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Aquatic product hydrolysates increase rearing performance in red seabream (*Pagrus major*), fed a low fish meal diet, in both controlled and stressed conditions: From growth to stress responses

Herault Mikael [1, *], Gunathilaka Buddhi E. [2], Fournier Vincent [1], Le Bris Hervé [3], Lee Kyeong-Jun [2], Sadoul Bastien [3]

[1] Symrise Aqua Feed, ZA du Gohelis, Elven 56250, France
[2] Department of Marine Life Sciences, Jeju National University, Jeju 63243, South Korea
[3] DECOD, Institut Agro, INRAE, Ifremer, Rennes 35042, France

* Corresponding author : Mikael Herault, email address : mikael.herault@symrise.com

Abstract :

Although aquaculture industry has made great efforts to reduce the levels of fish meal (FM) in aquaculture feeds, further efforts and investigations are still needed to increase aquaculture production, which has more than tripled in the last two decades. This study was implemented to Six replicate groups of fish (24.6 ± 0.1 g) were fed one of the experimental diets to apparent satiety twice daily for 15 weeks; half of them were subjected to a daily net chasing stress for 1 min in the morning, 1 h before feeding. At the end of the feeding trial, fish were challenged with Edwarselia tarda injection. LFM diet resulted in comparable growth and feed efficiency performance to HFM group. However, significant impairments were observed in apparent digestibility coefficients, condition factor, hematocrit and serum super oxide dismutase activity. Fish fed LFM showed much lower resistance to E. tarda challenge. Both TH and SH resulted in enhanced fish nutritional and health performance with significant improvements in growth performance, feed efficiencies, digestibility, serum non-specific immunity and gut morphometric parameters. Fish fed TH and SH also exhibited the highest survival after E. tarda challenge. Principal component analysis (PCA) showed significant differences between stress and dietary groups for dimension 1 (33.3% of the variability), which was mostly characterized by growth and health variables. SH and TH coordinates were significantly higher on dimension 1 compared to HFM and LFM ones. Daily repeated stress affected most of measured performance parameters of all dietary groups with very few interactions. The stressed fish groups fed TH or SH showed lower cortisol levels compared to those fed HFM and LFM ($p < 0.01$). Strong correlations were observed between feed efficiency and fish gut morphometrics as quantified by villi length, enterocyte height and goblet cell density. Dietary supplementation of aquatic protein hydrolysates such as TH and SH appeared to be essential for maintaining good fish performance when they were subjected to a daily husbandry stressor or to an infectious challenge.

Highlights

► APH mitigate stress adverse effects ► APH help to reduce FM and antibiotic uses

Keywords : Shrimp hydrolysate, Tilapia hydrolysate, FM replacement, Chronic stress, Growth performance, Digestibility, Disease challenge, Red seabream.

1. Introduction

Aquaculture production of major fed species has increased by an average of 10.5% per year, between 2000 and 2020 while FM usage for aquafeeds increased by only 2.46% per year for the same period (FAO, 2022). Facing scarcity and sustainability issues, the aquaculture feed industry has tested, and used, alternative protein sources to make significant FM substitution a reality. Thus, average FM use in marine fish feeds has been reduced from 50 % down to 14 % between 1997 and 2017 (Naylor et al., 2021). At the same time, aquaculture has become the first and main user of FM between 2000 (42% of the total volumes) and 2020 (83%) (FAO, 2022).

Soybean protein has been used as the main protein substitute to FM due to its nutritional characteristics, important production, global availability and reasonable cost (Gatlin et al., 2007; Boyd and McNevin, 2022). Soy protein concentrate (SPC) is one of its derivate commonly used in marine fish feeds because of its low content of antinutritional factors (Refstie et al., 2001; Gyan et al. 2019). FM replacement with SPC provided acceptable zootechnical performance in many fish species when dietary essential nutrients were kept above their nutritional requirements and palatability maintained to a certain level by limiting FM replacement or using animal-based proteins (Bureau et al., 1998; Gunathilaka et al., 2021).

Limitations in fishes, when higher FM contents in diets are replaced with plant proteins, are palatability resulting loss of zootechnical performance (Gatlin et al., 2007; Tola et al., 2019) along with compromised gut health (Green et al., 2013; Wang et al., 2020), possible adverse effects on immune status (Francis et al. 2001) and susceptibility to diseases (Khosravi et al., 2015b). There are few studies about impact of dietary FM substitutions on fish resilience to a stress challenge (Gause and Trushenski, 2011; Trushenski et al., 2013) and none of them

related to red seabream. Yet, capacities to correctly react to a stress challenge is of primary importance in a context of climate change in aquatic species facing rising temperature and increased frequencies of extreme events (Alfonso et al., 2020; Maulu et al., 2021), and in a societal context advocating for increased welfare in aquaculture conditions (Ashley, 2007). Also, it is generally accepted that correct responses to stress challenge allows quick recovery of homeostasis and reduces sensitivity to pathogens and promotes survival (Schreck and Tort, 2016).

Animal by-products are now considered as a good source of protein, especially if issued from seafood processing industries due to more appropriate amino acid profiles and flavors. These raw materials are still mainly converted in low-value products such as FM, fish oil and soluble, and sometimes used as fertilizers. Yet, protein hydrolysis process is a good way to add significant value to these coproducts by releasing protein-encrypted free amino acids, di/tri peptides, and short/medium sized peptides, adding functionalities to the final product - aquatic protein hydrolysates (APH) - (Kang et al., 2019) and the diets that are supplemented with. Recent studies confirmed the possibility of improving dietary palatability using APH at reasonable dosages i.e. between 2 to 5%, while enhancing most of zootechnical and health performance in several marine fish species (Khosravi et al., 2015b; Khosravi et al. 2018; Siddik et al., 2018).

Red seabream (*Pagrus major*) is cultured within East Asian region. It was the third largest cultured fish in South Korea in 2000s and remains the second one in Japan (Tabata and Taniguchi, 2000; FAO, 2022). Red seabream is a carnivorous species with high requirements in FM products, allowing for improvement through research and innovation. Red seabream is also part of the Sparidae family, which includes the Gilthead seabream (*Sparus aurata*), the

most cultured species in the Mediterranean (FAO 2022) and can therefore be used as a model species for the entire family.

In previous studies implemented on red seabream, significant impairments of zootechnical, digestive and health performance were reported when dietary FM contents was reduced to 25% with SPC (Khosravi et al., 2015b; Gunathilaka et al., 2021). In these studies, the dietary supplementation of low FM diets with 5% APH allowed to restore fish performance to higher levels than those observed for non-substituted dietary groups.

In this study, we investigated further whether the dietary FM reduction with SPC is possible, from 30 to 15%, and assessed the benefits of two APH origins when supplemented at 5% in red seabream juveniles by means of zootechnical, physiological and health parameters. These dietary changes were studied simultaneously to the impact of a daily repeated stress by a net chasing before feeding. The purpose of this repeated stress was to assess the resilience of fish when facing concomitant challenging dietary changes and husbandry stress.

2. Materials and Methods

All the experimental protocols for the feeding trial followed the guidelines of the Institutional Animal Care and Use Committee of Jeju National University (approval number: 2019-0042).

2.1. Experimental diets

Four experimental diets were formulated to be isonitrogenous (45.4% crude protein) and isocaloric (21.7kJ.g^{-1}), on dry matter basis. A control diet was formulated to contain 30% FM (Fair Average Quality brown FM, with 67.7% crude protein) and considered as a high FM (HFM) diet (Table 1). A low FM (LFM) diet was formulated by substituting half of the FM

content from HFM by SPC and balancing essential nutrients accordingly (lipids and total omega 3 fatty acids, amino acids, calcium and phosphorus). Two other experimental diets were prepared by including two protein hydrolysates, provided by Symrise Aqua Feed (part of Taste, Nutrition & Health segment of Symrise group, Elven, France), in LFM basal diet (Table 1).

Farmed Pacific white shrimp (*Litopenaeus vannamei*) cephalothorax coproducts were enzymatically hydrolyzed, spray dried (SHyd) and incorporated in the LFM diet (as SH). Farmed tilapia (*Oreochromis sp*.) co-products containing heads, bones, trims and viscera were enzymatically hydrolyzed, defatted, spray-dried (THyd) and included in LFM diet (as TH).

All dry ingredients were thoroughly mixed before addition of fish oil, soybean oil and 15-20% double-distilled water. The mixed dough was extruded through a pelletizer machine (SP-50, Gum Gang Engineering, Daegu, Korea) with diameters and lengths ranging from 2-3mm and 3-4mm respectively, before being freeze-dried at −40 °C for 24 h and coated with 50% of dietary oil mix (fish and soybean oil, Table 1). Diets were stored at −20 °C until use.

Proximate compositions and amino acid profiles of the experimental diets are presented in Table 1 and Table 2, respectively. Peptide profiles of THyd and SHyd were quantified by size exclusion chromatography method as issued by Guérard et al. in 2001 (Table 3).

2.2. Fish and feeding trial

Red seabream juveniles were purchased and reared in the Institute of Marine Sciences of Jeju National University (Jeju, South Korea). The transported fish were immediately treated with 100ppm oxytetracycline solution for 30 min. We fed the juvenile red seabream with a commercial diet (VOGUELUCK, Jeju, South Korea, 52% crude protein, 8% crude lipid) to acclimate them to the experimental conditions.

960 fish (initial mean BW, 24.6 ± 0.04g) were randomly stocked into 24 circular tanks (40 fish/ tank) of 215 L capacity supplied with filtered flow-through sea water at a flow rate of 3 L/min. All the tanks were aerated to maintain sufficient dissolved oxygen (7.5 ± 0.5 ppm). Water temperatures followed local seawater temperatures during summer season with observed minimum at 19.3°C and maximum at 27.8°C, and gradual changes due to seawater temperature inertia. Six tanks were randomly assigned to one of the dietary treatments. We fed the fish with the experimental diets twice daily to apparent satiation (09:30h and 18:30h) for 15 weeks. Uneaten feeds were carefully collected 20-30 min after every feeding, oven-dried for 4h at 125°C and reweighed for the exact calculation of the feed intake. For each dietary group, the fish in three tanks (n=3 per diet) were subjected to daily stress by chasing them with a net for one minute (at 08:30h). Measurement of fish bulk weight was conducted every 3 weeks after a 24h fasting period.

2.3. Sample collection

The fish were fasted for 24 h before sampling. All the fish in each tank were individually weighted and 8 fish were randomly selected from each tank. The selected fish were immediately euthanized with 500ppm 2-phenoxy ethanol solution immediately for tissue sampling.

Within 2 minutes following euthanasia, blood was collected from three fish per tank with heparinized syringes. After hematocrit (Ht) determination, whole blood was centrifuged at 5000×g for 10 min to separate plasma and then, stored at -70°C until analysis of hemoglobin (Hg), immunoglobulin (Ig), antiprotease activity (Ap), cortisol levels and other biochemical parameters. The fish were also sampled for viscera and muscle proximate compositions, muscle total antioxidant capacity (TAC) and malondialdehyde (MDA) level.

Three other whole blood samples were used to separate serum with non-heparinized syringes. The serum samples were stored at -70°C until the analyses of non-specific immune responses, such as lysozyme (LYS), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), myeloperoxidase (MPO) and phagocytic (NBT) activities. These fish were also sampled for mRNA expression of liver insulin-like growth factors (IGF-I) and their whole-body proximate compositions after storage at -20°C.

The last two fish were euthanized, sampled for the whole intestine and fixed in Bouin's solution, and their liver and carcass were stored at -20°C until proximate composition analysis. Condition factor (CF), hepatosomatic index (HSI) and viscerosomatic index (VSI) were calculated with the fish body index.

The list of performance indicators and physiological biomarkers used in this study is summarized in table 4.

2.4. Sample analyses

The proximate compositions of fish tissues were analyzed by the standard procedures (AOAC, 2005). Ht was determined by Brown (1980). Plasma Hb, glucose, triglyceride, cholesterol and total protein were analyzed with the same method described in Gunathilaka et al. (2021).

Phagocytosis estimated by an oxidative radical production during respiratory burst was determined through NBT (nitro-blue-tetrazolium) assay (Anderson and Siwicki, 1995). Serum lysozyme activity in serum was accessed by Khosravi et al. (2015a) and anti-protease activity was measured by the method (Ellis, 1990) with a slight modification (Magnadóttir et al., 1999). Serum MPO activity was measured by Quade and Roth (1997). With plasma samples, immunoglobulin (Ig) levels were determined by Siwicki and Anderson (1993).

Commercial kits (Biovision Inc., Moutainview, CA, USA) were used for catalase (K773-100), GPx (K762-100), total cholesterol (1010-225), low-density lipoprotein (LDL, K613-100) and high-density lipoprotein (HDL, K613-100) cholesterol and triglyceride levels (2200-225) in plasma and malondialdehyde (MDA, K739-100) in muscle. Total antioxidant capacity (TAC) in muscle and plasma serum superoxide dismutase (SOD) were also determined using commercial kits (Sigma; CS0790 and 19160 respectively).

Plasma and muscle cortisol levels were determined with a commercial kit (CUSABIO Fish cortisol ELISA Kit Cat; CSB-E08487f) using competitive inhibition enzyme immunoassay technique. Plasma samples were used for the assay after dilution (x100). Muscle samples (100mg) were homogenized in 1 ml of 1 x PBS and centrifuged at 5000g for 5min after two freeze-thaw cycles to prepare muscle homogenates. A dilution series of cortisol (0-10 ng/ml) was prepared for the standard curve according to manufacturer's instructions.

For the histological observation, the intestine samples were fixed in Bouin's solution, dehydrated, embedded, sectioned in the foregut and stained for the observation of the mucus secreting goblet cells (GC) (Gunathilaka et al., 2020). The ImageJ 1.44 analysis software was used to measure villus length (VL), intestine diameter (ID) and enterocyte height (EH) of foregut.

Total RNA of liver was isolated using TRIzol Reagent (Sigma-Aldrich) and isolated RNA was quantified at 260 nm before synthesis of cDNA using PrimeScript first-strand cDNA synthesis kit (TaKaRa Code. DRR047). The Expression levels of IGF-I were measured by real-time PCR using 18S rRNA as a housekeeping gene. Primers were designed based on cloned sequence of IGF-I (NCBI Genbank accession no: AF061278) and 18S rRNA (NCBI Genbank accession no: EF126037). Relative expression ratio of IGF-I was calculated according to Pfaffl's mathematical model (Pfaffl, 2001).

2.5. Estimation of apparent digestibility coefficients (ADC)

The experimental diets were added with 1% chromic oxide (Cr_2O_3) (Sigma-Aldrich, St. Louis, USA) that is an inert indicator for digestibility test. The digestibility diets were made following the same procedure explained above.

A modified Guelph system (Cho et al., 1982) consisted of four 300 L capacity fiberglass tanks to which each faecal collection column was attached. A red seabream stock (mean BW of 100 g) was distributed into each faecal collection tank (30 fish per tank). Then, the fish were fed one of the digestibility diets. The faecal collection tanks were supplied with cartridge-filtered seawater at a flow rate of 1 L min^{-1} and aerate. Fish were fed once daily at 18:00h. One hour later, bottom of each tank was brushed out to remove uneaten feeds and faecal residues. On the next day, faeces were collected from the faecal collection columns at 9:00h. Each digestibility trial lasted 10 days and repeated 2 more terms for three replicate sample collections for each diet. All faeces collected from each faecal tank (diet) in each period were pooled and frozen at −20 °C for chromic oxide analysis. Chromium oxide level was analysed by Divakaran et al. (2002). The ADCs of the diets were calculated through the following formula:

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

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

2.6. Bacterial challenge

After the feeding trial, 18 fish from each tank (54 fish per treatment) were randomly captured and intraperitoneally injected with 0.1mL of *Edwardsiella tarda* (ATCC 15947, American type culture collection) suspension (1×10^6 CFU mL^{-1}). *E. tarda* broth was prepared at desired concentration following the procedure described in Bui et al. (2014). The bacteria injected fish were then distributed into twenty-four 120L fiberglass tanks and their behaviour and mortality were carefully monitored and recorded for 17 days. The test was terminated when the survival rates became constant. No feeds were given to injected fish and water temperature was maintained at 25°C during the challenge trial. Tanks were siphoned and 75% of water was changed daily. Two additional 120L fiberglass tanks were prepared with 18 fish randomly taken from remaining fish of the stress and non-stressed experimental population to serve as non-infected PBS controls over the disease challenge trial. We used a 1× PBS solution diluting 10 × PBS solution (pH 7, T & I Co., Gangwon, South Korea) with distilled water.

2.7. Statistical analysis

All measures were analyzed at the tank level, using the mean value of all individuals in the tank. First, a PCA was performed on all variables in order to test whether diet and stress explain total variability between tanks. This also allowed to identify the variables that contributed the most to the variability between tanks. This was performed using R cran 4.1.1. Two-ways analysis of variance (ANOVA) was implemented using STATGRAPHICS Centurion software with diet, stress and associated interaction as main effects. An one-way ANOVA was also applied to diet x stress groups and Tukey's Honestly Significant Differences (HSD) multiple test was applied ($P < 0.05$) when differences were detected between groups.

Spearman correlation coefficients were also calculated between fish gut morphometrics and feed conversion ratio (FCR) using STATGRAPHICS Centurion software.

The disease challenge trial data were specifically analyzed using a Log-rank test followed by post-hoc Bonferoni multi-par analysis.

3. Results

All the four experimental diets were readily accepted by the red seabream with significant growth, feed assimilation and survival (Table 5). Stressed fish groups fed either HFM or LFM diet showed significantly lower growth performance than all other groups ($p < 0.001$). While FM replacement did not lead to significant changes in growth and feed assimilation performance, even under chronic stress condition ($p > 0.05$), addition of the hydrolysates (SH or TH) to LFM diet resulted in significantly improved growth and feed performance in both normal and stressed conditions ($p < 0.001$, Table 5). The IGF-I mRNA expression of fish fed SH or TH diets were significantly ($p<0.001$) higher than fish fed the LFM diet, even in the stress conditions. Chasing stress impacted IGF-I expression independently in the dietary groups ($p < 0.001$ and non-significant interaction, Table 5). Fish CF was negatively impacted by the dietary FM replacement while the hydrolysate supplementations partly restored CF (Table 5).

Dietary FM replacement with plant proteins significantly impacted ADC, especially to protein (Table 6). Dietary hydrolysate supplementations resulted in enhanced ADC for both dry matter and protein reaching similar or higher levels than those observed for HFM (Table 6). Amino acid availabilities were also significantly increased by the dietary APH supplementations compared to those observed for the basal LFM diet (Table 7). Lysine digestibility of SH group only was significantly lower than that of the LFM group ($p < 0.05$).

The multivariate analysis was performed using all physiological measures (40 variables) and it significantly separated tanks on the first axis based on the stress effect (Figure 1A). The first axis captured 33.3% of the total variability, while the second axis captured only 10.1%. The 7 variables contributing the most to the first axis were: two non-specific immune responses (the lysozyme and antiprotease activities), two antioxidant enzyme activities (glutathione peroxidase and superoxide dismutase), two markers of endocrine responses associated to growth and stress (IGF-I relative expression and plasma cortisol) and one indicator of oxidation in the muscle (malondialdehyde) (Figure 1B). The 4 diets were distributed on the first axis (Figure 1C), showing their coordinates significantly different (Figures 1D). The group LFM had the lowest coordinates on the first axis, while both hydrolysate diets (SH and TH) had the highest coordinates (Figure 1D).

All parameters of non-specific immune response confirmed the negative effect of stress (Table 8). The use of hydrolysates led to a significant increase of all non-specific immune responses in both controlled and stressed conditions (Table 8), except for myeloperoxidase activity comparable between diets. LFM diet had no consequence on non-specific immune response compared to HFM diets. A significant interaction between stress and diet was found for the antiprotease activity with a reduced difference between diets in stressed conditions (Table 8).

All markers of antioxidant capacities were significantly affected by stress, leading to a concomitant increase in lipid peroxidation as assessed by malondialdehyde levels (Table 9). The diets affected all antioxidant capacities, with increased capacities assessed for APH diets, except for catalase activity ($p=0.18$) and total antioxidant capacities ($p=0.06$) (Table 9).

Muscle and plasma cortisol levels were 8 to 18 times higher in stressed fish compared to the controls ($p < 0.001$, Table 10). A strong interaction between diet and stress condition was

observed for both measures of cortisol (Table 10), and this was the consequence of differences between diets in stressed fish only. Indeed, under stress condition, LFM group showed the highest cortisol values for both muscle and plasma tissues ($p < 0.01$), while dietary TH and SH supplementations resulted in the lowest values, significantly lower than LFM ($p < 0.001$) and HFM, but only for muscle cortisol levels ($p < 0.001$). Ht and Hb levels were negatively affected by the stress ($p < 0.01$) and dietary FM replacement ($p < 0.05$). However, the dietary APH supplementations positively affected both Ht ($p < 0.01$) and Hb levels ($p < 0.05$).

Significantly longer villi were observed in fish fed SH and TH diets compared to fish fed HFM and LFM diets ($p < 0.001$, Table 11), while intestinal diameter was constant. Similarly, fish fed APH supplemented diets showed significantly higher enterocytes ($p < 0.001$) than those fed LFM, or even HFM diet, regardless of the stress conditions ($p > 0.05$). Diets, but not stress, had a significant impact on fish gut goblet cell density ($p < 0.05$, Table 11) with the highest observed values for hydrolysate diets and the lowest for LFM. Analysis of Spearman correlation coefficients indicated a strong negative correlation between FCR and gut morphometrics, excluding fish gut ID (Table 12).

Higher liver vacuolation was observed in LFM group while the lowest one was shown in the hydrolysate groups, regardless of stress conditions (Figure S2).

Glucose, triglyceride, cholesterol, LDL, HDL and total protein levels of plasma were not significantly affected by the dietary and stress treatments (Table S1, $p > 0.05$). Yet, the two-way ANOVA revealed higher levels of circulating levels of TG and LDL in fish plasma for the groups with the stress ($p < 0.05$ and 0.001 respectively).

Proximate compositions of whole-body or carcass of fish were not significantly different after the feeding trial (Table S2 and S3). Also, viscera proximate composition, liver lipid content and muscle water retention rate were not significantly affected by the diets or chronic stress (Table S4). The two-way ANOVA still revealed that fish body lipid content was significantly lower in fish in the stress conditions ($p < 0.01$ and 0.05, respectively). Fish HSI and VSI were not significantly affected by the stress conditions and dietary changes ($p > 0.05$, Table S4).

Stressed and controlled fish injected with PBS did not show any mortality over the 17 days of *E. tarda* challenge (Figure 2). Kinetics and log-rank analysis clearly showed a negative effect of the chronic stress on fish survival over the challenge, but the dietary effect appeared to be much stronger; especially in the two LFM dietary groups showing the highest mortality rates and lower survival kinetics ($p < 0.001$). Compared to HFM group, the hydrolysate supplemented groups tended to increase disease resistance of fish to *E. tarda* challenge, but differences remained too low to be significant.

4. Discussion

4.1. Effects of dietary FM reduction on fish growth, health status and disease resistance.

Our results demonstrated that the use of a LFM diet in red seabream renders good growth performance even in stressful conditions. Indeed, reducing FM, from 30 down to 15%, in red seabream diet formulation allowed fish to perfectly survive, eat and grow with comparable performance to fish fed HFM diet. Negative impacts on red seabream growth were previously nevertheless observed when replacing FM with soy proteins concentrates (Khosravi et al., 2015b, Gunathilaka et al., 2021) in the earlier juvenile stage of the fish (IMW: 4.9g and 8.5g) indicating that LFM diet is better accepted in bigger red seabream juveniles.

Liver vacuolation, circulating HDL and TG are diagnosis parameters for fish liver steatosis, especially when carnivorous fish species are fed with plant proteins (Zhang et al., 2019; Siddik et al., 2021b). In this study, we may suspect a light liver steatosis in fish fed LFM without APH as their liver showed higher vacuolation compared to groups with APH, while differences observed for circulating HDL, LDL and TG remained non-significant. It is well documented that fish species store excess dietary fatty acids in their liver (Medagoda et al., 2022). In the present study, LFM diet contained comparatively higher SPC level than the APH diet (Table 1). Therefore, dietary fatty acids might be stored in liver of fish fed LFM diet because of a low feed utilization and muscle growth, as indicated by significantly lower CF and IGF-I (Table 2), in the group during the feeding trial. Further studies should be conducted to elucidate this phenomenon.

Despite good zootechnical performance, we may consider that fish fed LFM diet without APH can show nutritional and physiological stress signs in gut morphometrics, Ht level, non-specific immune responses, and serum antioxidant capacities. In healthy conditions, these adverse effects may remain "silent" without consequence to zootechnical performance of the fish. However, in less optimal conditions, as indicated by the induced bacterial challenge, consequences could be more critical for the survival rates. These results are in accordance with previous studies related to FM replacement in carnivorous marine fish species (Khosravi et al., 2015b; Gunathilaka et al., 2020; Siddik et al., 2021b).

Dietary FM reduction to 15% resulted in acceptable zootechnical performance but compromised resilience to disease challenge.

4.2. Effects of dietary APH supplementations on fish growth, health status and disease resistance.

APH supplementation resulted in significantly improved fish weight gain. This is in accordance with previous studies (Refstie et al., 2004, Bui et al., 2014, Khosravi et al., 2015b, Khosravi et al., 2015a), which explained higher growth performance as consequences of increased dietary palatability and protein digestibility resulting in a higher feed intake and thereby higher assimilation of the nutrients. The results in this study demonstrated that the two hydrolysates could perform better when the fish are in stressful conditions.

In the literature, feed utilization efficiency and digestibility are usually explained by differential gut morphometrics, digestive enzyme activities or, more recently, microbiota (Ganguly and Prasad, 2012; Egerton et al., 2018). EH and GC are generally considered as good indicators of fish gut health, especially because they are easily affected by higher plant protein inclusions (Khosravi et al., 2015b; Correa et al., 2019; Gupta et al., 2020; Gunathilaka et al., 2020). Consequently, in our study, the lower EH and densities of GC in fish fed the LFM diet indicate a worse gut health of the fish. GC is known to synthesize bioactive molecules secreted in the mucus, which are involved in immune function and digestive capacities (Shephard, 1994; Kim and Khan, 2013). Dietary APH supplementation resulted in much improved fish gut EH and GC, exceeding values observed for HFM control diet. The correlations between improved gut fish morphometrics and higher feed efficiencies was demonstrated in this study and such correlations were also found in our previous studies for red seabream (Khosravi et al., 2015b) and olive flounder, *Paralichthys olivaceus* (Gunathilaka et al., 2020). The improved gut health also resulted in increased ADCs of protein and dry matter. APH are pre-digested proteins rich in free amino acids, di/tri peptides, and functional medium sized peptides. These so-called bioactive peptides can have a direct nutritional effect on fish gut enterocytes or an indirect one by providing additional protection relative to a more appropriate microbiota due to antimicrobial peptides and antioxidative

functionalities (Robert et al., 2014; Robert et al., 2015; Siddik et al., 2021a; Aloo and Oh, 2022).

These functional peptides are also known to be responsible for improved immune functions (Siddik et al., 2021a) found in our study, but the mechanisms of actions related to immune modulations still remain unclear.

Both improved fish zootechnical condition, and enhanced non-specific immunity, most likely explain the higher resistance to disease infection observed for APH fed fish. Several studies have already demonstrated the increased disease resistance on APH fed fish, as reviewed by Siddik et al. (2021a) and more specifically reported in red seabream by our previous studies (Bui et al., 2014, Khosravi et al., 2015b, Gunathilaka et al., 2021).

APH supplementations restored performance observed in HFM groups, even exceeding it, whatever the stress conditions are.

4.3. Effects of APH raw material origin and process on their resulting benefits in fish.

SH was performing slightly better than TH. SH peptide profile shows a higher proportion of free AA, di/tri peptides (< 500da), which are known to drive palatability in marine carnivorous fish (Kasumyan and Døving, 2003). This could highlight the fact that dietary palatability could contribute to a large part of side benefits observed in APH groups. Interestingly, PCA analysis showed a lower fish response variability for SH group by reducing the observed differences between the stressed and control fish.

4.4. Effects of daily stress on fish zootechnical and health parameters.

Daily net chasing stress strongly affected fish growth rates, and to a lower extend, feed conversion ratio. It is widely described that husbandry stress, like net chasing, can drastically reduce fish feeding motivation leading to compromised zootechnical performance (Sadoul

and Vijayan, 2016). It is interesting to note that in our experimental conditions, daily net chasing did not really impact fish gut morphometrics. Also, it is worth noting that stressed fish showed higher circulating TG and LDL, while they showed lower body fat content. These changing physiological indicators have not been described in fish for acute handling stress (Young et al., 2019); they are likely the consequence of energetic mobilization for coping with stress.

Maybe something like:

While effects of hydrolysates on cortisol levels of carnivorous marine fish have previously been investigated (Mamauag et al. 2016; Resende et al. 2023), this is, to our knowledge, the first study investigating effects of on cortisol levels in red sea bream. Fish from stress groups showed muscle and plasma cortisol levels, respectively, 8 and 15 times higher than fish in non-stressed groups although the last netting stress took place 24 hours before the fish sampling for the cortisol analyses.

We assume that these results are the consequence of a conditional learning of the fish. Indeed, it is well known that fish are able to anticipate a specific event after having been conditionally trained using a neutral cue, often a change in the water flow or illumination of a light. The event can be positive, often a food reward, or aversive like a confinement stress (Moreira et al., 2004) or a net chase (Yue et al., 2008). While behavior is often used as the main indicator of an efficient learning process (Noakes and Jones, 2016), there is evidence that physiological markers can also be used such as heart rates (Brijs et al., 2018) or plasma cortisol (Moreira and Volpato, 2004; Moreira et al., 2004). The later studies respectively demonstrated that Nile tilapia, *Oreochromis niloticus*, and rainbow trout, *Oncorhynchus mykiss*, conditionally increase their plasmatic cortisol levels after 10 or 6 days of learning that they will face a confinement after a change in light or water flow, respectively. We suppose

that in our study, fish learned over the 15 weeks of the feeding trial that they would face a net chase 30 minutes after the morning light is turned on.

It is well documented that repeated stress, and cortisol secretion, can have long term deleterious impacts on fish growth, metabolism and immune functions (Yada and Tort, 2016). In this study, the impact of daily repeated stress was very severe for almost all tested non-specific immune parameters and antioxidative defenses.

Contrary to circulating cortisol, fish Ht may be considered as a more long-term indicator of fish welfare with a short-term increased level due to splenic contraction (Fazio et al., 2015) and long-term reduced levels following repeated handling stress as shown in jundia, *Rhamdia quelen*, (Barcellos et al. 2004) and in red seabream in the present study.

Considering the effects of repeated stress on zootechnical and health parameters in red seabream, such a low impact of chronic stress on the fish resistance to the bacterial challenge was not expected. The effect was visible for all dietary groups, and most of zootechnical & health related parameters, but not as critical as the effect of FM replacement.

4.5. Effects of diet x stress interactions.

Except for plasma cortisol level and antiprotease activity, no significant interactions between stress and dietary changes were seen in zootechnical performance and physiological parameters. It meant that daily net chasing stress had affected all dietary groups in the same way with the same amplitude. Even though APH fish groups were also affected by this daily stress, their final growth and feed conversion ratio came to an end at similar or better levels when compared to unstressed HFM fish group. We already explained this by improved nutrient absorption capacities (gut morphometrics) fully offsetting adverse effects of repeated stress resulting from a reduced feeding motivation.

As shown in turbot *Scophthalmus maximus* (Nagel et al., 2012) and in red seabream (Kader et al., 2012), we didn't note any change of basal cortisol levels (control stress condition) in fish fed LFM or APH diets compared to the ones fed HFM. In the stress condition, we found a negative impact of FM replacement with increased levels of muscle and plasma cortisol, while APH supplementations led to their reductions. These results are in accordance with reduced levels of serum cortisol as observed by Serradell et al. (2020) in European seabass, *Dicentrarchus labrax*, fed dietary prebiotics or phytogenics during a confinement stress and by Sadoul et al. (2016) in rainbow trout fed a plant-based diet 30 minutes after an isolation stress. This confirms that dietary changes could have beneficial or deleterious effects on the fish responses to daily stress that occurs frequently in farming conditions.

5. Conclusions and perspectives

This study confirms the possibility of 50% FM replacement with plant proteins in diets for red seabream grow-out without adverse effects when 5% APH are added as a feed palatability enhancer, growth promoter and a fish immunity enhancer.

The results from innate immunity and disease resistance indicated that the APH supplementation into low FM diets was necessary to maintain good fish welfare and health conditions, thus maximizing the zootechnical performance of red seabream. This is especially true in farming conditions where environmental and husbandry stressors can be faced on a daily basis. In this study, a daily repeated stress resulted in the loss of fish feeding motivation leading to compromised fish growth and health performance. Surprisingly, fish even anticipated this stress possibly amplifying resulting adverse effects. While both stress and fish meal reduction may impair fish feeding motivation, it therefore seems important to dietary supplement fish with APH to restore dietary palatability and / or increase fish feeding motivation thus preventing further negative consequences.

It is critical to secure optimized feed formulations with functional ingredients. Otherwise, this could lead to impaired immune functions with greater risks of bacterial diseases outbreaks, and subsequent antibiotic uses, while jeopardizing initiatives of going further in FM replacement. This study confirmed that the origin and process of APH could result in different performance, therefore highlighting the needs for increased process standardization and product characterization.

As APH supplementation provided performance exceeding the ones observed in HFM group, we could reasonably expect further FM reduction in the diets to be successful.

In our study, APH accounted for approximately 8% of the dietary crude protein content. Their positive effects on fish performance, therefore, could either be related to a better feed palatability and fish welfare, or health benefits associated with APH functional nutrients (stimulation of taste receptors and appetite metabolic pathways, modulation of antioxidative and nonspecific immunity, gut health and etc). Further investigations, by supplementing lower dietary doses of liquid APH, would allow to better quantify the contribution of the dietary palatability in the global carnivorous fish zootechnical and health performance, when FM levels were significantly replaced.

When writing this article, the average cost of SPC was 1,200 US$/mt, while fair average quality (FAQ) FM was approximately 1,800 US$/mt. Other plant or novel alternative proteins could also be considered to make LFM diet cost effective. APH could then be considered to secure marine fish zootechnical and health performance while bringing more flexibility to feed formulators. This can contribute to the efforts undertaken for last decades to limit the impact on marine FM.

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 co-participated in the design of the study, analyzed the results, and wrote the first draft of the article. Vincent Fournier co-participated in the design of the study. Bastien Sadoul analyzed the results and contributed to the first draft of the article. Hervé Le Bris contributed to the first draft of the article. Buddhi E. Gunathilaka mainly conducted the feeding trial and analyses, and Kyeong-Jun Lee designed the dietary formulation and consulted whole process of the study. All coauthors contributed to the writing of the manuscript.

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

Ingredients	Experimental diets			
	HFM	LFM	SH	TH
FM FAQ65[1]	30.00	15.00	15.00	15.00
Shrimp hydrolysate[2]			4.72	
Tilapia hydrolysate[3]				4.78
Soy protein concentrate[4]	15.00	26.40	22.20	21.60
Corn gluten meal[5]	12.00	12.00	12.00	12.00
Wheat flour[6]	30.00	30.00	30.00	30.00
Fish oil[7]	4.65	6.00	6.00	6.00
Soybean oil[8]	4.65	4.20	3.90	3.90
Mineral Mix[9]	1.00	1.00	1.00	1.00
Vitamin Mix[10]	1.00	1.00	1.00	1.00
Starch[11]	1.20	1.20	1.04	1.52
Choline chloride[12]	0.50	0.50	0.50	0.50
L-Lysine[13]	0.00	0.50	0.50	0.50
L-Methionine[14]	0.00	0.20	0.20	0.20
Taurine[15]	0.00	0.50	0.44	0.50
Di-calcium phosphate[16]	0.00	1.50	1.50	1.50
Proximate composition (%, dry matter)				
Crude protein	45.4	45.7	45.2	45.4
Crude lipid	16.8	17.1	17.3	16.8
Crude ash	5.76	5.00	5.39	5.26
Gross energy (MJ.kg^{-1})	21.78	21.59	21.84	21.65
Moisture	5.37	7.25	7.05	6.63

[1]Fish meal fair average quality (DM basis, 69.2% protein and 8.27% lipid), Orizon S,A., CO., Ltd., Chile.

[2]Shrimp hydrolysate (DM basis, 67.6% protein and 11.3% lipid), Symrise Aqua Feed, Ecuador.

[3]Tilapia hydrolysate (DM basis, 74.9% protein and 16.2% lipid), Symrise Aqua Feed, Costa-Rica.

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

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

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

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

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

[9] 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.

[10] 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.

[11]Starch, Samyang Co., Ltd., Seoul, South Korea.

[12]Choline chloride, Solton-Biochem Co., Ltd., Cheonan, South Korea.

[13]L-Lysine, Ajinomoto Amino Acid Co., Ltd., Shanghai, China.

[14]L-Methionine, Evonik Pharmaceutical Co., Ltd., China.

[15]Taurine, Qianjiang Yongan Pharmaceutical Co., Ltd., Qianjiang, China.

[16]Di-calcium phosphate, Sigma-Aldrich, Missouri, USA.

Table 2. Amino acid contents (% of the ingredient as dry matter basis) of the four experimental diets for red seabream.

	Experimental diets			
	HFM	LFM	SH	TH
Non-essential amino acids				
Aspartic acid	3.55	3.54	3.51	3.52
Alanine	2.32	2.11	2.23	2.26
Serine	2.10	1.92	1.90	1.92
Glutamic acid	8.77	8.24	8.14	8.23
Proline	1.67	1.95	1.88	1.93
Glycine	1.87	1.56	1.69	1.80
Tyrosine	1.20	1.07	1.15	1.13
Essential amino acids				
Arginine	2.52	2.30	2.30	2.34
Threonine	1.77	1.55	1.55	1.56
Valine	2.04	1.83	1.89	1.88
Phenylalanine	2.07	1.95	1.96	1.95
Isoleucine	1.82	1.67	1.69	1.69
Leucine	3.90	3.48	3.53	3.55
Histidine	1.01	0.96	0.96	0.96
Lysine	1.59	1.80	1.75	1.90
Methionine	0.98	0.90	0.94	0.95
Taurine	0.21	0.46	0.48	0.51

Table 3. Chemical compositions of shrimp hydrolysate (SHyd) and tilapia hydrolysate (THyd) (from product technical data sheets).

	SHyd	THyd
Dry matter, DM (%)	96.0	95.0
Protein (% DM)	67.6	74.9
Lipid (% DM)	11.3	16.2
Ash (% DM)	10.7	5.1
Soluble nitrogen (% N)	91.1	91.5
Essential amino acids (% products)		
Arginine	4.15	4.06
Histidine	1.55	1.36
Isoleucine	2.71	2.45
Leucine	4.28	4.38
Lysine	4.13	4.23
Methionine	1.26	1.58
Phenylalanine	2.95	2.42
Threonine	2.62	2.77
Valine	3.34	2.84
Molecular weight repartition (% of peptides)		
Peptides > 30,000 Da	<0.1	<0.1
Peptides 20,000 - 30,000 Da	<0.1	<0.1
Peptides 10,000 - 20,000 Da	0.1	3
Peptides 5,000 - 10,000 Da	<0.5	4
Peptides 1,000 - 5,000 Da	8	18
Peptides 500 - 1,000 Da	9	14
Peptides < 500 Da	83	61

Table 4. Performance indicators and physiological biomarkers used

Category	Performance indicator or physiological biomarker	Acronym	Interpretation	Indication	Number of samples per tank
Zootechnical	Insulin Growth Factor-I	IGF-1	Produced by liver upon Growth Hormone stimulation.	Growth potential	3
	Condition Factor	CF	Length-weight relationship	Fish feeding status and metabolic rate	2
Metabolic	Proximate composition	DM	Dry matter, crude protein and fat, ash of body compartments	Metabolic macro changes	3, pooled
	Liver lipid	LL	Liver fat content	Metabolic / nutritional disorders / changes	2, pooled
	Muscle water retention	MWR	Muscle water content	End user organoleptic quality parameter	2, pooled
	Hepato-somatic index	HSI	Liver weight over body weight	Fat metabolic disorders	2
	Viscera-somatic index	VSI	Viscera weight over body weight	Fat metabolic changes	2
Blood biochemistry	Glucose	-	Circulating glucose	Nutritional status and / or metabolic changes	3
	Triglyceride	TG	Energy sources and transporters of dietary fat		3
	Total cholesterol	-	Circulating cholesterol		3
	High density lipoprotein	HDL	Circulating lipoprotein	Nutritional and / or chronic stress	2
	Low density lipoprotein	LDL			2
	Total protein	-	Circulating protein consisting mainly in immunoglobulin & albumin	Protein metabolism disorders or health issue	3
Immune – non-specific	Antiprotease	Ap	Proteins inhibiting actions of virus and bacteria proteases	Dietary or environmental modulation on non-specific immunity	3
	Lysozyme	LYS	Enzyme produced by phagocytic cells in fish serum to lyse bacteria cells		3
	Myeloperoxidase	MPO	Enzyme produced by leukocytes during respiratory burst process to lyse bacteria cells		3
	Respiratory burst activity (NBT)	NBT	NBT assay assesses the leukocyte respiratory burst activity when phagocyting pathogens		3

	Total immunoglobulin	Ig	Glycoproteins produced by lymphocytes	3

Table 4 (continue). Performance indicators and physiological biomarkers used

Category	Performance indicator or physiological biomarker	Acronym	Interpretation	Indication	Number of samples per tank
Health	Catalase	CAT	Enzymatic activity of serum catalase, which catalyzes the H_2O_2 degradation into O_2 and H_2O	Nutritional and health status	2
	Super oxide dismutase	SOD	Enzymatic activity of serum SOD, which catalyzes the O_2^- degradation into O_2 and H_2O_2		3
	Glutathione peroxidase	GPx	Enzymatic activity of serum GPx, which catalyzes the ROOH degradation into ROH and H_2O		3
	Total antioxidant capacity	TAC	Muscle TAC quantifies the total non-enzymatic TAC resulting from non-enzymatic antioxidant molecular compounds		2
	Malondialdehyde	MDA	MDA is one of the final products of PUFA peroxidation in cells – an increase of free radicals causes overproduction of MDA		2
	Cortisol		1st response stress hormone	Short term answer of acute / chronic stress when found in serum or muscle	3
	Hematocrit	Ht	Ht provides an overview of the red cells proportion observed in fish plasma	Health and chronic, or repeated stress	3
	Hemoglobin	Hb	Hb content is another indication of red cells proportion		3
Gut morphometrics	Villi length	VL	Villi are essential elaborations of the gut epithelium than increase surface area for nutrient absorption	Nutritional changes or stressor	3
	Enterocyte height	EH	Enterocytes are gut epithelium cells responsible for nutrient absorption	Fish feeding status and they can be dietary modulated	3
	Intestinal diameter	ID	Measured as the inner diameter	Dietary changes	3
	Goblet cell count	GC	Goblet cells secrete mucus for protection and absorption purposes	Fish nutritional and health status.	3

Table 5. Growth performance and feed utilization parameters of red seabream fed the four experimental diets for 15 weeks.

	FBW[1]	SGR[2]	FI[3]	FCR[4]	IGF-I[5]	CF[6]	Survival (%)
HFM	131±0.97^{bc}	1.59±0.01^{bc}	148±3.19^{bc}	1.39±0.02^{abc}	1.00±0.00^{ab}	1.82±0.09^{a}	99.2±1.44
LFM	126±0.91^{c}	1.56±0.01^{c}	142±2.78^{c}	1.39±0.03^{ab}	0.70±0.05^{cd}	1.59±0.06^{b}	98.3±1.44
SH	150±2.16^{a}	1.72±0.02^{a}	158±2.48^{a}	1.26±0.04^{d}	1.20±0.18^{a}	1.70±0.03^{ab}	98.3±1.44
TH	147±5.51^{a}	1.71±0.04^{a}	157±1.16^{a}	1.28±0.05^{d}	1.10±0.03^{ab}	1.70±0.10^{ab}	100±0.0
HFM-S	116±1.08^{d}	1.48±0.01^{d}	131±0.23^{d}	1.42±0.02^{a}	0.81±0.11^{bc}	1.70±0.05^{ab}	99.2±1.44
LFM-S	114±2.20^{d}	1.46±0.02^{d}	130±2.57^{d}	1.44±0.05^{a}	0.40±0.19^{d}	1.57±0.07^{b}	96.7±1.44
SH-S	135±1.63^{b}	1.62±0.01^{b}	146±3.39^{bc}	1.31±0.02^{bcd}	1.01±0.06^{ab}	1.63±0.11^{ab}	98.3±1.44
TH-S	132±0.20^{bc}	1.60±0.00^{bc}	140±1.32^{c}	1.30±0.01^{cd}	0.85±0.05^{bc}	1.66±0.02^{ab}	99.2±1.44
Factorial ANOVA (P>F)							
Chasing stress	0.000	0.000	0.000	0.006	0.000	0.053	0.332
Dietary changes	0.000	0.000	0.000	0.000	0.000	0.005	0.088
Interactions	0.682	0.647	0.238	0.876	0.774	0.630	0.783

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]Final body weight (g); [2]Specific growth rate (%/d) = [Ln (final body weight (g)) -Ln (initial body weight (g))]/ feeding trial duration (d)] x 100; [3]Feed Intake (g/fish) = dry feed consumed (g)/fish; [4]Feed conversion ratio = dry feed fed/wet weight gain; [5]Liver insulin-like growth factors I (relative expression of mRNA); [6]Condition factor = (Fish weight/ Fish length^3) x 100

Table 6. Apparent digestibility coefficients (%, of ADC) for protein and dry matter of the four experimental diets for red seabream.

	HFM	LFM	SH	TH
ADCp[1]	87.2±0.60^{b}	83.2±0.9^{c}	89.6±0.80^{a}	87.3±0.91^{b}
ADCd[2]	71.0±1.37^{ab}	66.5±2.01^{b}	72.7±2.10^{a}	70.5±2.10^{ab}

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 coefficient of protein (%); [2] Apparent digestibility coefficient of dry matter (%)

Table 7. Apparent digestibility coefficients (%, of ADC) for amino acid composition of the four experimental diets for red seabream.

	Experimental diets			
	HFM	LFM	SH	TH
Non-essential amino acids				

Aspartic acid	84.3±0.74[a]	75.8±1.45[b]	84.3±1.21[a]	81.7±1.30[a]
Alanine	83.6±0.77[a]	73.0±1.62[c]	83.0±1.31[ab]	79.9±1.43[c]
Serine	83.6±0.77[a]	74.6±1.52[b]	83.7±1.25[a]	80.6±1.38[a]
Glutamic acid	84.5±0.73[a]	75.3±1.48[b]	85.1±1.15[a]	82.6±1.24[a]
Proline	85.0±0.70[a]	79.4±1.23[c]	80.6±1.49[bc]	83.0±1.20[ab]
Glycine	85.6±0.68[a]	76.0±1.44[b]	84.8±1.17[a]	84.6±1.09[a]
Tyrosine	84.7±0.72[a]	74.1±1.55[c]	85.4±1.13[a]	81.4±1.32[b]
Essential amino acids				
Arginine	85.9±0.67[a]	76.7±1.40[b]	85.9±1.08[a]	83.6±1.17[a]
Threonine	83.7±0.77[a]	73.9±1.56[c]	83.1±1.30[ab]	80.2±1.40[b]
Valine	83.8±0.76[a]	74.3±1.54[c]	84.3±1.21[a]	80.5±1.39[b]
Phenylalanine	82.9±0.81[ab]	73.9±1.56[c]	83.7±1.26[a]	79.8±1.44[b]
Isoleucine	83.6±0.77[a]	74.4±1.53[c]	84.0±1.23[a]	80.3±1.40[b]
Leucine	83.0±0.80[a]	72.8±1.63[c]	82.7±1.33[a]	78.5±1.53[b]
Histidine	86.9±0.62[a]	79.0±1.26[b]	86.1±1.07[a]	84.3±1.12[a]
Lysine	92.8±0.30[a]	90.3±0.58[b]	88.1±0.92[c]	90.9±0.65[b]
Methionine	86.2±0.65[ab]	78.8±1.27[c]	87.4±0.97[a]	84.6±1.05[b]
Taurine	94.1±0.30[c]	96.7±0.20[b]	98.6±0.11[a]	98.8±0.05[a]

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

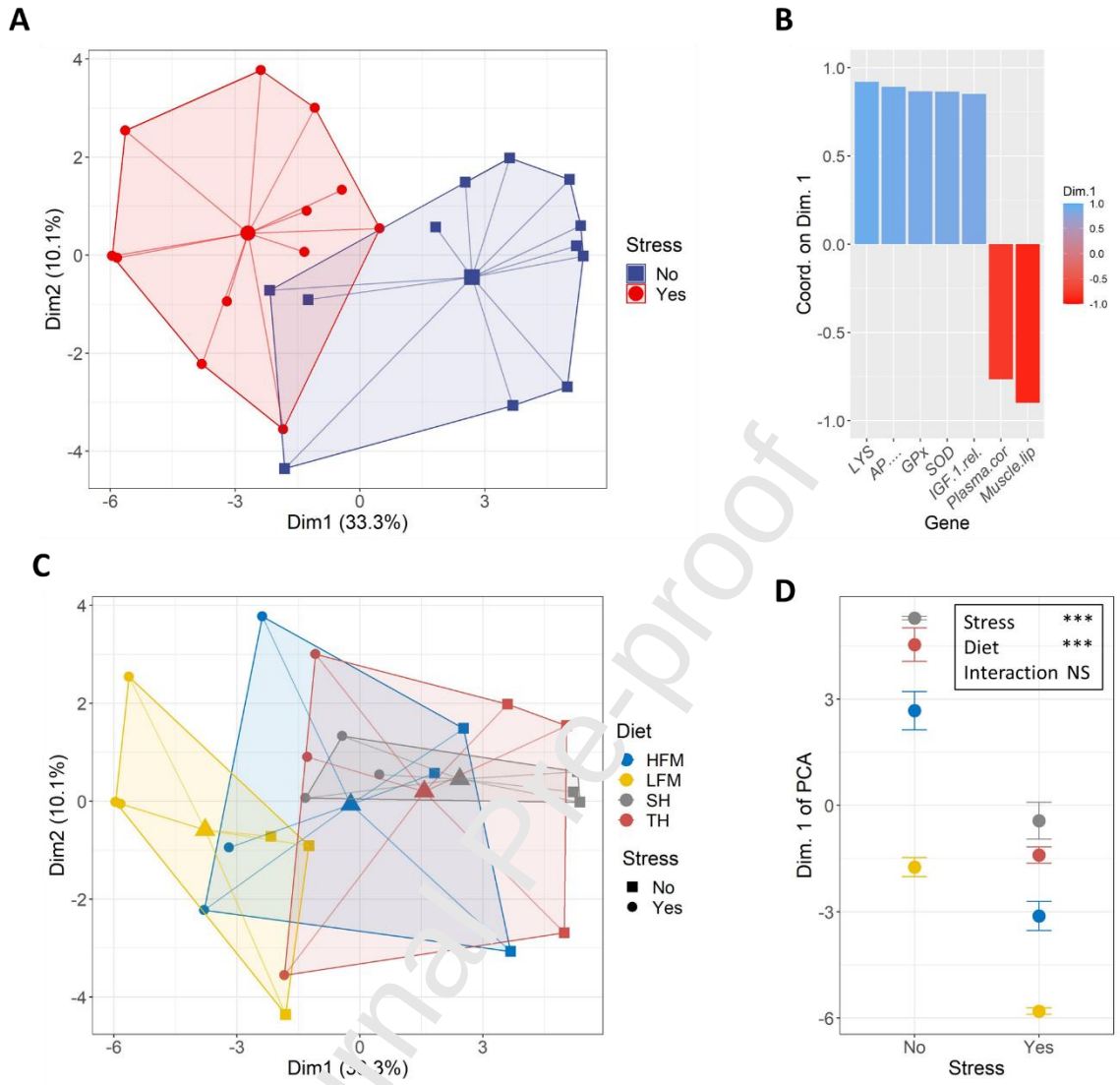

Figure 1. Principal component analyses (PCA) on physiological and health parameters performed on red seabream fed 4 different diets in a control or a stressful context. (A) Variability on the 2 first axes of the PCA between stressed vs. controlled individuals. (B) The coordinates on the first axis of the PCA for the 7 variables contributing the most to the first dimension (lysozyme, antiprotease, GPx, SOD, IGF.1, plasma cortisol, muscle lipid peroxidation). (C) Variability on the 2 first axes of the PCA between the 4 diets for stressed (points) or control conditions (squares). (D) Mean and standard error of the coordinates on the first axis (Dim. 1) of the PCA for each diet in controlled or stressed conditions. The asterisks in (D) illustrate that Stress and Diet have a significant effect ($p<0.001$) on the first-dimension coordinates.

Table 8. Non-specific immune response parameters of red seabream fed the four experimental diets for 15 weeks.

	Antiprotease[1]	Lysozyme[2]	MPO[3]	NBT[4]	Ig[5]
HFM	15.1±0.70^{b}	6.80±0.20^{bc}	1.42±0.19^{a}	1.44±0.06^{a}	31.2±0.50^{abc}
LFM	14.8±0.60^{bc}	6.53±0.31b^{cd}	1.22±0.04^{ab}	1.39±0.05^{ab}	29.1±1.69^{bc}
SH	17.6±0.50^{a}	7.64±0.32^{a}	1.41±0.03^{ab}	1.45±0.06^{a}	34.6±1.59^{a}
TH	17.8±0.50^{a}	7.44±0.58^{ab}	1.41±0.03^{ab}	1.44±0.04^{ab}	33.9±0.68^{a}
HFM-S	13.5±0.70^{bc}	5.80±0.10^{cd}	1.11±0.11^{ab}	1.37±0.07^{ab}	30.4±1.41^{abc}
LFM-S	13.3±0.50^{c}	5.65±0.07^{d}	1.09±0.15^{ab}	1.30±0.05^{b}	28.5±2.97^{c}
SH-S	14.8±0.80^{bc}	6.51±0.24^{cd}	1.11±0.06^{bc}	1.42±0.03^{ab}	33.4±1.40^{ab}
TH-S	13.8±0.10^{bc}	6.19±0.18^{bcd}	1.09±0.17^{b}	1.43±0.03^{ab}	30.6±1.57^{abc}
Factorial ANOVA (P>F)					
Chasing stress	0.000	0.000	0.000	0.030	0.046
Dietary changes	0.000	0.000	0.349	0.013	0.000
Interactions	0.006	0.721	0.414	0.507	0.463

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, tissue antioxidant capacity and malondialdehyde level of red seabream fed the four experimental diets for 15 weeks.

	Catalase[1]	SOD[2]	GPx[3]	TAC[4]	MDA[5]
HFM	50.1±1.26^{ab}	65.6±0.29^{bc}	100±12.04^{ab}	2.00±0.15^{a}	4.13±0.13^{de}
LFM	47.8±1.77^{ab}	57.9±1.10^{de}	92.1±4.53^{ab}	2.01±0.09^{a}	4.92±0.15^{cde}
SH	51.2±1.51^{a}	74.1±3.89^{a}	110±5.98^{a}	2.09±0.06^{a}	3.86±0.27^{e}
TH	51.0±1.01^{a}	70.6±2.87^{ab}	108±6.00^{a}	2.09±0.04^{a}	3.93±0.26^{e}
HFM-S	46.1±2.95^{a}	60.6±1.37^{cd}	82.5±5.60^{bc}	1.50±0.27^{b}	6.13±0.94^{ab}
LFM-S	47.4±0.86^{ab}	54.8±0.65^{e}	71.9±4.92^{c}	1.52±0.17^{b}	6.43±0.20^{a}
SH-S	47.7±1.30^{ab}	68.4±0.22^{b}	85.0±4.17^{bc}	1.81±0.16^{ab}	5.19±0.45^{bcd}
TH-S	47.0±1.27^{ab}	65.8±0.86^{bc}	85.6±3.46^{bc}	1.80±0.17^{ab}	5.70±0.13^{abc}
Factorial ANOVA (P>F)					
Chasing stress	0.001	0.000	0.000	0.000	0.000
Dietary changes	0.183	0.000	0.002	0.057	0.001
Interactions	0.147	0.690	0.791	0.438	0.522

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}).

Table 10. Muscle cortisol, plasma cortisol, hematological Ht and Hb levels of red seabream fed the four experimental diets for 15 weeks.

	Muscle cortisol[1]	Plasma cortisol[1]	Ht[2]	Hb[3]
HFM	11.7±1.36	8.70±0.52	39.3±2.89^{a}	6.70±0.44^{a}
LFM	11.6±1.39	9.20±0.57	33.9±0.69^{bc}	5.70±0.15^{abc}
SH	10.6±1.43	8.40±1.16	38.0±1.73^{ab}	6.33±0.18^{ab}
TH	11.6±0.45	8.70±0.31	37.2±2.22^{ab}	6.34±0.36^{ab}
HFM-S	90.4±1.10^{a}	154±4.50^{b}	34.3±0.67^{abc}	5.67±0.46^{bc}
LFM-S	97.3±2.42^{a}	169±12.4^{a}	31.3±2.08^{c}	5.29±0.18^{c}
SH-S	75.3±7.34^{b}	151±2.77^{b}	35.0±1.00^{abc}	5.39±0.62^{bc}
TH-S	82.1±3.12^{b}	150±8.34^{b}	36.7±2.03^{ab}	5.38±0.17^{bc}
Factorial ANOVA (P>F)				
Chasing stress	0.000	0.000	0.002	0.000
Dietary changes	0.000	0.002	0.002	0.033
Interactions	0.000	0.004	0.253	0.438

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 11. Morphometric parameters of red seabream intestine fed the four experimental diets for 15 weeks.

	VL[1]	EH[2]	ID[3]	GC[4]
HFM	1270±20^{d}	54.7±4.6^{ab}	3087±496	922±61
LFM	1223±32^{d}	50.5±3.0^{bc}	3133±465	837±59
SH	1615±48^{ab}	60.0±2.0^{a}	3535±281	969±57
TH	1623±40^{a}	61.2±1.7^{a}	3515±267	961±30
HFM-S	1300±86^{cd}	54.5±4.3^{ab}	3457±264	864±79
LFM-S	1240±81^{d}	49.4±4.1^{c}	3543±113	826±52
SH-S	1513±41^{ab}	59.5±1.6^{a}	3749±012	927±87
TH-S	1457±77^{bc}	59.8±1.1^{a}	3459±369	928±31
Factorial ANOVA (P>F)				
Chasing stress	0.027	0.559	0.096	0.161
Dietary changes	0.000	0.000	0.243	0.013
Interactions	0.029	0.994	0.604	0.921

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

	FCR	EH	GC	ID	VL
FCR		-0.6561	-0.6179	-0,2698	-0.8189
		0.0017	**0.0030**	**0.1958**	**0.0001**
EH	-0.6561		0.6222	0.1685	0.7252
	0.0017		**0.0028**	**0.4191**	**0.0005**
GC	-0.6179	0.6222		0.0435	0.6954
	0.0030	**0.0028**		**0.8348**	**0.0009**
ID	-0,2698	0.1685	0.0435		0.4528
	0.1958	**0.4191**	**0.8348**		**0.0299**
VL	-0.8189	0.7252	0.6954	0.4528	
	0.0001	**0.0005**	**0.0009**	**0.0299**	

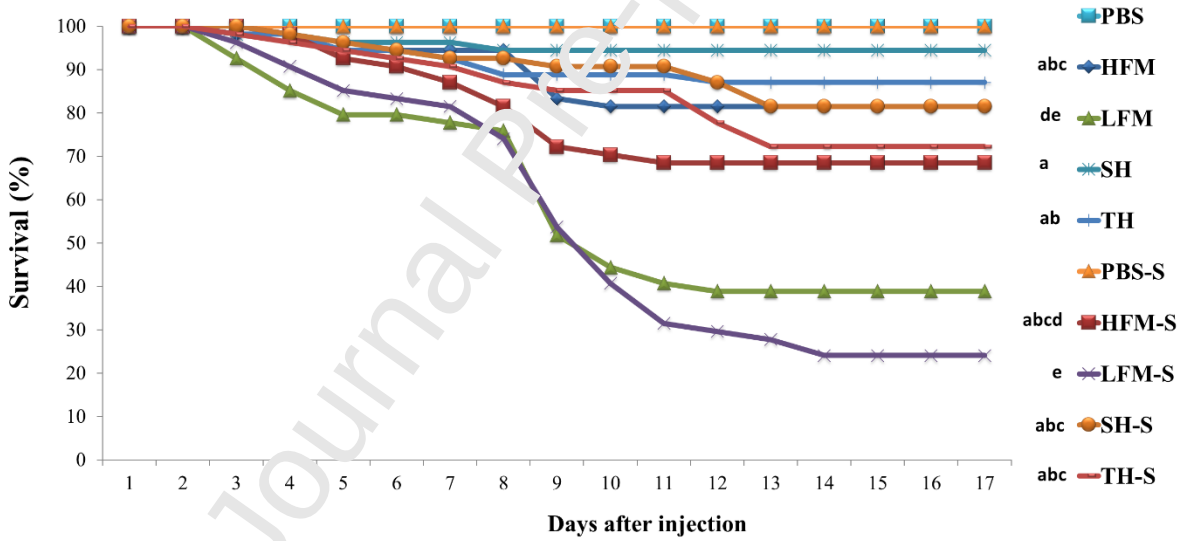

Figure 2. Survival of red seabream fed the four experimental diets after challenge with *E. tarda.* At the beginning, fish were injected with *E. tarda* suspension containing 1 x 10^6 CFU mL^{-1}. Different letters besides the legend denote significant differences between diet x stress groups at the $P < 0.001$ level (Log-rank analysis with Bonferroni post-hoc analysis).

Appendices

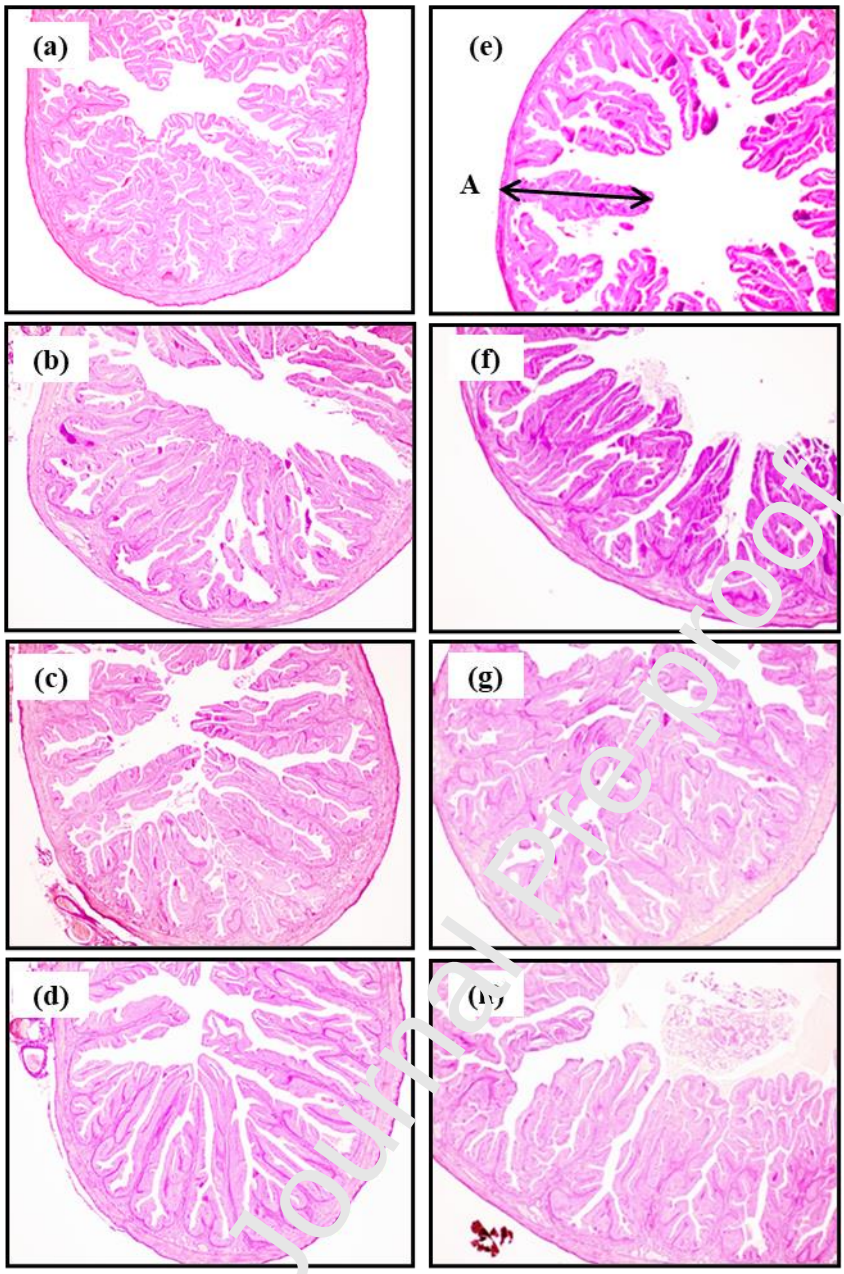

Figure S1. Intestine morphology of the red seabream fed the four experimental diets for 15 weeks (A. villus height; magnification, x 4). (a) HFM, (b) LFM, (c) SH, (d) TH, (f) HFM-S, (g) LFM-S, (h) SH-S, (i) TH-S

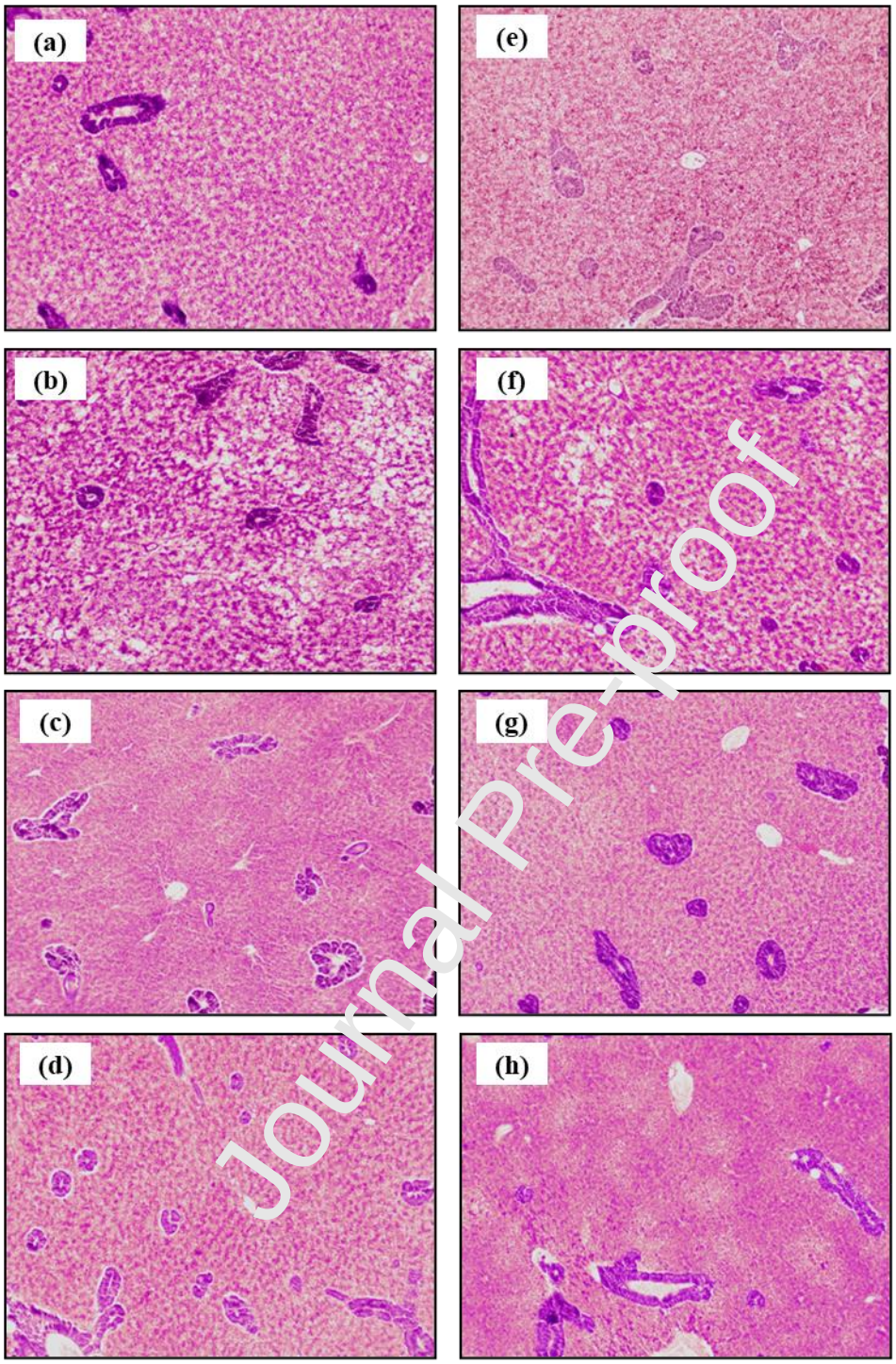

Figure S2. Liver morphology of the red seabream fed the four experimental diets for 15 weeks (A-blood vessels, B-vacuoles; magnification, x 10). (a) HFM, (b) LFM, (c) SH, (d) TH, (f) HFM-S, (g) LFM-S, (h) SH-S, (i) TH-S

Table S1. 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	56.4±9.16	98.7±6.10	3.70±0.26	166±24.4	95.7±1.51	57.0±7.86
LFM	55.9±3.24	104±3.01	4.00±0.75	150±26.7	88.6±3.66	63.8±2.17
SH	55.9±2.58	93.3±8.66	3.91±0.26	167±7.20	90.5±6.25	58.9±8.20
TH	56.7±2.60	92.3±8.59	4.12±0.58	151±8.00	90.2±7.91	61.8±4.41
HFM-S	55.4±6.96	109±9.39	3.98±1.14	160±22.4	91.0±5.35	74.4±8.49
LFM-S	49.4±6.15	107±10.7	3.62±0.07	149±14.1	94.3±1.76	75.2±9.64
SH-S	53.9±3.22	104±7.17	3.29±0.48	150±21.6	93.9±1.55	69.7±8.64
TH-S	53.9±4.16	106±5.72	3.50±0.47	162±26.4	95.2±1.61	73.6±3.76
Factorial ANOVA (P>F)						
Chasing stress	0.170	0.016	0.182	0.683	0.221	0.000
Dietary changes	0.743	0.408	0.885	0.725	0.911	0.618
Interactions	0.810	0.746	0.527	0.625	0.195	0.837

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 S2. Whole-body proximate composition (%, dry matter) of red seabream fed the four experimental diets for 15 weeks.

	Dry matter	Crude protein	Crude lipid	Crude ash
HFM	36.6±0.6	16.9±0.6	13.7±0.6^{ab}	4.1±0.8
LFM	35.9±1.1	17.7±1.1	12.2±0.9^{bc}	4.3±0.4
SH	35.6±0.7	16.7±1.1	14.8±1.2^{a}	4.6±0.6
TH	36.4±1.5	18.0±0.3	13.6±1.8^{ab}	4.0±0.9
HFM-S	34.8±2.0	18.9±2.1	11.8±0.4^{c}	4.4±0.2
LFM-S	35.5±0.6	17.5±1.1	12.1±0.9^{bc}	4.1±0.7
SH-S	35.9±1.2	17.4±1.1	12.1±0.5^{bc}	4.7±0.6
TH-S	34.8±0.9	17.6±1.6	13.2±1.3^{abc}	4.7±0.8
Factorial ANOVA (P>F)				
Chasing stress	0.077	0.297	0.008	0.448
Dietary changes	0.998	0.619	0.164	0.676
Interactions	0.333	0.353	0.145	0.658

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

Table S3. Whole-body eviscerated carcass proximate composition (%, dry matter) of red seabream fed the four experimental diets for 15 weeks.

	Dry matter	Crude protein	Crude lipid	Crude ash
HFM	32.3±3.1	18.8±1.3	8.2±1.7	5.4±0.6
LFM	32.0±2.3	18.6±1.8	8.5±0.8	4.9±0.2
SH	33.5±0.9	19.3±0.3	9.2±1.5	5.2±1.0
TH	34.2±1.0	19.8±0.6	9.8±0.3	5.1±0.4

HFM-S	32.7±0.6	19.0±0.7	8.0±0.5	5.0±0.5
LFM-S	32.3±1.6	20.0±0.1	7.7±1.0	5.3±0.6
SH-S	32.4±1.5	19.3±1.6	8.0±1.3	5.1±0.5
TH-S	33.1±2.4	18.8±0.7	7.5±1.4	4.7±0.3
Factorial ANOVA (P>F)				
Chasing stress	0.615	0.761	0.030	0.636
Dietary changes	0.552	0.883	0.788	0.676
Interactions	0.853	0.311	0.487	0.514

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

Table S4. Viscera proximate composition, muscle water retention and liver lipid content (%, wet basis) of red seabream fed the four experimental diets for 15 weeks.

	HSI[1]	VSI[2]	VP[3]	VL[4]	MWR[5]	LL[6]
HFM	1.42±0.32	6.47±0.68	17.2±1.15	61.3±3.[illegible]	73.3±0.9	13.5±4.23
LFM	1.47±0.18	5.57±0.82	17.0±1.27	59.6±4.6	73.8±0.8	17.5±2.21
SH	1.35±0.16	6.95±0.31	17.1±2.40	60.2±2.82	73.3±0.5	14.1±1.97
TH	1.44±0.30	6.01±1.10	17.5±2.15	59.3±2.64	72.9±0.9	16.9±5.05
HFM-S	1.35±0.24	6.65±1.25	17.4±1.19	58.5±4.52	73.2±1.6	13.9±3.65
LFM-S	1.52±0.08	5.97±0.26	17.2±1.46	61.9±2.75	73.4±0.9	17.4±2.39
SH-S	1.46±0.28	6.08±0.98	17.4±2.50	60.6±1.47	73.5±0.4	14.1±3.24
TH-S	1.47±0.36	6.24±1.35	16.1±1.99	60.5±1.99	73.7±0.5	16.9±3.41
Factorial ANOVA (P>F)						
Chasing stress	0.760	0.262	0.656	0.902	0.730	0.973
Dietary changes	0.865	0.436	0.920	0.971	0.908	0.181
Interactions	0.938	0.637	0.998	0.577	0.677	1.000

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

[1]Hepatosomatic index = (Liver weight/ Fish weight) x 100, [2]Viscerosomatic index = (Viscera weight/ Fish weight) x 10; [3]Viscera crude protein (%); [4]Viscera crude lipid (%); [5]Muscle water retention (%) and [6]Liver crude lipid (%).

Author contributions

Mikaël Herault co-participated in the design of the study, analyzed the results, and wrote the first draft of the article. Vincent Fournier co-participated in the design of the study. Bastien Sadoul analyzed the results and contributed to the first draft of the article. Hervé Le Bris contributed to the first draft of the article. Buddhi E. Gunathilaka mainly conducted the feeding trial and analyses, and Kyeong-Jun Lee designed the dietary formulation and consulted whole process of the study. All coauthors contributed to the writing of the manuscript.

Declaration of interests

☐The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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

Mikael Herault reports financial support was provided by Symrise Aqua Feed. Mikael Herault reports a relationship with Symrise Aqua Feed that includes: employment and funding grants. co-authors employed by Symrise Aqua Feed or have received a part of the founding for this study

- APH mitigate stress adverse effects
- APH help to reduce FM and antibiotic uses