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Luca Parma[a*], Serena Busti[a], Sara Ciulli[a], Enrico Volpe[a], Francesca Errani[a], Åge Oterhals[b], Odd Helge Romarheim[b], Tone Aspevik[b], Francesco Dondi[a], Pier Paolo Gatta[a], Alessio Bonaldo[a]

[a]Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra 50, 40064 Ozzano Emilia, Bologna, Italy

[b]Nofima, the Norwegian Institute of Food Fisheries and Aquaculture Research, 5141 Fyllingsdalen, Norway.

*Corresponding author: Luca Parma, Department of Veterinary Medical Sciences, University of Bologna, Viale Vespucci 2, 47042 Cesenatico, FC, Italy.

E-mail address: luca.parma@unibo.it

Abstract

The effect of bioactive peptides (BPs) derived from farmed Atlantic salmon (*Salmo salar*) by-products on growth, blood biochemistry and immune response was tested in European sea bass (*Dicentrarchus labrax*). Three diets with different levels of BPs (0% BP0, 5% BP5, 10% BP10) in substitution for fish meal (FM) were administered to triplicate fish groups over 58 days. At the end of the trial, fish were subjected to suboptimal environmental conditions (high water temperature, low oxygen) for 7 days. No significant differences ($p>0.05$) were observed in final body weight, specific growth rate, feed intake, and feed conversion while lipid efficiency was higher ($p<0.05$) in BP10. At the end of the growth trial, plasma profile remarked previous studies on this species; however cholesterol was higher in BP5 while glucose decreased with increasing BP content. In addition, a reduction in plasma total protein, cholesterol, high-density lipoprotein and triglycerides after environmental stressful conditions was observed only in BP5 supporting a better lipid utilization for energy purposes as a strategy to counteract high energy demand periods. The immune gene analysis

showed in BP5 a reduction of the pro-inflammatory interleukin 1β (IL-1β), IL-8, and a significant increase in the expression of the anti-inflammatory interleukin-10; on the contrary, BP10 led to an increase in the expression of IL-1β, and IL-8. In conclusion, data suggest that BPs from salmon by-products have a promising implication as circular and functional ingredients for European sea bass diets. In addition to being a valid alternative ingredient to fishmeal in terms of acceptability and growth, it could enhance physiological mechanisms related to lipid metabolism and immune response.

Keywords: European sea bass, fish protein hydrolysate, growth, plasma biochemistry, immune response

1. Introduction

Aquaculture is the fastest growing animal production sector and is currently about to play a key role as primary protein production for food security worldwide (FAO 2018, 2020). However, recognizing the capacity of aquaculture for further growth, but also the enormity of the environmental challenges the sector must face, the aquaculture sector is now challenging the needs of high-quality circular feed ingredients to reduce its dependence from wild-marine and land-based vegetable resources (Woodgate et al., 2022). In addition, in order to reduce the pressure of chemical treatments, to alleviate stressful management practices and to counteract negative climate change effects on animal welfare, the application of functional feed with immune stimulant effects are key topics of aquaculture research (Dawood et al., 2018).

Fish by-products are all raw materials (an average of 50% of the whole fish) edible or inedible generated during fish processing, and include heads, (containing the gills), trimmings (containing muscle, bone and skin), mince, frames, and viscera (liver, kidney and roe) (Gao et al., 2021). The increase in farmed fish production is creating increasing amounts of by-products from fish processing waste, and among the currently farmed species the salmon industry generates one of the largest

77 portions of fish processed globally (Idowu et al., 2019). Salmon leftovers have low market values and
78 are usually utilized as fertilizer or wasted even if they contain a considerable amount (15-60%) of
79 high value protein. Fish protein may be a suitable substrate to produce hydrolysates and bioactive
80 peptides, but only recently has this been evaluated in salmon waste (Idowu et al., 2019). Fish
81 hydrolysates have an excellent nutritional composition, favourable amino acid profiles, and beneficial
82 biological activities such as antihypertensive, antioxidative, antimicrobial and anti-inflammatory
83 activity making them suitable ingredients for functional human food and animal feed application (Nag
84 et al., 2022). Previous studies have demonstrated the beneficial role of the application of fish protein
85 hydrolysate (FPH) in fish nutrition as reviewed by Siddik et al. (2020). Positive effects are related to
86 the improvement of zootechnical fish performance such as feed intake, feed utilization and growth,
87 while other effect account for several beneficial health aspects stimulating various haematological
88 and immunological parameters and improving fish immunity and disease resistance (Siddik et al.,
89 2020). European sea bass is one of the major farmed species for the Mediterranean aquaculture
90 industry. In the past two decades its carnivorous habits have led the feed industry and academics to
91 search extensively for alternative high-value protein sources to replace fishmeal (FM). Most of the
92 studies have been carried out using plant sources (i.e. soy products) demonstrating great potential for
93 an almost total FM replacement (Kousoulaki et al., 2015; Bonvini et al., 2018a, 2018b; Torrecillas et
94 al., 2017; Parma et al., 2019). However, when included at a very high dietary level, plant ingredients
95 seem to affect immune and stress response and the general state of health, especially during stressful
96 husbandry or unfavourable environmental conditions (Montero et al., 2003; Machado et al., 2019;
97 Pelusio et al., 2022). Among Mediterranean fish, this species is particularly vulnerable to farming
98 stress such as chasing and confining (Samaras et al., 2018a; Serradell et al., 2020; Pelusio et al.,
99 2022). High water temperature and low oxygen conditions have also been related to altered stress
100 response in European sea bass, to change in antioxidant status, and to inducing inflammatory process
101 at gut mucosal level (Samaras et al. 2016, 2018b; Busti et al., 2020a). Recently, the utilization of
102 protein hydrolysates manufactured from aquaculture by-products (shrimp and tilapia) have shown

promising results as FM replacement meeting optimal growth but also promoting metabolic pathways related to immunity (Leduc et al., 2018). In addition, as future research, the authors suggested the necessity of assessing hydrolysate performances in fish grown under more challenging conditions such as low oxygen and high temperature. No study concerning the effect of farmed salmon bioactive hydrolysate on zootechnical performance and resistance to stressful environmental conditions has been tested on Mediterranean fish species. This study will thus explore the efficacy of bioactive hydrolysate derived from farmed Atlantic salmon (*Salmo salar*) by-products to replace dietary FM in European sea bass. Growth performance, plasma biochemistry, and immune-related gene expression in animals reared under normal conditions and after high temperature and low oxygen exposure are investigated.

2. Materials and methods

2.1 Protein hydrolysate and experimental diet

Atlantic salmon (*Salmo salar*) heads and backbones were purchased from Biomega (Øygarden, Norway). Ground raw material was mixed with tap water (1:1) and heated to 50 °C and chymotrypsin was added (0.1 % w/w; Enzyme Supplies, UK). Hydrolysis was run for 60 min before inactivation of the enzyme activity at T > 90 °C for 10 min. The water phase (hydrolysate) was separated from the lipid and bone phases and dried in a NIRO P-6.3 spray drier (GEA, Denmark) with inlet and outlet temperature of 200 and 92°C, respectively, to a dry powder. Three isoproteic and isolipidic experimental diets were produced by Nofima AS, Tromsø, Norway, with different inclusion levels of bioactive peptide (BP): BP0 (control diet, 0% of BP), BP5 (5% of BP), and BP10 (10% of BP) to replace an equal amount of FM. Diets were conditioned in an atmospheric double differential preconditioner (Wenger Manufacturing Inc., Sabetha, KS) prior to extrusion on a TX-52 twin-screw extruder (Wenger), and expanded over 2.5 mm dies to give 3.2 mm pellets. Pellets were dried in a hot air dual layer carousel dryer (Model 200.2, Paul Klockner GmbH, Nistertal, Germany) at constant

129 air temperature and vacuum oil coated to reach the final lipid level. Total and free amino acid
130 composition, non-protein amino acid and molecular weight of BP are shown in Table 1. Dietary
131 ingredients and proximate composition are shown in Table 2.
132
133 *2.2 Fish and feeding trial*
134 The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary
135 Medical Sciences of the University of Bologna (Cesenatico, Italy). European sea bass specimens were
136 obtained from Italian hatcheries and adapted to the laboratory facilities for a week. Thereafter, 60 fish
137 (initial average weight: 73.0 ± 0.3 g) per tank were randomly distributed into nine 800 L square tanks
138 with a conical base. Each diet was administered to triplicate groups randomly assigned, over 58 days.
139 Tanks were provided with natural seawater and connected to a closed recirculation system (RAS).
140 (overall water volume: 15 m^3), RAS utilized and water flow rate according to Busti et al. (2020b).
141 Oxygen level was maintained at 8.0 ± 1.0 mg L^{-1} through a liquid oxygen system connected to a
142 software controller (B&G Sinergia snc, Chioggia, Italy); temperature was kept at 24 ± 1.0 °C during
143 the entire trial, Salinity (25 g L^{-1}) was measured by a salt refractometer (106 ATC), photoperiod was
144 held constant at 12 h day through artificial light. Ammonia (total ammonia nitrogen ≤0.1 mg L−1)
145 and nitrite (≤ 0.2 mg L−1) were spectrophotometrically monitored once a day (Spectroquant Nova
146 60, Merck, Lab business, Darmstadt, Germany), and sodium bicarbonate was added daily to keep pH
147 at 7.8–8.0. Feed was provided to satiation by oversupplying feed via automatic feeders by
148 approximately 10% of the daily ingested ration, twice a day: the first 60% of the daily ration was
149 administered at 0830 h and the remaining 40% at 1600 h for six days a week (Pelusio et al., 2022).
150 Each meal lasted 1 hour, after which the uneaten feed of each tank was gathered, dried overnight at
151 105°C, and weighed for overall calculation of feed eaten.
152
153 *2.3 Sub-optimal rearing conditions*

To explore the effect of BP during unfavorable rearing conditions, after the end of the growth trial, fish were maintained at high temperature (30.5 ± 0.03 °C) and low oxygen (4.51 ± 0.39 mg L^{-1}, 64.6 ± 5.68% saturation level) for 7 days while keeping the same feeding conditions. Specifically, the temperature was increased at a rate of 2-degree day^{-1} while oxygen concentration was decreased from 8.0 ± 1.0 mg L^{-1}, 105.0 ± 1.0% saturation level to 4.51 ± 0.39 mg L^{-1}, 64.6 ± 5.68% within 24 h. Temperature and oxygen levels were constantly monitored through an automatic system regulated by a software program (B&G Sinergia snc, Chioggia, Italy). The methodology was conducted according to Busti et al. (2020a). Fish were daily monitored to check for any mortality.

2.4 Sampling

At the beginning and the end of the experiment, all the fish in each tank were anesthetized or euthanised with tricaine methanesulfonate (MS-222) at 100 or 300 mg L^{-1} and individually weighed. Specific growth rate (SGR), feed intake (FI), and feed conversion ratio (FCR) were calculated. The proximate composition of the carcasses was determined at the beginning of the trial on a pooled sample of 10 fish and a pooled sample of 5 fish per tank at the end of the trial. Protein efficiency ratio (PER), gross protein efficiency (GPE), lipid efficiency ratio (LER) and gross lipid efficiency (GLE) were calculated. At the beginning (day 0, 9 fish in total), at the end of the feeding trial (T1, day 58, 3 fish per $tank^{-1}$), and after the high rearing temperature/low oxygen period (T2, 3 fish per $tank^{-1}$), liver and distal intestine were sampled for cytokine gene expression. At T1 and T2, 5 fish per tank were also sampled for plasma biochemistry analyses. At T1 tissues/organs from 3 fish per tank were collected for analysis of somatic indexes.

All experimental procedures were evaluated by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes (ID 113/2020-PR).

2.5. Performance parameters calculation

The formulae employed were as follows:

Specific growth rate (SGR) (% day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights). Feed intake (FI) (% ABW^{-1} day^{-1}) = ((100 ∗ total ingestion)/(ABW))/days)) (where average body weight, ABW = (IBW + FBW)/2; Feed conversion ratio (FCR) = feed intake / weight gain. Protein efficiency ratio (PER) = weight gain / protein intake. Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)] / total protein intake fish. Gross lipid efficiency (GLE) (%) = 100 * [(% final body lipid * FBW) - (% initial body lipid * IBW)] / total lipid intake fish. Lipid efficiency ratio (LER) = weight gain/lipid intake.

2.6 Analytical methods

Diets and whole body were analysed for proximate composition which were used for GPE and GLE calculation. Moisture content was obtained by weight loss after drying samples in an oven 105 ◦C overnight. Crude protein was determined as total nitrogen (N*6.25) after performing the Kjeldahl method. Total lipids were determined according to Bligh and Dyer (1959) extraction method. Ash content was estimated by incineration in a muffle oven at 450 ◦C overnight. Total amino acid composition was measured by HPLC after hydrolysing in 6 N HCl for 22 h at 110 °C, using the Waters Accq-Tag method and fluorescence detection with excitation/emission at 250/395 nm (Liu et al., 1995). Free amino acids were measured by HPLC using Waters Pico-Tag method and UV-detection at 254 nm (Bidlingmeyer et al., 1987).

2.7 Plasma biochemistry

Analyses of plasma were conducted according to Parma et al. (2020) on individual plasma aliquot of 500 µL previously collected at T1 and T2. The measures of total protein (TP), urea, uric acid, albumin (ALB), triglycerides (TRIG), cholesterol, high-density lipoprotein (HDL), glucose (GLU), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine, transaminase (ALT), creatine

206 kinase (CK), lactate dehydrogenase (LDH), creatinine, cortisol, calcium (Ca^{+2}), sodium (Na^{+}),
207 chloride (Cl), potassium (K^{+}) phosphorus (P), magnesium (Mg), iron (Fe), lactate were performed by
208 automated analyzer (AU 480; 220 Olympus/Beckman Coulter, Brea, CA, United States) using
209 specific methods (Olympus system 221 reagent, OSR).
210
211 *2.8 Immunological analyses*
212 Immunological analyses were conducted as described in Busti et al. (2020a). Briefly, the total
213 RNA was extracted from 30 mg of tissue (liver and distal intestine) using the NucleoSpin RNA kit
214 (Macherey-Nagel) according to the manufacturer's instructions. The RNA was then back-transcribed
215 using GoScript Reverse Transcriptase (Promega) and the concentration of the obtained cDNA was
216 quantified using the Qubit fluorometer (ThermoFisher). Real time PCRs were conducted with the
217 ABI PRISM 7300 instrument (Applied Biosystems) using the BRYT Green GoTaq qPCR system
218 (Promega). The primers used for the amplification of the housekeeping gene (HK) and of the target
219 genes are reported respectively in Table 3. All samples were tested in triplicate. The expression of
220 the FER, HEP and C3 genes was measured in liver samples, while the expression of the IL-1 β, IL-8,
221 IL-10, IFN-1, Mx and TGF-β genes were measured in the bowel specimens. For each sample, the
222 gene expression was normalized towards the 18S rRNA gene (HK) and expressed as $2^{-\Delta\Delta Ct}$, where
223 the ΔCt was obtained by subtracting the cycle threshold (Ct) of the 18S rRNA gene from the Ct value
224 of the target gene. Gene expression in the samples of treated and untreated subjects collected at times
225 T1 and T2 was expressed as a multiplicative factor relating to the samples collected at time zero (T0).
226
227 *2.9 Statistical analysis*
228 All data are presented as mean ± standard deviation (SD). The tank was used as the experimental
229 unit for analyzing growth performance and nutritional indices. Individual fish were used for analyzing
230 somatic indices, plasma biochemistry and cytokine gene expression. At the end of the feeding trial
231 (T1, 58 days), data of growth performance, nutritional indices and somatic indices were analyzed by

a one-way analysis of variance (ANOVA) and in case of significance ($P<0.05$) Tukey's post hoc test was performed. In order to provide the effect of diets before and after the exposure to suboptimal environmental condition, plasma biochemistry and cytokine gene expression results were analyzed by a two-tier statistical approach analyzing first the data at T1 by one-way ANOVA and followed by a two-way ANOVA considering sampling points and diets as factors. When statistically significant differences were found among groups ($P<0.05$), Tukey's post hoc test was performed to determine differences among experimental groups. Prior to ANOVA analyses, all data were checked for normality and homogeneity of variance by means of the Shapiro Wilk and Levene tests, respectively. All the statistical analyses were conducted using GraphPad Prism and Statistical Package for Social Science (SPSS) for Windows version 20.0.

3. Results

3.1 Growth

Results on growth performance are reported in Table 4. No significant differences among treatments were observed for final body weight, weight gain, SGR, FI and FCR. Results on nutritional and somatic indices are reported in Table 5. LER was higher in BP10 compared to the other treatments while no significant differences were reported in the values of PER, GPE, GLE, HSI, VSI, MFI, K.

3.2 Plasma biochemistry

Plasma biochemistry results are summarized in Table 6. At the end of the growth trial (T1, one-way ANOVA), cholesterol was higher in BP5 than BP0 and BP10. GLU was higher in BP0 than BP10 while K was higher in BP5 than BP10. Plasma results in relation to dietary treatments and time before and after stress (T1, T2) are also presented in Table 6 (Two-way ANOVA). TP displayed a significant interaction with higher values in BP5 than BP0 at T1. Urea was affected by time with higher values at T1 than T2. A significant time and interaction were observed for TRIG, resulting in lower values at T2 than T1 only in BP5. GLU displayed significant diet effect with higher values in

BP0. Cholesterol displayed significant diet and interaction effect with higher values in BP5 compared to BP10 and BP0 at T1. ALP, ALT, Creatine, Cl, Fe and Mg showed a significant time effect with higher values at T2 than T1. Conversely, P was lower in T2 than T1. K showed both time and diet effect; specifically, values were lower in BP10 compared to the other treatments and lower in T1 than T2. Lactate was affected by diet with higher values in BP0.

3.3 Cytokine gene expression

Gene expression analysis is shown in Figure 1. At the end of the growth trial (T1, one-way ANOVA), IL-1β was higher in BP10 compared to BP0 and BP5 (Figure 1d). At the same time IL-8 was higher in BP10 than BP5 (Figure 1e) and IL-10 was higher in BP5 than BP0 (Figure 1f).

Gene expression results in relation to dietary treatments and time before and after stress (T1 vs T2) are also presented in Figure 1 (Two-way ANOVA). IL-8, Mx protein, ferritin and C3 displayed a significant time factor with lower values at T2 compared to T1 (Fig1 a, b, e, h). The effect of the diet was instead significant only for the interleukin 1β gene, showing in this case also an interaction between time and diet. Specifically, at T1 interleukin 1β was higher in BP10 then the other treatments (Figure 1d).

4. Discussion

Several studies have assessed the possibility of replacing FM using FPH in a wide number of fish species. Most of the FPH previously tested derived from wild caught fish species such as pollock, tuna, herring and krill, others were obtained from cultured species such as carp, tilapia and shrimp, while no data obtained from farmed salmon raw materials are available (Siddik et al., 2021). In the present study, no significant differences were obtained among treatments in growth parameters (FBW, SGR) and feed intake showing the possibility of almost totally replacing wild cached FM, including BPs from salmon by-products in a plant-based diet. Dietary inclusion of FPH has been recently shown to enhance fish growth in several species especially when included at moderate level

(5-10%) as partial FM replacement (Siddik et al., 2018, 2019; Leduc et al., 2018; Gisbert et al., 2021). The increase in FI due to higher feed palatability together with the improved availability and subsequent uptake of free amino acids or bioactive growth promoter compounds, is generally recognized as responsible for growth enhancement. In the present study the absence of differences in low FM diet compared to the control diet agree with previous studies on European sea bass juveniles (2.2-13.3g) where dietary inclusion of 5% of hydrolysate from aquaculture by-product (tilapia and shrimp, 50:50 mixture) in a 5% FM diet, restored growth performance to the same level as a 20 % FM control diet (Leduc et al., 2018). Gisbert et al. (2018) did not find differences in growth performance in specimens of European sea bass fed a 5% FM diet with krill hydrolysate against a control FM diet (20 % FM). In turbot, Xu et al. (2016) observed similar growth in the FPH supplemented group compared to the FPH free group. The authors hypothesized that the lack of growth improvement was due to the lower level of FM used in that study (15 % FM) compared to previous work, which may have masked the growth stimulating activity of FPH to some extent. Concerning FCR in the present study no differences occurred suggesting similar digestibility and utilization of BP compared to FM. However, bioactive peptide tends to increase lipid utilization, data which are also partially supported by an increase of plasma cholesterol observed in BP5. Few studies have assessed the effect of FPH on lipid metabolism and deposition in fish tissue. In turbot, Xu et al. (2016) observed a decrease in lipid content at the increasing dietary FPH level in several exemplars of fish tissue with a concomitant reduction of serum cholesterol and triglycerides. Lower plasma triglycerides and cholesterol were also observed in barramundi, *Lates calcarifer* (Siddik et al., 2019). On the contrary, in the same species different hydrolysate sources promoted the increase of plasma cholesterol in comparison to a control diet (Chaklader et al., 2020). The role of FPH on lipid metabolism is not fully clear; however previous authors suggested that FPH is reached in taurine, carnitine and choline which may affect lipid metabolism (Chaklader et al., 2020). These hypotheses agree with the present work where taurine and creatine were present in considerable amounts in the BP tested. According to Martins et al. (2018) a minimum quantity of taurine is necessary for European

sea bass, its function related to the production of bile salts and consequent effect in enhancing lipid metabolism (availability and absorption) (Li et al., 2022).

Blood biochemistry profile is commonly utilized as a valuable tool for determining fish health in response to a novel dietary ingredient or under stressful environmental conditions (Peres et al., 2014; Parma et al., 2020; Pelusio et al., 2022). In general plasma profile confirms previous studies on this species fed FM as a reference protein source (Peres et al., 2014; Bonvini et al., 2018b; Pelusio et al., 2022) except for triglycerides which were considerably higher than in previous observation. At the end of the growth trial (T1) FPH did not affect most of the plasma parameters considered except cholesterol, potassium and glucose. The decrease of glucose at increasing FPH has been previously observed in barramundi and red sea bream (*Pagrus major*) fed FPH at 10-20% and 3%, respectively (Khosravi et al., 2015; Siddik et al., 2018). In this context, Siddik et al. (2019) postulated a bioactive effect of FPH modulating insulin secretion and positively affecting fish welfare.

The exposure to low oxygen and high temperature modified several plasma parameters including urea, triglycerides, ALP, ALT, Creatinine, Cl, K, P, Mg and Fe, and different patterns were observed in relation to dietary treatments. Specifically, a reduction in TP, cholesterol, HDL and triglycerides after environmentally stressful conditions were observed only in BP5 as shown by a significant time x diet interaction. No studies concerning the effect of dietary FPH in this species exposed to high temperature and low oxygen are reported; however this data may suggest a different response to stressful conditions in relation to a different FPH inclusion level. In fact a reduction of these metabolites in BP5 may indicate a better utilization of lipids for energy purposes as a strategy to counteract high energy demand periods. Fish protein hydrolysates are known for their potential immune-modulating effect as reviewed by Siddik et al. (2021). However, to the best of our knowledge no studies reporting the effect from salmon by-products in Mediterranean species are available. Under current experimental conditions the increase of pro-inflammatory cytokines IL-1B and IL-8 were observed only at 10% BP inclusion. In general, the increase of BP content agrees with previous observation in *Lates Calcarifer* (Siddik et al., 2020). The authors reported in fact an enhancement of

mRNA expression level of pro-inflammatory cytokines (IL-1β, TNF-α) in the distal intestine when tuna hydrolysate was included at 6% in fermented poultry by-product meal based-diet. However, the increase of pro-inflammatory cytokines in the intestinal mucosa in absence of immune stimulant could also indicate the activation of an inflammatory process at intestinal level in response to the higher BP dose tested since these cytokines are known to be up-regulated during infective and inflaming reactions. Concerning anti-inflammatory response, IL-10 was significantly up-regulated at BP5 compared to control while this effect was not statistically significant at BP10. Anti-inflammatory cytokines such as IL-10 and TGF-β are essential for a balanced inflammatory response aimed to clear an infection without excessive host damage (Secombe et al., 2016). These cytokines are known to antagonise the effect of pro-inflammatory cytokines playing multiple homeostatic roles during the resolution of inflammatory events and immunoregulatory processes (Rebl and Goldammer, 2018). Furthermore, a beneficial local effect on intestinal epithelial integrity associated with the increase of anti-inflammatory cytokines was pointed out (Al-Sadi et al., 2009). Previous studies using in vitro and in vivo models for evaluating the functional proprieties of fish hydrolysates showed an anti-inflammatory effect of hydrolysates (Ahn et al., 2012; Giannetto et al., 2020; Gisbert et al., 2021). Similarly, in our study an anti-inflammatory effect was indicated for salmon hydrolysates in European sea bass in the form of an up-regulation of the anti-inflammatory IL-10 cytokine suggesting their beneficial effect. This anti-inflammatory effect at BP5 is strengthened by a slight down-regulation of pro-inflammatory cytokines. However, the hydrolysate effect seems to be affected by dose, as the anti-inflammatory effect manifested at BP5 was not present at the higher BP dose tested.

5. Conclusion

Dietary inclusion levels of 5 and 10% of BP derived from farmed Atlantic salmon by-products did not affect feed intake and growth in European sea bass, showing the possibility of almost totally replacing wild cached FM in a plant-based diet. However, BP enhanced lipid efficiency, an effect that could be related to the content of taurine and creatinine. Although most of the plasma profile were

not affected by BP, after the exposure to high temperature and low oxygen a reduction in plasma total protein, cholesterol, high-density lipoprotein and triglycerides was observed only in BP5 supporting a better lipid utilization for energy purposes as a strategy to counteract high energy demand periods. In addition, intestinal IL-10 was significantly up-regulated only at BP5 supporting a better immunoregulatory processes at mucosal level under this treatment. Overall, data suggest that BP from salmon by-products have a promising implication as circular and functional ingredients for European sea bass diet. In addition to being a valid alternative ingredient to fishmeal in terms of acceptability and growth, it could enhance physiological mechanisms related to lipid metabolism and immune response.

Declaration of Competing Interest

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

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Table 1. Amino acid composition, g/100g (total, free and non-protein) and molecular weight distribution (%) of the bioactive peptide obtained from salmon raw material

Proximate composition, %		
Dry matter	98.3	
Crude Protein	82.3	
Fat	11.2	
Ash	6.3	
Amino acid, g/100g	*Total amino acid*	*Free amino acid*
Aspartic acid	6.5	0.15
Glutamic acid	9.4	0.47
Hydroxyproline	1.8	0.02
Serine	3.2	0.13
Glycine	7.6	0.21
Histidine	1.7	0.18
Arginine	4.7	0.85
Threonine	3.0	0.17
Alanine	4.9	0.4
Proline	4.1	0.05
Tyrosine	2.0	0.32
Valine	3.2	0.26
Methionine	2.1	0.18
Isoleucine	2.7	0.19
Leucine	4.6	0.56
Phenylalanine	2.6	0.36
Lysine	5.4	0.9
Asparagine	n.d.	0.02
Glutamine	n.d.	0.02
Tryptophan	n.d.	0.08
Cysteine	n.d.	< 0.01
Creatinine	0.60	n.d.
β-alanine	0.15	n.d.
Taurine	0.56	n.d.
4-aminobutanoic acid	<0.01	n.d.
Citrulline	0.01	n.d.
Carnosine	<0.01	n.d.
Molecular weight distribution (Da), %		
> 20000	0.1	
20000-15000	<0.1	
15000-10000	0.1	
10000-8000	0.2	
8000-6000	0.9	
6000-4000	3.9	
4000-2000	16.5	
2000-1000	24.3	
1000-500	19.4	
500-200	13.9	
< 200	20.7	

N.d.: not determined

Table 2. Ingredients and proximate composition of the three experimental diets

	Experimental Diets		
	BP0	BP5	BP10
Ingredients, %			
Salmon bioactive peptides	0.00	5.00	10.00
Fish meal	15.00	10.00	5.00
Soybean meal	15.00	15.00	15.00
Wheat	15.28	16.02	16.87
Wheat gluten	12.20	11.30	10.30
Corn gluten	8.00	8.00	8.00
Soy protein concentrate	5.40	5.40	5.40
Fish oil	10.00	9.90	9.80
Rapeseed oil	5.00	5.00	5.00
Horse beans	10.00	10.00	10.00
Lecithin from rapeseed	1.00	1.00	1.00
*Vitamin premix	0.50	0.50	0.50
*Mineral premix	0.50	0.50	0.50
Monosodiumphosphate	3.00	3.00	3.00
L-Lysine	0.40	0.40	0.40
DL-Methionin	0.05	0.05	0.05
Water adjustment	-1.33	-1.07	-0.82
Proximate composition, %			
Moisture	6.41	6.46	6.57
Protein	39.90	39.50	39.22
Lipids	19.20	18.75	18.04
Ash	6.79	6.29	5.73

*Vitamins and mineral premix (IU or mg kg^{-1} diet): vitamin A: 3000IU; vitamin D3: 3800 IU; vitamin E: 300 mg; vitamin K3: 30 mg; vitamin B1: 30 mg; vitamin B2: 45 mg; vitamin B6: 38 mg; vitamin B12: 0.08 mg; niacin: 300 mg; Ca-D-pantonat: 90 mg; biotin: 1.5 mg; folic acid: 15 mg; vitamin C: 300 mg; Fe: 60 mg; Mn: 30 mg; Zn:130 mg; Cu: 6 mg; I: 5 mg; Co: 0.05 mg; Se: 0.3 mg.

Table 3. Primers used for the amplification of immune-related genes

Gene	Abbreviation	GenBank ID	Primer sequence (5'- 3')	References
18s rRNA	18S	AM490061	AGGGTGTTGGCAGACGTTAC CTTCTGCCTGTTGAGGAACC	Sepulcre et al., 2007
Interleuchin 1β	IL-1β	AJ311925	ATCTGGAGGTGGTGGACAAA AGGGTGCTGATGTTCAAACC	Sepulcre et al., 2007
Interleuchin 8	IL-8	AM490063	GTCTGAGAAGCCTGGGAGTG GCAATGGGAGTTAGCAGGAA	Sepulcre et al., 2007
Interleuchin 10	IL-10	DQ821114	CGACCAGCTCAAGAGTGATG AGAGGCTGCATGGTTTCTGT	Sepulcre et al., 2007
Transforming growth factor β	TGF-β	AM421619	GACCTGGGATGGAAGTGGAT CAGCTGCTCCACCTTGTGTTG	Faliex et al., 2008
Mx protein	Mx	AM228977, HQ237501, AY424961	GAAGAAGGGCTACATGATCGTC CCGTCATTGTAGAGAGTGTGGA	Chaves-Pozo et al, 2012
Interferon Iα	IFN-I	AM765847	GGCTCTACTGGATACGATGGCT CTCCCATGATGCAGAGCTGTG	Scapigliati et al., 2010
Ferritin	FER	NP_001117129, P49946, AAB34575	ATGCACAAGCTCTGCTCTGA TTTGCCCAGGGTGTGTTTAT	Sarropoulou et al., 2009
Hepcidin	HEP	DQ131605	CCAGTCACTGAGGTGCAAGA TCAGAACCTGCAGCAGACAC	Sarropoulou et al. 2009; Rodrigues et al., 2006
Complement C3	C3	DN832026	TATGCCCTTCTTGCTCTGGT GCCTGAGTTGATCCATAGCC	Bado-Nilles et al., 2011

Table 4. Growth performance of *Dicentrarchus labrax* fed the experimental diets overall 58 days.

	Experimental diets			*p-value*
	BP0	BP5	BP10	
IBW(g)	73.2 ± 0.57	73.3 ± 1.61	73.3 ± 0.33	0.999
FBW(g)	145.1 ± 1.76	147.1 ± 3.79	149.2 ± 2.65	0.271
Weight gain(g)	71.8 ± 2.33	73.8 ± 2.29	75.9 ± 2.32	0.996
SGR	1.18 ± 0.03	1.20 ± 0.01	1.23 ± 0.02	0.146
FI	1.47 ± 0.06	1.53 ± 0.01	1.53 ± 0.02	0.112
FCR	1.30 ± 0.03	1.33 ± 0.02	1.31 ± 0.02	0.291

Values are indicated as mean (n=3) ± SD.
FBW= Final body weight
IBW= Initial body weight
SGR (% day^{-1}) (Specific growth rate) = 100 * (ln FBW- ln IBW) / days
FCR feed conversion rate = feed intake (g)/weight gain (g).
FI (% ABW^{-1} day^{-1}) (Feed intake) = ((100 ∗ total ingestion)/ (ABW))/days

Table 5. Nutritional and somatic indices of European sea bass fed experimental diets over 58 days.

	BP0	BP5	BP10	*p-value*
Nutritional indices				
PER	1.94 ± 0.04	1.91 ± 0.02	1.96 ± 0.04	0.394
GPE	34.0 ± 1.81	34.3 ± 1.04	35.1 ± 0.71	0.587
LER	4.03 ± 0.08[a]	4.03± 0.04[a]	4.25 ± 0.08[b]	**0.013**
GLE	88.2 ± 5.67	93.4 ± 2.39	103.8 ± 12.4	0.128
Somatic indices				
HSI	2.66 ± 0.26	2.78 ± 0.40	2.53 ± 0.35	0.365
MFI	6.42 ± 1.33	6.64 ± 1.46	6.55 ± 1.87	0.954
VSI	12.2 ± 1.05	12.6 ± 1.57	12.3 ± 1.74	0.839
K	1.24 ± 0.06	1.25 ± 0.05	1.25 ± 0.08	0.832

Data are given as the mean (n=3 for nutritional indices; n=9 for somatic indices) ± SD PER = Protein efficiency ratio = weight gain/protein intake; Gross protein efficiency (GPE, %) = 100*[(% final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish; Gross lipid efficiency (GLE, %) = 100*[(% final body lipid*FBW) - (%initial body lipid*IBW)]/total lipid intake fish; Lipid efficiency ratio (LER) = weight gain/lipid intake; Hepatosomatic index (HSI, %) = 100*(liver weight/FBW); Mesenteric Fat Index (MFI, %) = 100*(mesenteric fat weight/FBW); Viscerosomatic index (VSI, %) = 100*(viscera weight/FBW); Fulton's condition factor (K) = 100*(FBW/length^3). SD = Standard deviation.

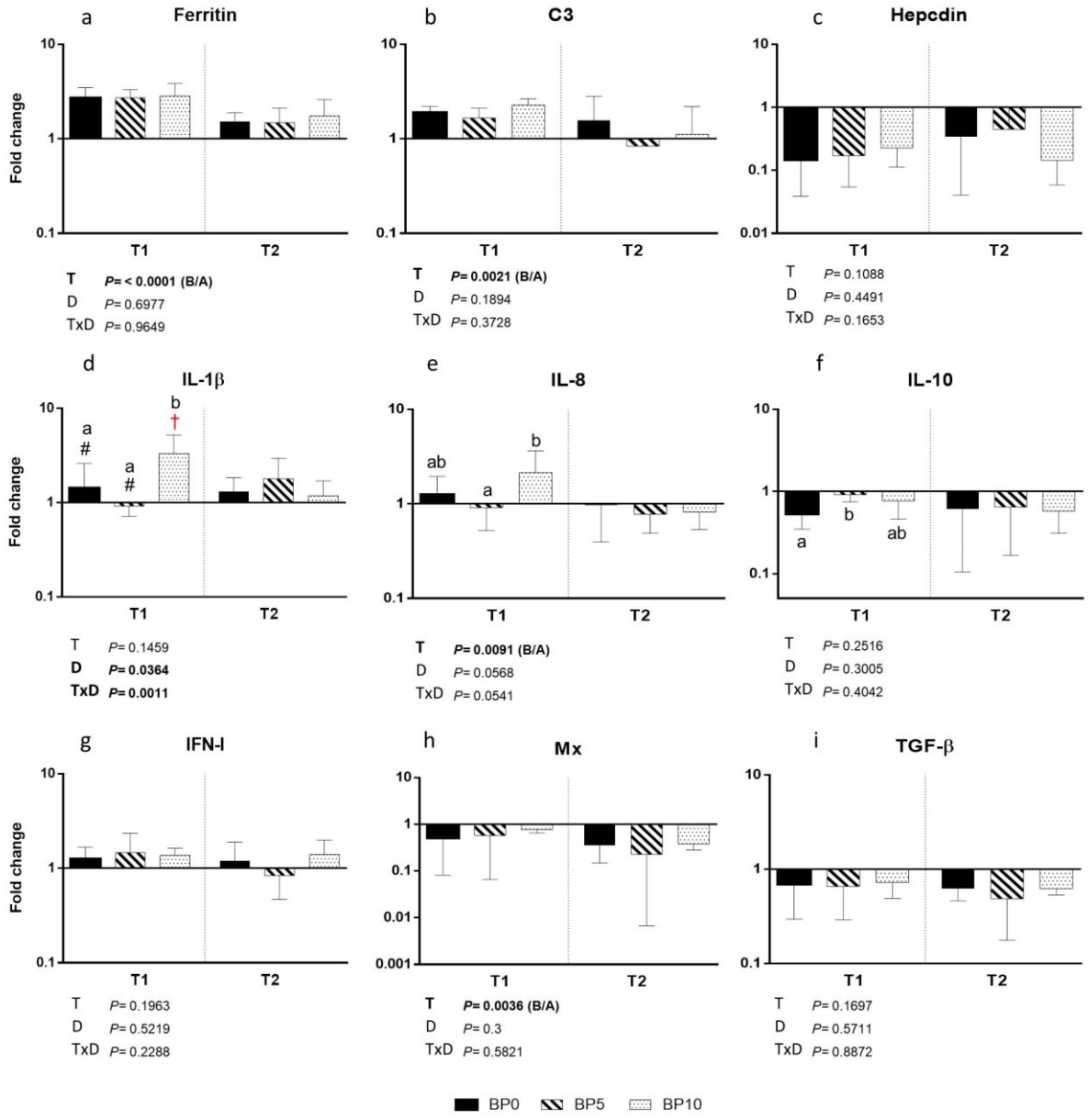
Table 6 Plasma biochemistry values for European sea bass fed the experimental diets and exposed to high temperature and low oxygen.

Analytes	Time	BP0	BP5	BP10	Two-way ANOVA			One-way ANOVA
					Diet	Time	Interaction	
TP (g dL^{-1})	T1	4.3 ± 0.4#	4.8 ± 0.4†	4.6 ± 0.7$^{\#\dagger}$	0.9749	0.6559	**0.0091**	0.088
	T2	4.7 ± 0.4	4.3 ± 0.4	4.7 ± 0.4				
Urea (mg/dL)	T1	8.61 ± 1.49	9.01 ± 1.82	9.68 ± 1.66	0.233	**<0.001 (B/A)**	0.430	0.174
	T2	5.47 ± 1.16	5.28 ± 1.80	5.59 ± 0.88				
Uric acid (mg dL^{-1})	T1	0.12 ± 0.07	0.11 ± 0.10	0.13 ± 0.10	0.460	0.07	0.559	0.915
	T2	0.27 ± 0.39	0.13 ± 0.11	0.22 ± 0.22				
ALB (g dL^{-1})	T1	1.15 ±0.17	1.27 ± 0.18	1.19 ± 0.20	0.932	0.245	0.194	0.350
	T2	1.20 ± 0.17	1.11 ± 0.13	1.15 ± 0.25				
TRIG (mg dL^{-1})	T1	1442.2 ± 250	1620.1 ± 225^{b}	1364.8 ± 47	0.7434	**0.0008 (A/B)**	**0.0457**	0.241
	T2	1142.0 ± 325.9	1105.0 ± 311.8^{a}	1340.2 ± 220.3				
Cholesterol (mg dL^{-1})	T1	153.9 ± 28.9#	212.5 ± 27.8†	154.4 ±48.7#	**0.049**	0.319	**0.001**	**0.0020 (A/B/A)**
	T2	179.2 ± 32.9	173.5 ± 35.7	194.3 ± 25.2				
HDL (mg dL^{-1})	T1	44.4 ± 11.4	55.5 ±13.9	47.2 ± 13.1	0.651	0.045	**0.023**	0.147
	T2	55.8 ± 10.3	49.7 ± 9.8	59.0 ± 11.7				
GLU (mg dL^{-1})	T1	155.4 ± 50.9†	139.4 ± 31$^{\dagger\#}$	114.5 ± 18.3$^{\#}$	**0.0101**	0.0884	0.0771	**0.015 (B/AB/A)**
	T2	151.8 ± 25.2	165.2 ± 42.8	154.8 ± 35.1				
ALP (U L^{-1})	T1	77.8 ± 11.5	90.5 ± 21.2	82.9 ± 16.3	0.1595	**0.008 (A/B)**	0.7434	0.233
	T2	91.9 ± 15.1	97.3 ± 15.9	93.0 ± 14.6				
AST (U L^{-1})	T1	68.1 ± 56.7	176.8 ± 178	144.8 ± 96.7	0.055	0.494	0.1505	0.151
	T2	149.6 ± 70.5	188.4 ± 119.2	104.4 ± 42.6				
ALT (U L^{-1})	T1	4.45 ± 5.15	7.40 ± 5.08	8.64 ± 7.00	0.126	**0.034 (A/B)**	0.126	0.226
	T2	8.54 ± 3.67	12.6 ± 7.68	8.17 ± 4.47				
CK (U L^{-1})	T1	986 ± 1166	1037 ± 914	1593 ± 912	0.451	0.140	0.1719	0.376
	T2	2182 ± 1199	1277 ± 940	1487 ± 1552				
LDH (U L^{-1})	T1	111 ± 163	139 ± 140	121 ± 108	0.627	0.849	0.587	0.897
	T2	140 ± 71.7	127.6 ± 48.4	88.5 ± 39.5				
Creatinine (mg dL^{-1})	T1	0.2 ± 0.06	0.2 ± 0.1	0.2 ± 0.07	0.413	**0.0031 (A/B)**	0.6009	0.446
	T2	0.3 ± 0.03	0.3 ± 0.03	0.3 ± 0.06				
Cortisol (µg dL^{-1})	T1	14.4 ± 7.64	8.3 ± 2.6	16.9 ± 9.3	0.431	0.12	0.3663	0.156
	T2	10.6 ± 6.8	11.0 ± 6.2	13.0 ± 7.2				
Ca^{+2} (mg dL^{-1})	T1	13.1± 0.35	13.5 ± 0.50^{b}	13.1 ± 1.25	0.395	0.487	0.021	0.40
	T2	13.4 ± 0.69	12.7 ± 0.55^{a}	13.3 ± 0.33				
Na^{+} (mEq L^{-1})	T1	182.4 ± 4.70	185.1 ± 7.7	184.0 ± 6.01	0.160	0.861	0.437	0.703
	T2	180.4 ± 16.5	189.0 ± 13.3	182.6 ± 2.93				
Cl (mEq L^{-1})	T1	149.6 ± 4.15	151.7 ± 7.02^{a}	149.2 ± 3.32	0.065	**0.0053 (A/B)**	0.347	0.495
	T2	151.8 ± 13.2	160.3 ± 11.5^{b}	154.1 ± 2.53				
K^{+} (mEq L^{-1})	T1	3.98 ± 0.60^{a}	4.65 ± 0.76	3.85 ± 0.95	**0.0004(B/B/A)**	**0.0001**	0.419	**0.043 (AB/B/A)**
	T2	5.04 ± 0.71^{b}	5.30 ± 0.57	4.39 ± 0.68				
P (mg dL^{-1})	T1	11.5 ± 0.92	11.3 ± 1.12	11.6 ± 1.88	0.595	**0.0001 (B/A)**	0.814	0.936
	T2	9.0 ± 1.03	8.8 ± 0.66	8.4 ± 0.75				
Mg (mg dL^{-1})	T1	2.98 ± 0.40^{a}	3.23 ± 0.52	3.16 ± 0.34	0.234	**0.0001 (A/B)**	0.397	0.391
	T2	3.70 ± 0.51^{b}	3.68 ± 0.56	3.98 ± 0.44				
Fe (µg dL^{-1})	T1	86.2 ± 23.4	82.4 ± 23.9	85.9 ± 24.7	0.350	**<0.0001 (A/B)**	0.540	0.923
	T2	162.5 ± 60.6	143.3 ± 38.4	171.5 ± 34.6				
Lactate (mmol L^{-1})	T1	69.3 ± 16.3	60.0 ± 9.17	60.0 ±16.9	**0.036(B/A/A)**	0.410	0.380	0.322
	T2	69.2 ± 10.3	59.2 ± 9.64	69.0 ± 8.66				

Data are given as the mean (n = 15 $diet^{-1}$) ± standard deviation SD. The uppercase letters in the parenthesis represent significant differences in two-way ANOVA or one-way ANOVA. In the two-way ANOVA, the sets of letters in a parenthesis correspond to the time (T1/T2) or diet (BP0/BP5/BP10) groups, respectively, and data value corresponding to "A" are lower than those corresponding to "B." Different symbols stand for significant differences among dietary treatments for the same time while lowercase letters stand for significant differences between times for the same diet. BP0 = 0 g kg^{-1} bioactive peptide (BP); BP5 = 50 g kg^{-1}BP; BP10 = 100 g kg^{-1} BP. T1, time point before suboptimal environmental condition; (T2); time point after 7 days exposure to high temperature and low oxygen. TP, total protein; ALB, albumin; TRIG, triglycerides; HDL, high-density lipoprotein; GLU, glucose; ALP, alkaline phosphatase AST, aspartate aminotransferase; ALT, alanine transaminase; CK, creatine kinase; LDH, lactate dehydrogenase; Ca^{+2}, calcium; Na^{+} sodium; Cl, chloride; K^{+}, potassium; P, inorganic phosphorus; Mg, magnesium; Fe, iron.

Figure 1. Gene expression observed in sea bass specimens under normal rearing conditions (T1) and after suboptimal rearing conditions (T2). Lowercase letters stand for significant differences in the one-way ANOVA analyses. In the two-way ANOVA, the sets of letters in a parenthesis correspond to the time (T1/T2) or diet (BP0/BP5/BP10) groups, respectively, and data value corresponding to "A" are lower than those corresponding to "B." Different symbols stand for significant differences among dietary treatments for the same time.

Figure 1


a
Ferritin
Fold change
T1
T2
T P= < 0.0001 (B/A)
D P= 0.6977
TxD P= 0.9649
b
C3
T P= 0.0021 (B/A)
D P= 0.1894
TxD P= 0.3728
c
Hepcdin
T P= 0.1088
D P= 0.4491
TxD P= 0.1653
d
IL-1β
T P= 0.1459
D P= 0.0364
TxD P= 0.0011
e
IL-8
T P= 0.0091 (B/A)
D P= 0.0568
TxD P= 0.0541
f
IL-10
T P= 0.2516
D P= 0.3005
TxD P= 0.4042
g
IFN-I
T P= 0.1963
D P= 0.5219
TxD P= 0.2288
h
Mx
T P= 0.0036 (B/A)
D P= 0.3
TxD P= 0.5821
i
TGF-β
T P= 0.1697
D P= 0.5711
TxD P= 0.8872
BP0
BP5
BP10