conditions. This suggests an additive detrimental effects of dietary and
333 handling stressors, also illustrated by survival kinetics. Such additive effects were previously
334 observed despite a more pronounced stressor (Herault et al. 2023). Consequently, these results
335 demonstrate that the diet, including PE addition, cannot mitigate absolute effects of stress.

336 *4.3. PE supplementation as an effective mitigator of reduced fish meal in the diet.*

337 The tested PE, combining shrimp hydrolysate and sensory flavors, aimed to enhance dietary
338 palatability in marine fish. This was overall not the case for HFM diet, in both stressed and
339 control diet, illustrated by similar food intake levels. However, the supplementation with PE
340 increased feed intake and growth rates in red seabream groups fed the LFM diet, restoring the
341 levels observed for the HFM groups. Similarly, Tola et al. (2019) reported improved FCR in

red seabream on an SPC-based diet with 0.5% L-glutamic acid and 1.8% fish hydrolysate. Also, Tola et al. (2022) found that a 2.5% liquid PE made from tuna hydrolysate enhanced the protein efficiency ratio (PER) in Asian seabass fed a 40% SBM diet.

As observed in Herault et al. (2023), these improvements in FCR were likely related to better gut morphology (VL, EH, ID, GC) compared to the LFM groups. Rahimnejad et al. (2023) confirmed positive effects on the gut morphology of pikeperch (*Sander lucioperca*) using the same PE, while Khosravi et al. (2018) saw similar results in olive flounder (*Paralichthys olivaceus*) with various protein hydrolysates at higher doses. Notably, ADC for DM, CP, and amino acids improved within 10 days of PE supplementation, indicating rapid gut remodeling. Calo et al. (2024) also reported improved ADC for crude protein and energy in rainbow trout (*Oncorhynchus mykiss*) supplied with a LFM feed containing umami PE but saw no FCR enhancement. Their study, along with Tola et al. (2019, 2022), did not find changes in stomach or intestine digestive functions while the impact of PE supplementation on fish microbiota would deserve further investigations.

Fish supplemented with 2% PE showed enhanced health status, including significantly enhanced non-specific immune responses, antioxidants capacities and reduced levels of stress markers compared to the LFM diet, restoring values observed for the HFM groups. Khosravi et al. (2015) also reported improved non-specific immunity in red seabream and olive flounder fed HFM diets with 2% top-coated tuna or krill hydrolysates. In their study, possibly due to higher dietary FM levels (52 and 50% respectively), only red seabream receiving the tuna hydrolysate PE supplementation showed a significantly higher survival at the end of 21 days of bacterial injection challenge with 1 x 10^5 CFU/mL^{-1}.

Dietary supplementation of a shrimp hydrolysate-based PE helped fish to cope with reduced FM content, and mitigated bacterial challenge impacts in LFM groups through a significant reduction of fish mortality. This is consistent with a variety of research on hydrolysates use in aquatic species (Siddik et al., 2021). However, despite the well-documented benefits of functional peptides, such as anti-microbial peptides (AMP), which have been shown to be effective at low doses (Wang et al., 2023), it remains challenging to conceive such exhaustive list of benefits with a liquid product (PE) applied at maximum 0.45% dry matter or 0.25% crude protein. A recent study (Herault et al., unpublished), which followed similar methodology in red seabream juveniles, showed a higher global performance resulting from the dietary top-coating versus inclusion of the shrimp hydrolysate-based PE. These results suggest that top-coating application of a PE makes it more available to fish gustative receptors compared to when it is included in the feed matrix. The full mechanistics between a higher feeding motivation, and enhanced fish health performance would deserve further investigations. Part of it could rely on lower expressed cortisol levels, known to be immune-suppressant when chronically high (Yada and Tort, 2016) and a higher contribution to fish optimum nutritional requirements.

As described in Fournier (2013), PE dietary dosage can be reduced with fish pellet size due to a lower volume-specific surface area. This will result in even lower environmental and economic cost-in-use as most of aquafeed volumes consist in pellet size higher than 5mm length.

Ensuring optimal feed palatability for juvenile fish by applying a 2% top-coating of shrimp hydrolysate liquid PE has resulted in restored zootechnical and health performance, compensating for the reduced FM content. Interestingly, zootechnical performances of PE supplementation outperformed the HFM diet for both diets (HFM and LFM). Similar effects,

more pronounced were observed with a 5% supplementation of powdered APH (Herault et al., 2023). However, the difference in efficiency is counterbalanced by the lower economic and environmental costs of the PE tested in the present study.

4.4. Assessment of the potential sparing of wild fish using a shrimp hydrolysate-based PE.

In 2019, 22.52 million tons of fish - 86% being pelagic fish - were reportedly used as animal feed (Tacon et al., 2023) with 10% of them used -as FM and FO- in marine fish diets (Naylor et al., 2021). As mentioned before, a typical marine fish diet formulation currently contains 14% FM (Naylor et al., 2021) but this number hides great disparities between species, growth stages, markets but also feed manufacturers. While it is admitted that most of fish feed volumes consists in feed sizes longer than 5mm, shorter size feeds are containing higher contents of fish meal due to both higher nutritional requirements and conservatism among feed producers. In our research, we effectively decreased the proportion of FM derived from wild fish to 15% in a 3 to 4 mm diet produced for sub-adult red seabream.

Considering that the applied stress condition is more representative of commercial fish farming, we can assume that the FMC ratio to produce 1kg of crude fish biomass can reasonably be reduced to a maximum of 0.26 instead of 0.50 for red seabream fed HFM diet (Figure 3A). This 52% FMC reduction can be considered as realistic, and conservative, as fish groups receiving LFM+PE outperformed fish groups supplied with HFM control diet. Extending this FMC ratio to the entire Sparidae farming industry, which is mostly represented by gilthead seabream (282,1 kT in 2020; FAO 2022), and assuming similar nutritional properties as in red sea bream, it could result in a saving of more than 67.7 kT of FM derived from wild sources. A rough extrapolation to the entire marine fish farming industry would result in a saving of 594.8 kT per year (Naylor al., 2021).

412 eFIFO can be considered as the most relevant indicator for assessing the sparing of wild fish
413 biomass (Kok et al., 2020) in aquafeed industry. Because eFIFO accounts for the levels of oil
414 included in the diet, and integrates that fish oil as the limiting ingredient on the market, the FM
415 replacement resulted in a 25% eFIFO reduction in the stressed fish group while PE
416 supplementation resulted in an additional 6% reduction. As a result, the eFIFO required to
417 produce 1 kg of fish, from 27 to 125 g, was reduced from 2.05 down to 1.41 in farm-alike stress
418 conditions.
419 This value, estimated for juveniles, can be compared with the 0.94 eFIFO calculated by Kok et
420 al. (2020) for the entire marine fish farming industry. We can reasonably assume that this could
421 also be reduced by 31% by generalizing the practice of PE coating on diets where 50% of FM
422 is substituted. This would further support marine fish aquaculture in being a net fish producer.
423 Extrapolating it to Sparidae, or whole marine fish industry, this would respectively represent a
424 sparing of 87.5 kT and 768.3 kT of wild fish biomass.
425 Last but not least, it is interesting to note that a more ambitious study has recently been
426 conducted in red seabream by Takakuwa et al. (2023) who fully substituted dietary FM with a
427 mix of plant proteins and land-based proteins, during a full production cycle in commercial
428 conditions. They observed a moderate, but significant, loss of growth that they explained with
429 a lower feed intake and bile acid production. We may imagine that these issues would have
430 been mitigated with the adequate use of a PE.
431

432 ## *5.* Conclusions and perspectives

433 This research supports that it is possible to obtain fully satisfying zootechnical and health
434 performance in red seabream juvenile fed diets containing 47% plant protein and only 15% of
435 FM, when top-coated with liquid PE formulated mainly from a sustainable source of shrimp

hydrolysate made from coproducts. However, we also confirmed, that it is mandatory to supplement FM-substituted diets with a functional ingredient in order to offset the underlying effects of plant proteins silently affecting fish welfare and health defenses.

Additionally, PE supplementation was essential to maintain satisfying fish performance under intermittent periods of repeated husbandry stress, more representative of what fish would normally experience in commercial farming operations.

Our results also suggest that FM content in diets could be further reduced, as juvenile fish fed the PE-supplemented diet outperformed the control group. This would allow the Sparidae, and possibly other marine fish, farming industries to further progress on their reliance on wild fish. Such supplementations can therefore help alleviate concerns regarding aquaculture's use of wild fish, though it is important to consider other potential environmental impacts. Therefore, additional research is required to comprehensively evaluate the environmental footprint of using these types of PE in aquaculture.

Declaration of competing interest and funding source

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Mikaël Herault and Vincent Fournier are working as Performance Measurement Manager and R&D Manager at Symrise Aqua Feed.

Buddhi E. Gunathilaka and Kyeong-Jun Lee declare no conflict of interests.

Hervé Le Bris and Bastien Sadoul declare no conflict of interests.

Author contributions

Mikaël Herault participated to the conceptualization, formal analysis, and writing - original draft of the study. Vincent Fournier participated to the conceptualization of the study and funding acquisition. Bastien Sadoul participated to the formal analysis and writing - original draft of the study. Hervé Le Bris participated to the writing - original draft of the study. Buddhi E. Gunathilaka participated to the data curation, formal analysis and investigation of the study. Kyeong-Jun Lee participated to the conceptualization, project administration and supervision of the study. All coauthors contributed to the writing - reviewing & editing.

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Table 1. Formulation and proximate composition of the experimental diets for red seabream (*Pagrus major*) (%, dry matter basis)

Ingredients	Experimental diet			
	HFM[1]	LFM[2]	HFM+PE	LFM+PE
FM FAQ65[3]	30.00	15.00	30.00	15.00
PE[4] / coating			2.00	2.00
Soy protein concentrate[5]	14.33	20.20	14.33	20.20
Wheat Gluten[6]	9.00	12.00	9.00	12.00
Corn gluten meal[7]	10.00	15.00	10.00	15.00
Wheat flour[8]	22.00	21.00	22.00	21.00
Fish oil[9]	4.95	6.85	4.95	6.85
Soybean oil[10]	4.98	3.94	4.98	3.94
Mineral Mix[11]	1.00	1.00	1.00	1.00
Vitamin Mix[12]	1.00	1.00	1.00	1.00
Starch[13]	2.14	1.04	2.14	1.04
Choline chloride[14]	0.50	0.88	0.50	0.88
L-Lysine[15]	0.00	0.37	0.00	0.37
L-Methionine[16]	0.00	0.08	0.00	0.08
Taurine[17]	0.10	0.14	0.10	0.14
Mono-calcium phosphate[18]	0.00	1.50	0.00	1.50
Proximate composition (%, dry matter)				
Crude protein	50.4	49.4	50.1	49.2
Crude lipid	13.3	12.5	13.6	13.0
Crude ash	7.07	6.31	7.14	6.22
Gross energy ($MJ.kg^{-1}$)	21.78	21.59	21.84	21.65
Moisture	9.67	8.41	9.81	9.65

[1]High fish meal

[2]Low fish meal

[3]Fish meal fair average quality (DM basis, 69.2% protein and 8.3% lipid), Orizon S,A., Co., Ltd., Chile.

[4]Palatability enhancer - Extrapal L410 (crude basis, 12.8% protein and 1.3% lipid), Symrise Aqua Feed, Ecuador.

[5]Soy protein concentrate (DM basis, 72.0% protein and 0.14% lipid), CJ CheilJedang Co., Ltd., Seoul, South Korea.

[6]Wheat gluten (DM basis, 80.7% protein and 1.5% lipid), Royal Ingredients Group, Alkmaar, Netherlands.

[7]Corn gluten meal (DM basis, 68.9% protein and 11.9% lipid), Daebong LF Co., Jeju, South Korea.

[8]Wheat flour (DM basis, 12.8% protein and 6.2% lipid), CJ CheilJedang Co., Ltd., Seoul, South Korea.

[9]Fish oil, E-wha Oil Industry, Busan, South Korea.

[10]Soybean oil, Ottogi Co., Ltd., Anyang, South Korea.

[11]Mineral premix (g kg^{-1} of mixture): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[12] Vitamin premix (g kg^{-1} of mixture): L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-$_D$-pantothenate,

12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.
[13]Starch, Samyang Co., Ltd., Seoul, South Korea.
[14]Choline chloride, Solton-Biochem Co., Ltd., Cheonan, South Korea.
[15]L-Lysine, Ajinomoto Amino Acid Co., Ltd., Shanghai, China.
[16]L-Methionine, Evonik Pharmaceutical Co., Ltd., China.
[17]Taurine, Qianjiang Yongan Pharmaceutical Co., Ltd., Qianjiang, China.
[18]Mono-calcium phosphate, Sigma-Aldrich, Missouri, USA.

Table 2. Amino acid contents (% of the ingredient as dry matter basis) of the four experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) for red seabream.

	Experimental diets			
	HFM	LFM	HFM+PE	LFM+PE
Non-essential amino acids				
Aspartic acid	3.35	2.77	3.64	2.67
Alanine	2.29	1.97	2.69	1.85
Serine	1.85	1.81	2.08	1.72
Glutamic acid	9.54	9.64	10.5	9.21
Proline	2.78	2.53	2.99	2.63
Glycine	1.87	1.50	2.07	1.43
Tyrosine	1.22	1.19	1.45	1.12
Essential amino acids				
Arginine	2.37	2.04	2.63	1.93
Threonine	1.58	1.35	1.79	1.29
Valine	2.07	1.77	2.34	1.68
Phenylalanine	2.05	1.96	2.32	1.85
Isoleucine	1.87	1.63	2.1	1.56
Leucine	3.56	3.68	4.22	3.46
Histidine	1.13	0.90	1.23	0.86
Lysine	1.99	1.49	2.23	1.41
Methionine	0.93	0.81	1.10	0.76
Taurine	0.35	0.24	0.40	0.23

Table 3. Chemical compositions of Extrapal Shrimp, a palatability enhancer (PE) formulated from a shrimp liquid hydrolysate (from product technical data sheets).

	PE
Dry matter, DM (%)	≥ 21
Protein (%)	≥ 11
Lipid (% DM)	≤ 5
Ash (% DM)	≤ 8
Soluble nitrogen (% N)	91.1
Molecular weight repartition (% of peptides)	
Peptides > 30,000 Da	<0.1

Peptides 20,000 - 30,000 Da	<0.1
Peptides 10,000 - 20,000 Da	<0.1
Peptides 5,000 - 10,000 Da	<0.5
Peptides 1,000 - 5,000 Da	8
Peptides 500 - 1,000 Da	9
Peptides < 500 Da	83

Table 4. Physiological biomarkers used and methods

Category	Physiological biomarker	Acronym	Number of samples per tank	Sampled fish ID	Sampled tissues	Methods
Zootechnical	Insulin Growth Factor-I	IGF-1	3	4, 5, 6	L	Gunathilaka et al. (2021), primers: Hossain et al. (2016)
	Condition Factor	CF	2	7, 8	WB	x
Metabolic	Proximate composition	DM	3, pooled	4, 5, 6	WB	AOAC (1995)
	Proximate composition	DM	3, pooled	7, 8, 9	C	AOAC (1995)
	Liver lipid	LL	2, pooled	7, 8, 9	L	AOAC (1995)
	Viscera lipid	VL	2, pooled	1, 2	V	AOAC (1995)
	Muscle water retention	MWR	2, pooled	1, 2	M	AOAC (1995)
	Hepato-somatic index	HSI	2	7, 8, 9	L	x
	Viscera-somatic index	VSI	2	7, 8, 9	V	x
Blood biochemistry	Glucose	-	3	1, 2, 3	P	Gunathilaka et al. (2021)
	Triglyceride	TG	3	1, 2, 3	P	CK#2200-225 from Biovision Inc. (Moutainview, USA)
	Total cholesterol	-	3	1, 2, 3	P	CK#1010-225 from Biovision Inc. (Moutainview, USA)
	High density lipoprotein	HDL	2	1, 2, 3	P	CK#K613-100 from Biovision Inc. (Moutainview, USA)
	Low density lipoprotein	LDL	2	1, 2, 3	P	CK#K613-100 from Biovision Inc. (Moutainview, USA)
	Total protein	-	3	1, 2, 3	P	Gunathilaka et al. (2021)
Immune – non-specific	Antiprotease	Ap	3	1, 2, 3	P	Ellis (1990) with slight modification (Magnadóttir et al., 1999)
	Lysozyme	LYS	3	4, 5, 6	S	Khosravi et al. (2015a)
	Myeloperoxidase	MPO	3	4, 5, 6	S	Quade and Roth (1997)
	Respiratory burst activity	NBT	3	4, 5, 6	S	NBT, Anderson and Siwicki, 1995
	Total immunoglobulin	Ig	3	1, 2, 3	P	Siwicki and Anderson (1993)

Table 4 (continued). Physiological biomarkers used and methods

Category	Physiological biomarker	Acronym	Number of samples per tank	Sampled fish ID	Sampled tissues	Methods
Health	Catalase	CAT	2	4, 5	S	CK#K773-100 from Biovision Inc. (Moutainview, USA)
	Super oxide dismutase	SOD	3	4, 5, 6	S	CK#19160 from Sigma
	Glutathione peroxidase	GPx	3	4, 5, 6	S	CK#K762-100 from Biovision Inc. (Moutainview, USA)
	Total antioxidant capacity	TAC	2	1, 2	M	CK#CS0790 from Sigma
	Malondialdehyde	MDA	2	1, 2	M	CK#K739-100 from Biovision Inc. (Moutainview, USA)
	Cortisol		3	1, 2, 3	P/M	CK# Fish CSB-E08487f from Cusabio
	Hematocrit	Ht	3	1, 2, 3	HB	Gunathilaka et al. (2021)
	Hemoglobin	Hg	3	1, 2, 3	P	Gunathilaka et al. (2021)
Gut morphometrics	Villi length	VL	3	7, 8, 9	V	Gunathilaka et al., 2020
	Enterocyte height	EH	3	7, 8, 9	V	Gunathilaka et al., 2020
	Intestinal diameter	ID	3	7, 8, 9	V	Gunathilaka et al., 2020
	Goblet cell count	GC	3	7, 8, 9	V	Gunathilaka et al., 2020

HB: heparinized blood, P: plasma, S: Serum
M: Muscle, V: viscera, L: Liver
WB: Whole Body, C: carcass
CK: commercial kit

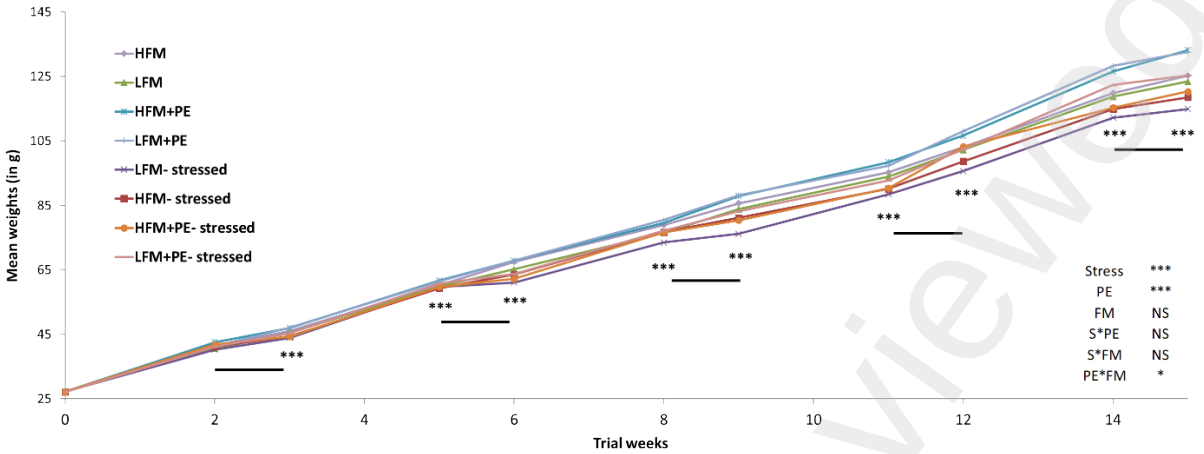
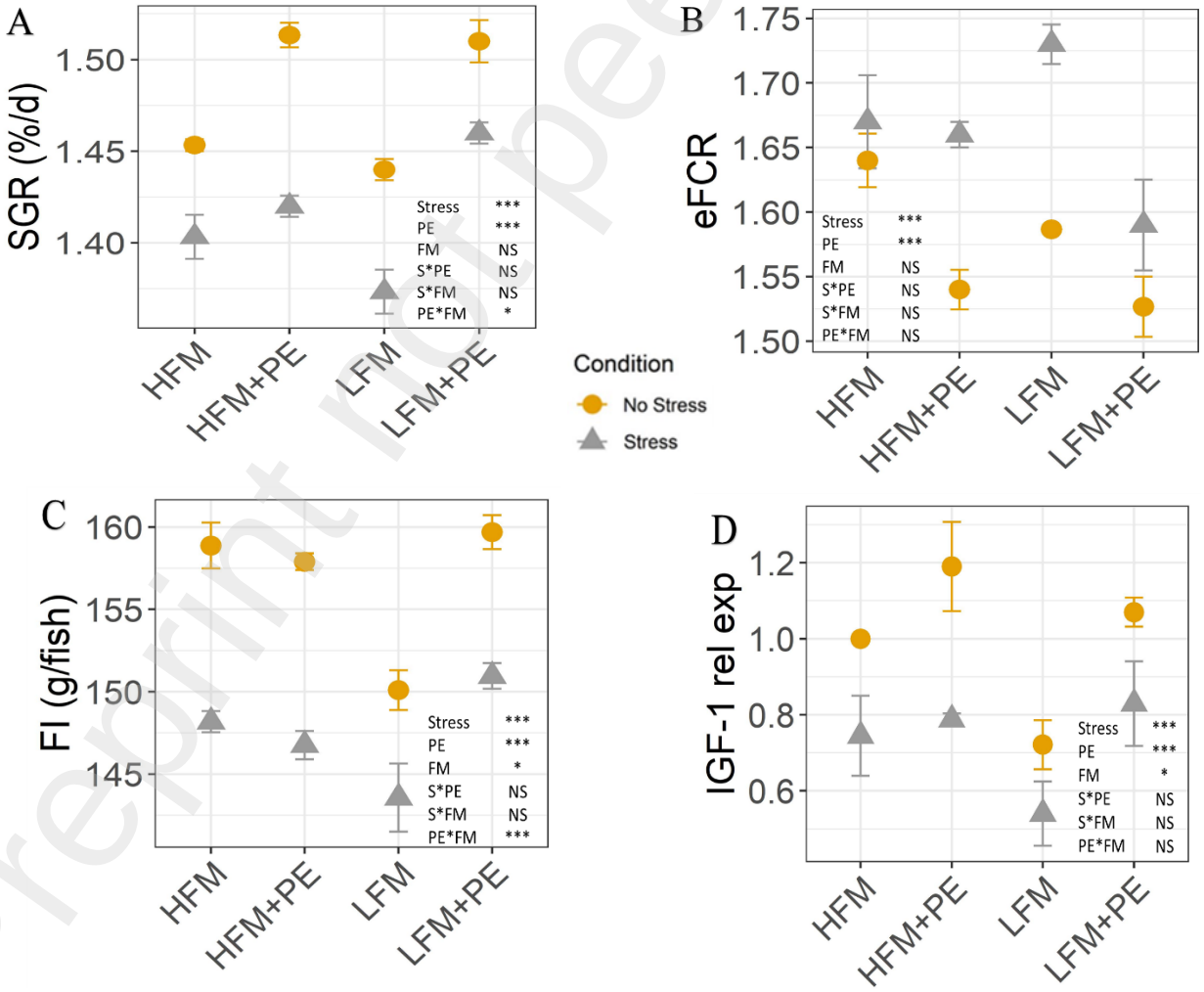
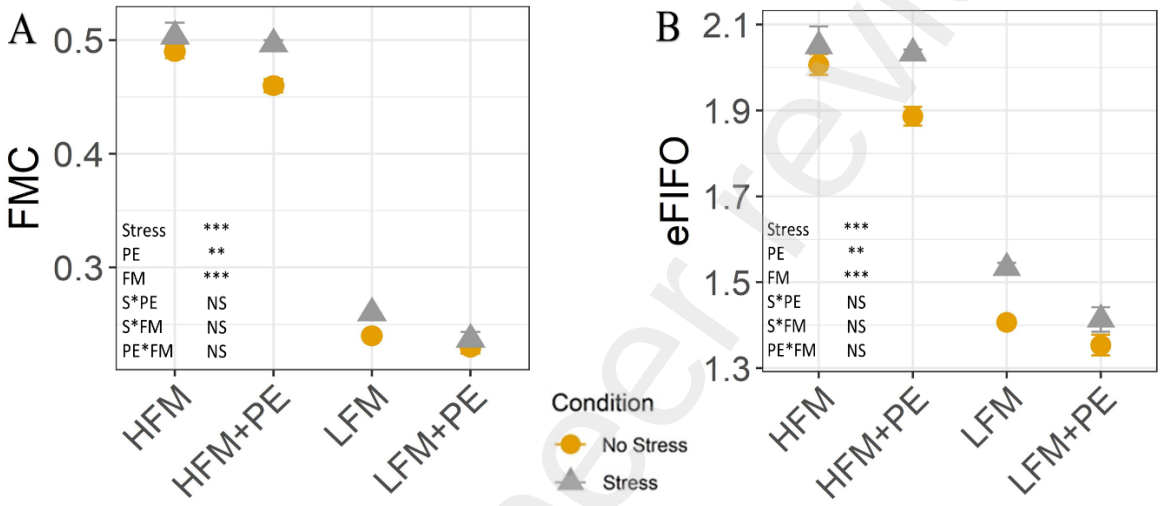


Figure 1. Mean weight kinetics of red seabream juveniles fed experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) for 15 weeks and submitted to control or periodical stress conditions (net chased daily during periods identified with the horizontal black line). Statistical significance is indicated with asterisks (1-way and 2-way ANOVA) and NS when p > 0.05).



Figures 2. Zootechnical parameters of red seabream groups fed the experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) for 15 weeks and submitted to control or periodical stress conditions (1 week daily net chasing every 2 weeks, S) 15 weeks feeding trial. **A**. Specific Growth Rates (%/d), **B**. Economic feed conversion ratio **C**. Feed intakes (g/fish), **D.** Liver insulin-like growth factors I (relative expression of mRNA). Statistical significance is indicated with asterisks (2-way ANOVA) and NS when p > 0.05).



Figures 3. Fisheries resources efficiency metrics of red seabream groups after 15 weeks fed on experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) and submitted to control or periodical stress conditions (1 week daily net chasing every 2 weeks, S). **A**. Fish meal consumption (FMC, kg of FM/kg of produced biomass), **B**. Economic fish in fish out ratio (eFIFO). Statistical significance is indicated with asterisks (2-way ANOVA) and NS when p > 0.05).

Table 5. Apparent digestibility coefficients (%, of ADC) for protein and dry matter of the four experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) for red seabream.

	ADCd[1]	ADCp[2]	ADCa[3]
HFM	62.2±1.28^{b}	86.7±0.45^{b}	81.7±3.69^{b}
LFM	61.3±1.36^{b}	82.5±0.61^{c}	72.1±1.44^{a}
HFM+PE	69.2±3.00^{a}	90.8±0.90^{a}	87.5±2.17^{b}
LFM+PE	73.0±2.62^{a}	91.3±0.85^{a}	82.9±2.12^{b}

Factorial ANOVA (P>F)			
PE coating	0.001	0.000	0.000
FM reduction	0.303	0.003	0.001
Interactions PExFM	0.105	0.001	0.124

Data are presented as mean of triplicate tanks ± S.D. Values with different superscripts in the same column are significantly different ($P < 0.05$). [1]Apparent digestibility coefficients of dry matter (%); [2]Apparent digestibility coefficients of protein (%);[3] Apparent digestibility coefficients of total amino acids (%) based on their dietary ponderation.

Table 6. Morphometric parameters of red seabream intestine fed the four experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) and submitted to control or periodical stress conditions (1-week daily net chasing every 2 weeks, S) for 15 weeks.

	VL[1]	EH[2]	ID[3]	GC[4]
HFM	1456±38^{ab}	57.7±2.38^{a}	3512±402	876±8.8^{abc}
LFM	1196±84^{b}	47.8±1.49^{c}	2855±101	855±55^{bc}
HFM+PE	1395±65^{ab}	54.6±1.43^{ab}	3516±206	910±54^{abc}
LFM+PE	1572±117^{a}	56.5±3.08^{a}	3715±325	974±23^{a}
HFM-S	1224±149^{b}	48.7±3.90^{bc}	2891±521	835±32^{c}
LFM-S	1203±68^{b}	47.6±2.26^{c}	2804±309	809±17^{c}
HFM+PE-S	1379±157^{ab}	53.2±1.58^{abc}	3343±428	884±53^{abc}
LFM+PE-S	1445±36^{ab}	52.3±0.53^{abc}	3471±144	959±24^{ab}
Factorial ANOVA (P>F)				
Chasing stress	0.056	0.005	0.065	0.049
PE coating	0.001	0.005	0.002	0.000
FM reduction	0.840	0.045	0.460	0.143
Interactions SxPE	0.653	0.460	0.648	0.430
Interactions SxFM	0.485	0.210	0.378	0.952
Interactions PExFM	0.010	0.019	0.069	0.006

Data are presented as mean of triplicate tanks ± S.D. Values with different superscripts in the same column are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments. [1]Villi length (µm); [2]Enterocyte height (µm); [3]Intestinal diameter (µm) and [4]Goblet cell count.

Table 7. Muscle and plasma cortisol levels of red seabream fed the four experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) and submitted to control or periodical stress conditions (1-week daily net chasing every 2 weeks, S) for 15 weeks.

	Muscle cortisol	Plasma cortisol

HFM	3.37±2.03[b]	5.08±1.00
LFM	13.60±0.64[a]	6.06±1.16
HFM+PE	4.59±1.10[b]	5.05±0.93
LFM+PE	4.27±1.00[b]	5.49±1.04
HFM-S	31.1±6.48[b]	14.3±4.00
LFM-S	83.6±21.1[a]	16.3±0.36
HFM+PE-S	44.4±12.7[b]	12.0±2.02
LFM+PE-S	34.5±10.2[b]	13.2±1.26
Factorial ANOVA (P>F)		
Chasing stress	0.000	0.000
PE coating	0.041	0.052
FM reduction	0.017	0.123
Interactions SxPE	0.181	0.113
Interactions SxFM	0.119	0.537
Interactions PExFM	0.002	0.656

Data are presented as mean of triplicate tanks ± S.D. Values with different superscripts in the same column are significantly different ($P < 0.05$) – cortisol values were distinguished for the 1-way ANOVA between stressed and non-stressed dietary groups due to very different values between the 2 stress conditions groups. Cortisol (ng mL^{-1}).

Table 8. Non-specific immune response parameters of red seabream fed the four experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) and submitted to control or periodical stress conditions (1-week daily net chasing every 2 weeks, S) for 15 weeks.

	AP[1]	LYS[2]	MPO[3]	NBT[4]	Ig[5]
HFM	15.0±0.5[abc]	5.65±0.56[abc]	0.41±0.04[ab]	1.06±0.06	22.7±2.87[bcd]
LFM	14.4±1.2[bcd]	4.24±0.39[bc]	0.38±0.01[abcd]	1.01±0.05	19.0±0.95[d]
HFM+PE	16.2±0.6[ab]	6.28±0.62[a]	0.43±0.05[a]	1.08±0.02	27.0±0.42[a]
LFM+PE	16.8±1.5[a]	6.37±0.10[a]	0.41±0.01[abc]	1.08±0.02	25.8±1.15[ab]
HFM-S	12.5±0.6[d]	5.78±0.61[abc]	0.32±0.05[bcd]	0.99±0.02	21.0±2.19[cd]
LFM-S	12.6±0.9[d]	4.04±0.50[c]	0.34±0.01[bcd]	0.99±0.05	18.8±0.93[d]
HFM+PE-S	12.8±0.4[cd]	6.19±1.37[a]	0.32±0.01[cd]	1.02±0.03	24.2±0.24[abc]
LFM+PE-S	13.6±0.6[cd]	5.96±0.45[ab]	0.31±0.03[d]	1.04±0.01	25.1±0.93[abc]
Factorial ANOVA (P>F)					
Chasing stress	0.000	0.601	0.000	0.007	0.034
PE coating	0.002	0.000	0.795	0.015	0.000
FM reduction	0.519	0.006	0.298	0.533	0.016
Interactions SxPE	0.100	0.693	0.106	0.812	0.499
Interactions SxFM	0.476	0.542	0.259	0.323	0.122
Interactions PExFM	0.163	0.011	0.610	0.265	0.027

Data are presented as mean of triplicate tanks ± S.D. Values in the same row having different superscript letters are significantly different ($P < 0.05$). [1]Antiprotease (% inhibition); [2]Lysozyme activity (μg mL^{-1}); [3]Myeloperoxidase level; [4]Nitro blue tetrazolium activity and [5]Total immunoglobulin (mg mL^{-1}).

Table 9. Antioxidant enzyme activities, muscle antioxidant capacity and malondialdehyde level of red seabream fed the four experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) and submitted to control or periodical stress conditions (1-week daily net chasing every 2 weeks, S) for 15 weeks.

	CAT[1]	SOD[2]	GPx[3]	TAC[4]	MDA[5]
HFM	39.2±3.30	68.3±1.22^{ab}	120±1.04^{ab}	1.91±0.15^{ab}	0.46±0.05^{c}
LFM	38.3±2.90	63.1±2.04^{bcd}	118±3.44^{bc}	1.67±0.06^{cd}	0.77±0.03^{a}
HFM+PE	40.1±4.25	70.4±0.98^{a}	128±3.04^{a}	2.00±0.07^{a}	0.54±0.06^{bc}
LFM+PE	42.3±3.95	70.5±1.77^{a}	127±2.85^{a}	2.05±0.09^{a}	0.50±0.02^{bc}
HFM-S	34.6±2.53	61.3±3.25^{cd}	113±4.73^{bc}	1.63±0.06^{cd}	0.78±0.01^{a}
LFM-S	33.9±0.31	59.0±1.99^{d}	111±4.56^{c}	1.56±0.03^{d}	0.82±0.12^{a}
HFM+PE-S	39.0±5.58	67.2±1.01^{abc}	118±1.14^{bc}	1.83±0.02^{abc}	0.70±0.12^{ab}
LFM+PE-S	38.5±4.11	66.6±3.16^{abc}	118±1.90^{bc}	1.77±0.06^{bcd}	0.68±0.07^{ab}
Factorial ANOVA (P>F)					
Chasing stress	0.030	0.000	0.000	0.000	0.000
PE coating	0.030	0.000	0.000	0.000	0.008
FM reduction	0.969	0.031	0.349	0.038	0.053
Interactions SxPE	0.481	0.253	0.412	0.762	0.734
Interactions SxFM	0.677	0.515	0.963	0.594	0.093
Interactions PExFM	0.578	0.059	0.450	0.046	0.008

Data are presented as mean of triplicate tanks ± S.D. Values in the same row having different superscript letters are significantly different ($P < 0.05$). [1]Catalase activity (mU ml^{-1}); [2]Superoxide dismutase (% inhibition); [3]Glutathione peroxidase activity (mU ml^{-1}); [4]Total antioxidant capacity (mM) and [5]Malondialdehyde level (nmol mg^{-1}).

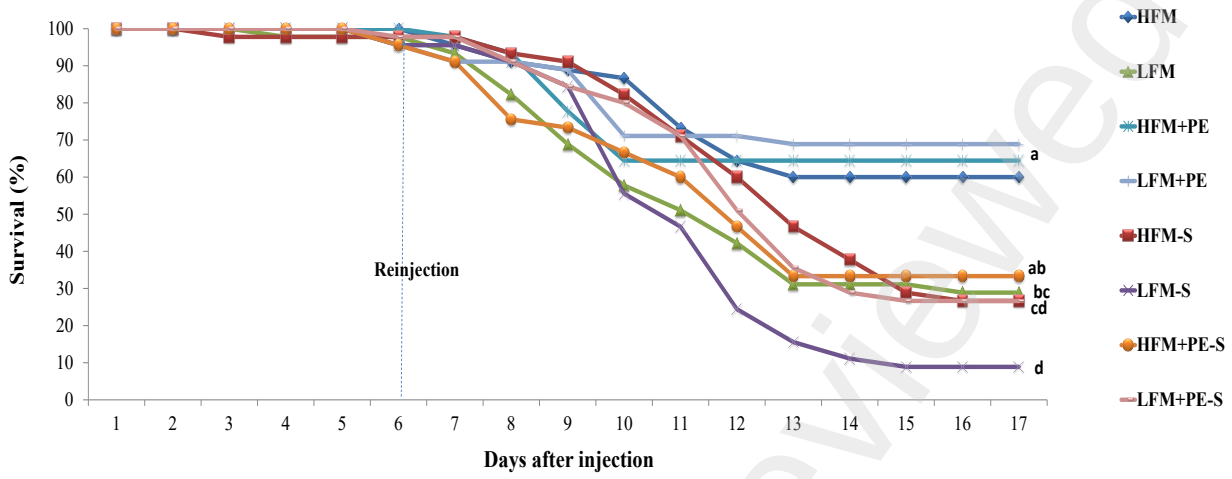


Figure 4. Survival of red seabream fed the four experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) and submitted to control or periodical stress conditions (1-week daily net chasing every 2 weeks, S) after challenge with *E. tarda.* At the beginning, fish were injected with *E. tarda* suspension containing 1 x 10^6 CFU mL^{-1}. Different letters besides the legend denote significant differences between diet x stress groups at the $P < 0.001$ level (Log-rank analysis with Bonferroni post-hoc analysis).

Appendices

Table S1. Whole-body proximate composition (%, dry matter) of red seabream fed the four experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) and submitted to control or periodical stress conditions (1-week daily net chasing every 2 weeks, S) for 15 weeks.

	Dry matter	Crude protein	Crude lipid	Crude ash
HFM	35.8±1.51	17.9±0.22	12.3±0.87	4.16±0.65
LFM	34.6±1.43	19.7±1.79	12.9±2.20	4.13±1.01
HFM+PE	34.4±1.44	18.9±1.89	12.0±1.67	4.12±0.56
LFM+PE	34.6±0.99	19.6±0.70	12.6±1.65	4.48±0.77
HFM-S	35.6±1.26	18.6±1.49	12.6±1.66	4.46±0.50
LFM-S	35.3±1.05	18.7±1.79	12.0±1.75	4.43±0.96
HFM+PE-S	35.1±0.46	19.1±1.70	12.3±2.19	4.35±0.71
LFM+PE-S	34.9±1.49	19.2±1.28	12.3±1.06	4.25±0.68
Factorial ANOVA (P>F)				
Chasing stress	0.379	0.836	0.850	0.625
PE coating	0.260	0.444	0.817	0.991
FM reduction	0.481	0.271	0.848	0.860

Interactions SxPE	0.787	0.964	0.800	0.621
Interactions SxFM	0.828	0.369	0.526	0.717
Interactions PExFM	0.477	0.678	0.842	0.796

Data are presented as mean of triplicate tanks ± S.D.

Table S2. Whole-body eviscerated carcass proximate composition (%, dry matter) of red seabream fed the four experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) and submitted to control or periodical stress conditions (1-week daily net chasing every 2 weeks, S) for 15 weeks.

	Dry matter	Crude protein	Crude lipid	Crude ash
HFM	36.3±1.23	19.0±1.22	11.3±1.17	4.69±0.33
LFM	36.0±1.27	19.5±0.76	11.0±0.99	4.83±0.79
HFM+PE	35.4±1.85	20.1±0.63	11.6±0.75	4.66±0.96
LFM+PE	35.5±0.65	19.2±1.68	11.5±1.15	4.68±0.68
HFM-S	36.3±0.56	18.9±0.82	11.7±1.19	4.64±0.31
LFM-S	36.2±0.84	19.9±1.75	11.4±0.92	4.79±0.25
HFM+PE-S	35.8±1.32	20.1±1.10	11.1±0.79	4.82±0.36
LFM+PE-S	36.1±1.43	20.2±1.08	11.4±0.95	4.76±0.40
Factorial ANOVA (P>F)				
Chasing stress	0.599	0.485	0.908	0.883
PE coating	0.331	0.236	0.846	0.982
FM reduction	0.995	0.767	0.779	0.781
Interactions SxPE	0.673	0.738	0.425	0.709
Interactions SxFM	0.883	0.462	0.798	0.942
Interactions PExFM	0.686	0.207	0.602	0.731

Data are presented as mean of triplicate tanks ± S.D.

Table S3. Viscera proximate composition, muscle water retention and liver lipid content (%, wet basis) of red seabream fed the four experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) and submitted to control or periodical stress conditions (1-week daily net chasing every 2 weeks, S) for 15 weeks.

	CF[1]	HSI[2]	VSI[3]	VL[4]	MWR[5]	LL[6]
HFM	1.93±0.03^{b}	1.45±0.17	7.52±0.95	39.6±2.32	73.1±0.79^{a}	29.1±7.06
LFM	1.95±0.07^{ab}	1.52±0.02	7.46±0.54	39.1±4.04	73.0±0.45^{ab}	28.1±1.87
HFM+PE	2.00±0.06^{ab}	1.44±0.07	7.14±0.10	41.6±3.25	73.4±0.79^{a}	30.8±3.90
LFM+PE	2.09±0.07^{a}	1.44±0.22	7.10±0.84	41.1±3.03	73.2±0.85^{a}	27.1±6.19
HFM-S	1.92±0.07^{bc}	1.26±0.09	6.19±0.12	41.6±3.69	73.1±0.68^{a}	23.6±1.75
LFM-S	1.87±0.03^{b}	1.28±0.17	6.13±0.06	40.8±1.93	71.0±0.68^{b}	23.5±3.04
HFM+PE-S	1.91±0.03^{b}	1.21±0.07	7.07±0.62	41.7±1.33	73.0±0.80^{ab}	23.9±1.58
LFM+PE-S	1.93±0.03^{b}	1.21±0.07	6.18±0.57	39.3±4.07	72.8±0.61^{ab}	23.5±1.76

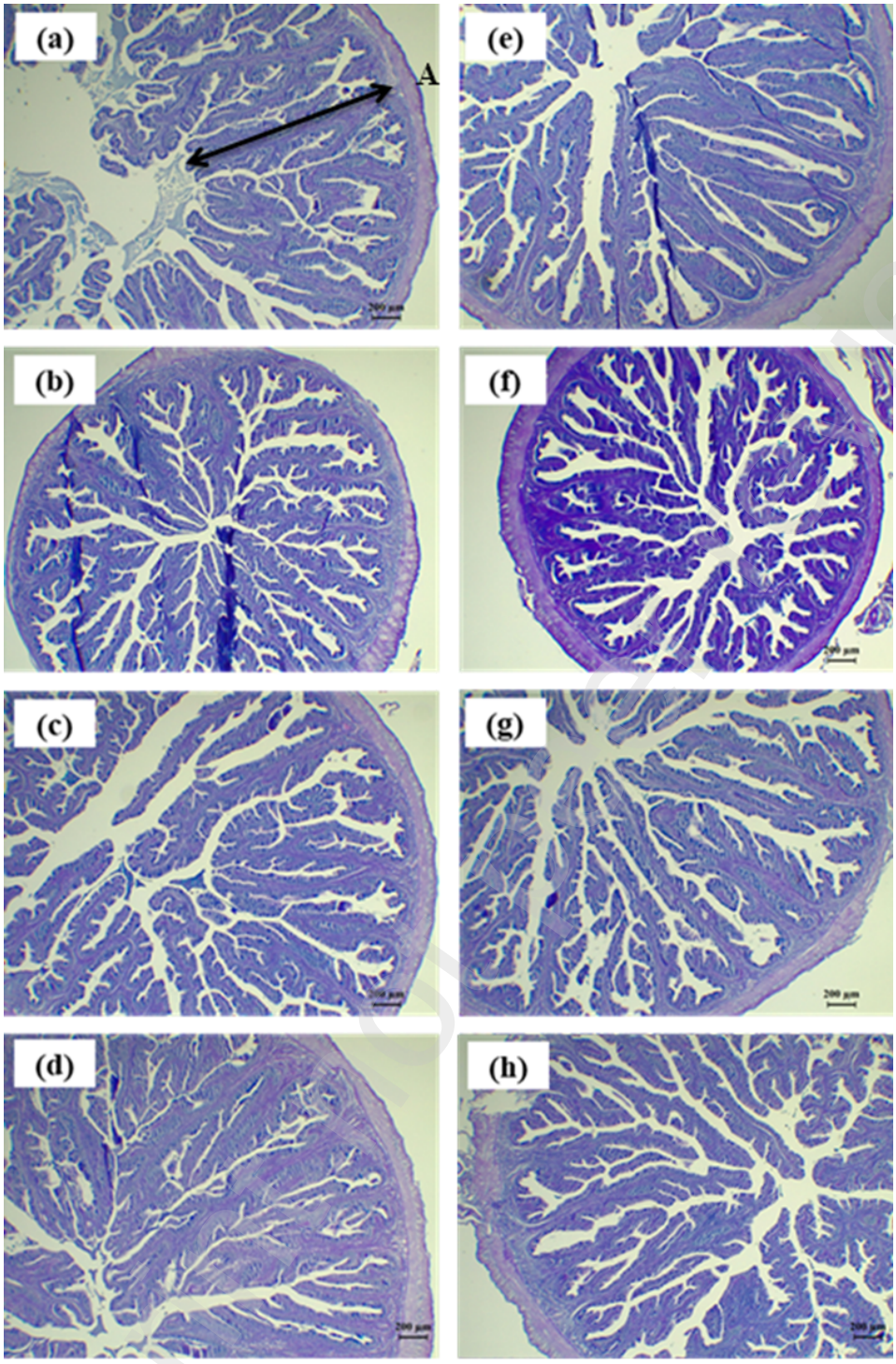
Factorial ANOVA (P>F)						
Chasing stress	0.001	0.001	0.001	0.897	0.037	0.004
PE coating	0.005	0.311	0.839	0.440	0.083	0.894
FM reduction	0.362	0.670	0.277	0.286	0.049	0.420
Interactions SxPE	0.084	0.862	0.095	0.184	0.318	0.957
Interactions SxFM	0.084	0.813	0.383	0.862	0.146	0.515
Interactions PExFM	0.150	0.670	0.413	0.572	0.141	0.648

Data are presented as mean of triplicate tanks ± S.D.
[1]Condition factor, [2]Hepatosomatic index = (Liver weight/ Fish weight) x 100, [3]Viscerosomatic index = (Viscera weight/ Fish weight) x 10; [4]Viscera crude lipid (%); [5]Muscle water retention (%) and [6]Liver crude lipid (%).

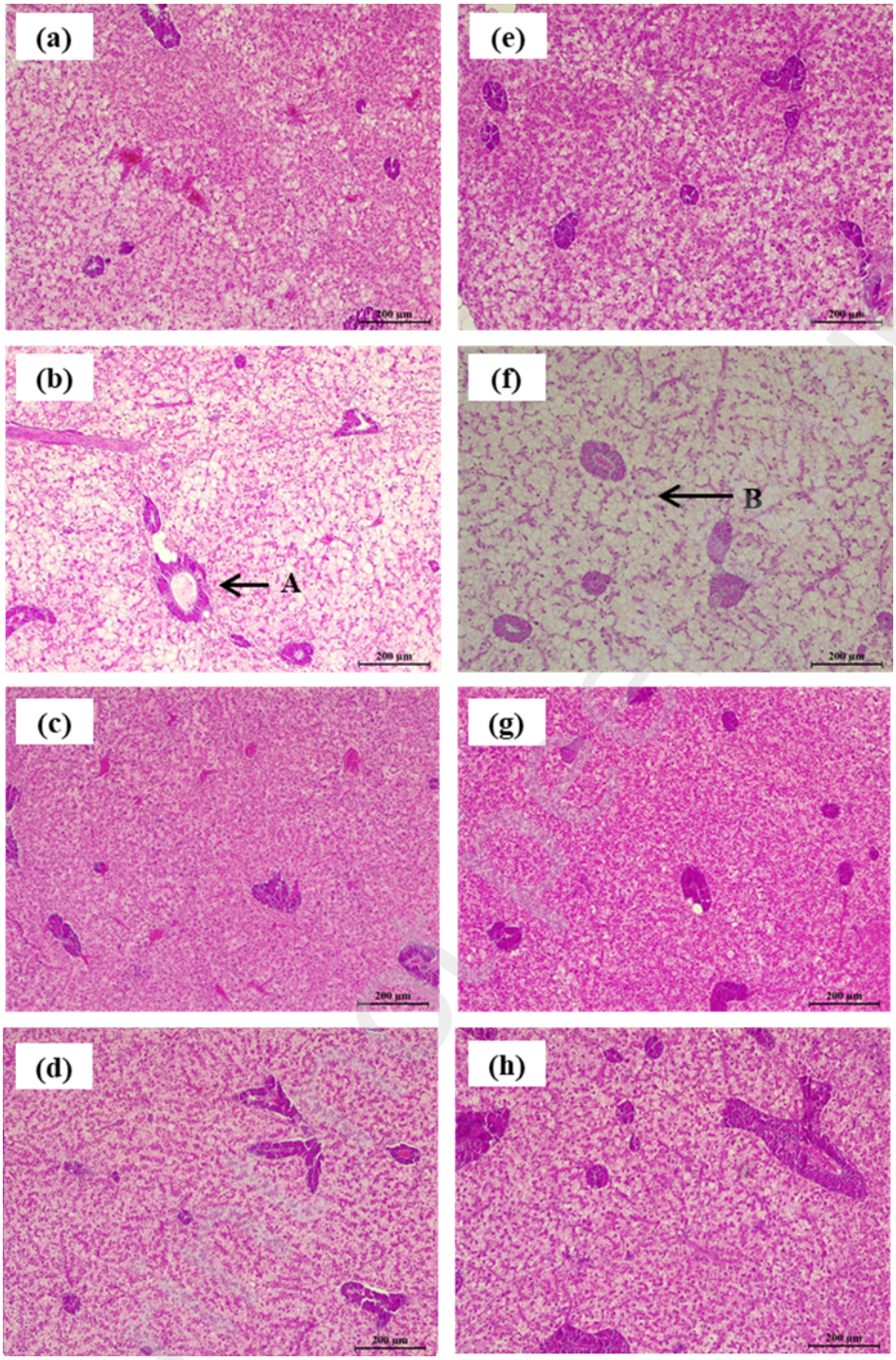
Table S4. Apparent digestibility coefficients (%, of ADC) for amino acid composition of the four experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) and submitted to control or periodical stress conditions (1-week daily net chasing every 2 weeks, S) for red seabream.

	Experimental diets			
	HFM	LFM	HFM+PE	LFM+PE
Non-essential amino acids				
Aspartic acid	82.5±4.16^{a}	72.1±3.01^{b}	86.3±2.21^{a}	82.1±1.97^{a}
Alanine	77.4±2.88^{b}	66.4±0.52^{c}	85.6±2.20^{a}	78.9±2.77^{b}
Serine	78.3±3.11^{b}	70.4±0.70^{c}	86.0±3.34^{a}	81.5±2.82^{ab}
Glutamic acid	83.7±4.28^{a}	74.2±1.72^{b}	88.8±2.68^{a}	84.8±2.27^{a}
Proline	91.6±6.4	80.0±6.42	91.3±1.75	89.1±6.53
Glycine	80.8±3.12^{a}	68.8±1.43^{b}	86.7±2.43^{a}	82.6±2.35^{a}
Tyrosine	77.4±3.04^{b}	68.2±0.95^{c}	87.1±2.82^{a}	81.6±2.88^{ab}
Essential amino acids				
Arginine	81.0±3.28^{b}	71.3±0.53^{c}	88.2±3.01^{a}	83.0±2.95^{ab}
Threonine	78.6±3.28^{b}	69.1±0.60^{c}	85.6±2.91^{a}	80.7±2.81^{ab}
Valine	80.5±3.99^{a}	70.2±1.07^{b}	86.8±2.03^{a}	81.2±2.50^{a}
Phenylalanine	78.7±3.44^{b}	70.3±1.19^{c}	86.2±2.36^{a}	81.0±2.67^{ab}
Isoleucine	80.0±4.13^{a}	70.0±1.30^{b}	86.5±2.12^{a}	81.0±2.64^{a}
Leucine	77.2±3.51^{b}	70.6±0.93^{c}	86.0±2.22^{a}	80.7±2.67^{ab}
Histidine	83.0±3.36^{a}	73.5±0.51^{b}	88.5±2.30^{a}	83.2±2.70^{a}
Lysine	86.1±4.11^{a}	76.5±2.44^{b}	88.9±1.73^{a}	84.9±0.72^{a}
Methionine	79.0±3.60^{b}	71.4±0.24^{c}	87.2±2.58^{a}	82.3±2.58^{ab}

Data are presented as mean of triplicate tanks ± S.D. with different superscripts in the same row are significantly different ($P < 0.05$).



Figures S1. Intestine morphology of the red seabream fed the four experimental diets (high or low - H and L- fish meal -FM- with or without palatability enhancer -PE-) and submitted to control or periodical stress conditions (1-week daily net chasing every 2 weeks, S) for 15 weeks (A. villus height; magnification, x4). (a) HFM, (b) LFM, (c) HFM+PE, (d) LFM+PE, (e) HFM-S, (f) LFM-S, (g) HFM+PE-S, (h) LFM+PE-S



Figures S2. Liver morphology of the red seabream fed the four experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) and submitted to control or periodical stress conditions (1-week daily net chasing every 2 weeks, S) for 15 weeks (A- blood vessels, B- vacuoles; magnification, x10). (a) HFM, (b) LFM, (c) HFM+PE, (d) LFM+PE, (e) HFM-S, (f) LFM-S, (g) HFM+PE-S, (h) LFM+PE-S

Table S5. Biochemical parameters of red seabream fed the four experimental diets for 15 weeks.

	Glucose[1]	Triglyceride[2]	T. Protein[3]	Cholesterol[4]	HDL[5]	LDL[6]
HFM	58.1±5.54^{ab}	136±22.8^{a}	3.70±0.48	179±12.8^{ab}	82.5±4.27	58.4±5.92^{ab}
LFM	50.5±12.3^{b}	107±11.2^{abc}	3.44±0.39	167±8.83^{abc}	83.1±2.28	51.8±10.9^{abc}
HFM+PE	58.6±3.68^{ab}	118±13.5^{ab}	3.36±0.46	179±12.6^{ab}	82.9±2.74	58.4±20.0^{ab}
LFM+PE	56.6±3.38^{ab}	119±16.6^{ab}	3.65±0.69	192±11.3^{a}	81.9±1.07	61.5±10.7^{a}
HFM-S	50.7±5.88^{b}	88.7±4.40^{bc}	3.50±0.23	161±7.41^{bc}	85.2±2.08	33.0±3.01^{bc}
LFM-S	68.3±3.30^{a}	84.9±5.87^{bc}	3.61±0.24	150±1.69^{c}	85.8±1.29	30.7±1.72^{bc}
HFM+PE-S	59.2±0.57^{ab}	88.7±13.2^{bc}	3.88±0.34	162±10.7^{bc}	87.2±1.13	25.7±8.25^{c}
LFM+PE-S	54.9±5.09^{ab}	71.6±3.91^{c}	3.43±0.56	151±5.47^{c}	84.1±2.29	27.4±4.28^{c}
Factorial ANOVA (P>F)						
Chasing stress	0.435	0.000	0.723	0.000	0.006	0.000
PE coating	0.889	0.424	0.938	0.143	0.870	0.950
FM reduction	0.751	0.047	0.704	0.197	0.450	0.799
Interactions SxPE	0.334	0.743	0.672	0.192	0.752	0.210
Interactions SxFM	0.064	0.752	0.629	0.164	0.591	0.859
Interactions PExFM	0.179	0.502	0.986	0.138	0.178	0.390

Data are presented as mean of triplicate tanks ± S.D. Values with different superscripts in the same column are significantly different ($P < 0.05$). The lack of superscript letter indicates no significant differences among treatments.; [1]Glucose (mg dL^{-1}); [2]Triglyceride (mg dL^{-1}); [3]Total protein (g dL^{-1}); [4]Total cholesterol (mg dL^{-1}); [5]High-density lipoprotein (mg dL^{-1}) and [6]Low-density lipoprotein (mg dL^{-1}).

Table S6. hematological Ht and Hb levels of red seabream fed the four experimental diets (high or low -H and L- fish meal -FM- with or without palatability enhancer -PE-) and submitted to control or periodical stress conditions (1-week daily net chasing every 2 weeks, S) for 15 weeks.

	Ht[2]	Hb[3]
HFM	39.1±0.69^{ab}	4.93±0.15
LFM	36.1±2.22^{ab}	4.39±0.53
HFM+PE	36.8±2.83^{ab}	4.54±0.24
LFM+PE	40.6±4.17^{a}	4.58±0.45
HFM-S	39.6±0.96^{ab}	4.54±0.81
LFM-S	33.6±1.71^{b}	4.02±0.16
HFM+PE-S	38.9±1.17^{ab}	4.64±0.26
LFM+PE-S	38.8±3.10^{ab}	4.56±0.42
Factorial ANOVA (P>F)		
Chasing stress	0.645	0.326
PE coating	0.097	0.548
FM reduction	0.178	0.129
Interactions SxPE	0.528	0.230

Interactions SxFM	0.087	0.871
Interactions PExFM	0.004	0.153

Data are presented as mean of triplicate tanks ± S.D. Values with different superscripts in the same column are significantly different ($P < 0.05$) – cortisol values were distinguished for the 1-way ANOVA between stressed and non-stressed dietary groups due to very different values between the 2 stress conditions groups. [1]Cortisol (ng mL^{-1}); [2]Hematocrit (%) and [3]Hemoglobin (g dL^{-1}).

Table S7. Spearman correlation coefficients between fish gut morphometrics and FCR (n=24 individuals, p-values are mentioned below the coefficients).

	FCR[1]	EH[2]	GC[3]	ID[4]	VL[5]
FCR		-0.3514	-0.6540	-0,4161	-0.4158
		0.092	**0.002**	**0.046**	**0.046**
EH	-0.3514		0.4933	0.7790	0.8049
	0.092		**0.018**	**0.000**	**0.000**
GC	-0.6540	0.4933		0.5026	0.6797
	0.002	**0.018**		**0.016**	**0.001**
ID	-0,4161	0.7790	0.5026		0.8216
	0.046	**0.000**	**0.016**		**0.000**
VL	-0.4158	0.8049	0.6797	0.8216	
	0.046	**0.000**	**0.001**	**0.000**	

[1]Feed Conversion Ratio; [2]Enterocyte height (µm); [3]Goblet cell count.; [4]Intestinal diameter (µm) and [5]Villi length (µm).