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

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Abstract

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

Keywords: Porcine spray-dried plasma, porcine protein hydrolysate, antibacterial activity, antioxidant power, cortisol, nutrition.

48

49 **1. Introduction**

50 Diet formulation is a critical issue in terms of fish performance. Several studies reported that diet promotes
51 and regulates specific metabolic pathways and resistance to environmental stressors and pathogenic
52 organisms; thereby, improving survival, growth, development, health, welfare and reproductive capacity in
53 fish [1,2]. During the lasts years, the feed manufacture industry focused in developing feed formulations
54 that promotes fish health and welfare without compromising somatic growth or product final quality [3].
55 Thus, the development of the so-called functional feeds is nowadays part of the business plan for all
56 aquafeed companies. Moreover, functional feeds aim to reduce the use of antibiotics, which its use as
57 growth promoters was banned in Europe (Regulation (EC) 1831/2003 of the European Parliament and of
58 the Council). A long and growing list of functional additives and ingredients exists in the field of animal
59 production, among which a lot of them have been tested and validated due to their properties in functional
60 feeds for aquatic animals as immunomodulators [4,5]. Their effects may vary among fish species depending
61 on the composition, route, dose and duration of administration, and association/interaction with other feed
62 components [5]. In the current study, we focused in two by-products from the pig industry, such as the
63 porcine spray-dried plasma (SDPP) and pig protein hydrolysate (PPH). SDPP has been reported to promote
64 fish growth due to its high digestibility and the improvement of feed intake and feed efficiency parameters
65 [6,7]. Moreover, SDPP has been recommended for livestock nutrition as a source of immunological support
66 due to its content in immunoglobulins and bioactive peptides [8]. Recently, the effects of SDPP on gilthead
67 sea bream skin mucosa and mucus composition was investigated [9]. We reported that the nutritional
68 stimulus from SDPP supplementation favoured the stimulation of the skin mucosa cell protein turnover and
69 the activation of the exudation machinery, resulting in putatively higher antioxidant and antimicrobial
70 properties. The benefits of the dietary inclusion of fish protein hydrolysates have been extensively studied
71 in several fish species. The benefits of such ingredients have been reported in terms of fish growth, survival,
72 feed utilization, immune response and disease resistance [10]. Regarding the use of livestock by-products
73 like plasma protein hydrolysates as functional feed additives, they have not extensively evaluated in fish
74 [11], regardless their promising properties as functional ingredients due to their content in bioactive
75 peptides with antioxidant and antimicrobial properties [12,13].

76 Studies evaluating functional feeds and their efficacy are long, complex and expensive, especially
77 at the bench level when biological samples have to be processed and analysed. Thus, novel tools are
78 required to preliminarily evaluate the efficiency of functional feeds, and these tools should be user-friendly
79 (*i.e.*, quick, and easy to use). It is well known that feeding regimes and diet composition modulate plasma
80 haematological and biochemical parameters, and these could be used as potential biomarkers for assessing

81 the functional and nutritional status of the organism [14]. In this sense, the analysis of the skin mucus
82 components and their properties could be an alternative reliable methodology, minimally invasive, as it was
83 recently proposed during evaluation of the effects of stress and other physiological responses in fish [15–
84 20]. However, there is limited information about the use of skin mucus biomarkers when evaluating
85 functional diets [21].

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

101 The current study aims to determine the adequacy of skin mucus analyses, as a minimally invasive
102 and injurious sampling procedure, to study the effects of functional diets based on blood porcine by-
103 products on meagre (*Argyrosomus regius*). Meagre is a commercial fish species, with great new importance
104 in the Mediterranean aquaculture, characterised by its fast growth, adaptation to domestication and high
105 tolerance to wide ranges of salinity and temperature [30]. Meagre aquaculture started in the late nineties
106 (30-35 t), from 2008 its production remarkably increase (1,712 t), reaching a maximal production in 2018
107 (7,032 t) [31]. Regardless of this, there is still fragmented information about the nutritional requirements
108 and diet formulation for this species and this issue becomes more evident when dealing with new diet
109 ingredients and functional feeds. Two different functional diets containing porcine by-products were
110 assayed, the porcine spray-dried plasma (SDPP) and pig protein hydrolysate (PPH), for a period of two
111 months. To evaluate the impact of the above-mentioned diets, the skin mucus was collected and analysed
112 for several specific biomarkers such as total soluble protein, glucose and lactate, as main markers of
113 nutritional status and cortisol levels and antioxidant power, as markers of stress. The expected benefits on

114 fish defensive systems were indirectly evaluated by the antibacterial power of the skin mucus via *in vitro*
115 co-culture challenges. Moreover, main digestive enzymes activities, together with filet proximal
116 composition were analysed to determine the effects on digestive function and commercial value of final
117 product, respectively. Our data indicate that the applied skin mucus methodology by testing all those
118 parameters is powerful, rapid and reliable to monitor fish condition and can be used as a preliminary and
119 minimally invasive tool to study functional diets in aquaculture.

120 **2. Material and methods**

121 *2.1. Animals, diets, and experimental procedures*

122 Unvaccinated meagre fingerlings (body weight, BW = 7.4 ± 1.3 g, mean ± standard deviation) were
123 obtained from a commercial hatchery (Piscimar, Andromeda Group, Burriana, Spain) and transported by
124 road to research facilities (IRTA, Sant Carles de la Ràpita, Spain), where they were acclimated in 2 x 2,000-
125 L tanks for two weeks. After acclimation, all fish were anesthetized (tricaine methanesulfonate, MS-222,
126 150 mg · L^{-1}) and individually weighted for initial BW (BWi) and measured for standard length (SLi) to
127 the nearest 0.1 g and 1 mm, respectively. Then, fish were distributed into twelve 500-L cyclindroconical
128 tanks at a density of 50 fish per tank. Experimental tanks were connected to an IRTAmar® water
129 recirculation unit to guarantee adequate water quality through ultraviolet, biological and mechanical
130 filtration.

131 Functional diets consisted of replacing 2% of fishmeal (FM) and 3.4% of fish protein hydrolysate
132 (CPSP 90; Sopropêche, Willime, France) from the control diet (CD) by 5% of spray-dried porcine plasma
133 (SDPP-diet, Appetein™ GS, APC Europe SA, Granollers, Spain) or 5% hydrolysed pig protein (PPH-diet,
134 Pepteiva, APC Europe SA). The level of SDPP and PPH inclusion in experimental diets was chosen
135 according to previous results on gilthead seabream [6]. The main ingredients and proximate composition
136 of experimental diets are shown in Table 1. Diets were manufactured by Sparos Lda. (Olhão, Portugal) as
137 described in Gisbert et al. (2015) [6]. Each diet was assayed in four replicate-tanks during a period of 68
138 days. Fish were fed four times per day (08:00, 11:00, 14:00 and 17:00 h) over the experimental period with
139 automated feeders (ARVO-TEC T Drum 2000™, Arvotec, Finland) at a 4.5% feeding rate of the stocked
140 biomass, which approached apparent satiation. During this time, water quality parameters were: 23.1 ± 1.1
141 °C, 7.5 ± 0.2 ppm of dissolved oxygen (OXI330, Crison Instruments, Barcelona, Spain) and pH values of
142 7.5 ± 0.1 (pH meter 507, Crison Instruments). Water flow rate in the experimental tanks was maintained at
143 approximately 9.0 L min^{-1} via recirculation that maintained adequate water quality (total ammonia and

144 nitrite were ≤ 0.10 and 0.4 mg L^{-1}, respectively). No signs of bacterial infections were found along the trial,
145 and the overall health condition of animals was considered as good.

146

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

	Experimental diets		
Ingredients (%)	**CD**	**SDPP**	**PPH**
Fishmeal LT70 (Norvik)	35.00	33.20	33.65
CPSP90	3.40		
Appetein GS (APC) *		5.00	
Pepteiva (APC) **			5.00
Soy protein concentrate (Soycomil)	10.00	10.00	10.00
Wheat gluten	6.60	6.60	6.60
Corn gluten	8.00	8.00	8.00
Soybean meal 48	5.00	5.00	5.00
Rapeseed meal	4.50	4.50	4.50
Wheat meal	13.63	13.48	13.08
Sardine oil – Sopropeche	12.35	12.70	12.65
Vitamin & Mineral Premix INVIVO 1%	1.00	1.00	1.00
Antioxidant powder (VERDILOX)	0.20	0.20	0.20
Sodium propionate	0.10	0.10	0.10
L-Taurine	0.20	0.20	0.20
Total	**100.00**	**100.00**	**100.00**

Proximate composition	**CD**	**SDPP**	**PPH**
Crude protein, % feed	50.42	50.37	50.35
Crude fat, % feed	16.24	16.18	16.17
Ash, % feed	8.00	7.90	8.20
Gross Energy, MJ/kg feed	22.10	22.10	22.10

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

147

148 The IRTA facilities are certified and have the required authorisation for the breeding and husbandry
149 of animals for scientific purposes. All procedures involving the handling and treatment of the fish were
150 approved as far as the care and use of experimental animals are concerned, by the European Union
151 (86/609/EU), the Spanish Government (RD 1201/2005) and the University of Barcelona (Spain).

152 *2.2. Sample collection*

153 At the end of the experimental period, all animals in each tank were measured in BW and SL, and condition
154 factor were calculated as well as the daily weight gain, specific growth rate (SGR) and Fulton's condition
155 factor (K). The former parameters were measured using the following formulae: Specific growth rate
156 (SGR; % BW day^{-1}) = (ln BWf– ln BWi) x 100 / days; Fulton's condition factor (K) = (BWf / SL^{3}f) x 100,
157 where BWf and BWi were the final and initial BW in grams, and SLf the final SL in cm. Fish survival was
158 also recorded. Twenty animals were randomly sampled for each dietary condition (5 per tank) and slightly
159 anaesthetized with tricaine methane sulfonate (MS-222, 150 mg · L^{-1}). Skin mucus was individually and
160 minimally-invasively collected as described in Fernandez-Alacid et al. (2018) [15]. Briefly, skin mucus
161 was collected in a very fast process (less than 2 min) using sterile glass slides from the over-lateral line in
162 a front to caudal direction and the epidermal mucus was carefully pushed and collected in a sterile tube (2
163 mL), avoiding the contamination with blood and/or urine-genital and intestinal excretions. Mucus samples
164 were homogenised using a sterile Teflon implement to desegregate mucus mesh before centrifugation at
165 14,000 x *g* for 15 min at 4 ºC. The resultant skin mucus supernatants were collected, avoiding the surface
166 lipid layer, aliquoted and stored at -80 ºC for further analyses. Sampled fish were subsequently sacrificed
167 with an overdose of MS-222. Muscle and liver samples and their digestive tract were individually collected,
168 immediately frozen in liquid nitrogen and stored at -80 ºC until further analyses.

169 *2.3. Mucus biomarkers*

170 The soluble protein concentration of homogenized skin mucus was determined using the Bradford assay
171 [32] with bovine serum albumin (BSA; Sigma-Aldrich, Madrid, Spain) as a standard. The optical density
172 (OD) was determined at λ = 596 nm. Protein values were expressed as mg mL^{-1} of skin mucus. Glucose
173 and lactate concentrations of homogenized skin mucus were determined by the respective enzymatic
174 colorimetric tests (SPINREACT®, Barcelona, Spain) following the manufacturer's instructions for plasma
175 determinations, but with slight modifications [15]. The OD was determined at λ = 505 nm. The glucose and
176 lactate values were expressed as μg mL^{-1} of skin mucus and as μg mg^{-1} of mucus protein.

177 Skin mucus cortisol levels were measured using an ELISA kit RE52611 (IBL International,
178 Germany) with lower cross-reactivity with other corticosteroid compounds in fish. The methodology of the
179 kit was previously adapted for fish skin mucus samples [15]. Briefly, 50 μL of mucus extract or standard
180 solutions was mixed with enzyme conjugate (100 μL) and incubated for 2 h at room temperature. After
181 rinsing the wells with a wash solution, the substrate solution (100 μL) was added and incubated for 30 min.

182 The reaction was stopped by adding 100 µL of stop solution and the OD was determined at λ = 450 nm.
183 The cortisol values were expressed as ng mL^{-1} of skin mucus and ng · g^{-1} of mucus protein.

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

192 *2.4. Evaluation of skin mucus antibacterial activity by co-culture challenges*

193 The study of mucus antibacterial activity was performed using three different bacteria: a non-pathogenic
194 bacterium for fish, *Escherichia coli* (DSMZ423), and two pathogenic bacteria for fish marine species,
195 *Vibrio anguillarum* (CECT522T) and *Pseudomonas anguilliseptica* (CECT899T). *E. coli* were grown in
196 Tryptic Soy Broth culture media (TSB, Conda, Spain), while *V. anguillarum* and *P. anguilliseptica* were
197 grown in Marine Broth culture media (MB, Difco Laboratories, Detroit). The effect of skin mucus on
198 bacterial viability was determined by monitoring the absorbance of bacterial cultures grown in flat-
199 bottomed 96-well plates. For the co-culture challenge, each well was loaded with 50 µl of bacterial
200 suspension (optical density, OD = 0.2) in the appropriate culture media (1X) plus 100 µL of skin mucus (2
201 µg μL^{-1} of mucus protein) and 50 µL of culture media (3X) to obtain a 200 µL final volume. Controls
202 without bacterial suspension were prepared by adding 100 µL of culture media (2X) and 100 µL of skin
203 mucus (2µg μL^{-1} of mucus protein). Bacterial growth without mucus (control) were prepared by adding
204 50 µL of bacterial suspension (OD = 0.2) in culture media (1X) and 150 µL of culture media (1X). Blanks
205 (control bacterial growth without bacteria and mucus) were prepared by adding 200 µL culture media (1X).
206 The absorbance of the bacteria was measured at λ = 400 nm every 30 min for 14 h at 25 °C in flat-bottomed
207 96-well plates. Average absorbance of controls without bacteria [skin mucus at 2µg μL^{-1} of protein (100
208 µL) plus 100 µL of medium] was subtracted from the absorbance from co-culture (bacteria plus skin mucus)
209 samples. All assays were done in triplicate (methodological replicates). Data are presented as growth curves
210 (increased absorbance at λ = 400 nm per unit of time) and as percentage of inhibition with respect to
211 bacterial growth for each two hours of co-culture.

212 *2.5 Proximal composition of the muscle*

213 Pools of five individual meagre muscle samples per tank were homogenized, and small aliquots were dried
214 (120 °C for 24 h) to estimate their water content. The total fat content from feed and fish tissues was
215 gravimetrically quantified after fat extraction in a chloroform-methanol solution (2:1) and evaporation of
216 the solvent under a stream of nitrogen followed by vacuum desiccation overnight [33]. Protein and
217 carbohydrate contents were determined according to Lowry et al. (1951) [34] and Dubois et al. (n.d) [35],
218 respectively. Ash contents were determined by keeping the sample at 500 to 600 °C for 6 h in a muffle
219 furnace [36]. All chemical analyses were performed in triplicate per pooled fish and feed samples.

220 *2.6 Activity of digestive and antioxidative stress enzymes*

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

229 Total alkaline protease activity was measured using azocasein (0.5%) as substrate in Tris-HCl 50
230 nmol L^{-1} (pH = 9). One unit (U) of activity was defined as the nmoles of azo dye released per minute and
231 per mL of tissue homogenate (λ = 366 nm) [39] Trypsin activity was assayed using BAPNA (N-benzoyl-
232 DL-arginine p-nitroanilide) as substrate; one unit of trypsin per mL (U) was defined as 1 µmol BAPNA
233 hydrolyzed min^{-1} mL^{-1} of enzyme extract (λ = 407 nm) [40]. Alpha-amylase activity was determined using
234 0.3% soluble starch as substrate, and its activity (U) was defined as the amount of starch (mg) hydrolyzed
235 during 30 min per mL of homogenate (λ = 580 nm) [41]. Bile salt-activated lipase activity was assayed for
236 30 min using p-nitrophenyl myristate as substrate; and its activity (U) was defined as the amount (nmol) of
237 substrate hydrolysed per min per mL of enzyme extract (λ = 405 nm) [42]. Pepsin was quantified using 2%
238 hemoglobin as substrate in 1 N HCl buffer as substrate, and its activity (U) defined as the nmol of tyrosine
239 liberated per min per mL of tissue homogenate (λ = 280 nm) [43]. Alkaline phosphatase was quantified
240 using 4-nitrophenyl phosphate (PNPP) as substrate. One unit (U) was defined as 1 µmol of pNP released
241 min^{-1} mL^{-1} of brush border homogenate at λ = 407 nm [44]. All digestive enzyme activities were measured
242 at 23 °C and expressed as specific activity defined as units per mg of protein (U mg $protein^{-1}$).

243 Quantification of lipid peroxidation in the intestine and liver was conducted using the
244 thiobarbituric acid reactive substances method described by Solé et al. (2004) [45]. In brief, lipid
245 peroxidation was measured using 200 μL of the homogenate mixed with 650 μL of methanol and 1-methyl-
246 2-phenylindole (10.3 mM) in acetonitrile:methanol (1:3; vol/vol) and 150 μL of 37% HCl. This mixture
247 was incubated for 40 min at 45 °C, cooled on ice for 10 min, and centrifuged at 21,000 × *g* for 10 min at 4
248 ºC to remove protein precipitates. Absorbance was read at $\lambda = 586$ nm, and the amount of peroxidized lipids
249 (in nmol malondialdehyde/100 g tissue; wt/wt) was evaluated by means of a calibration curve made of a
250 standard solution of 1,1,3,3-tetramethoxypropane (10 mM). Homogenized samples, prepared for the
251 determination of the levels of lipid peroxidation, were also used to measure antioxidant enzyme activities.
252 Catalase activity was measured in sampled tissues by the decrease in absorbance at $\lambda = 240$ nm (extinction
253 coefficient, e = 40 M^{-1} cm^{-1}) using 50 mM H_2O_2 as substrate [46]. Glutathione S-transferase (GST) activity
254 was assayed by the formation of glutathione chlorodinitrobenzene adduct at $\lambda = 340$ nm (e = 9.6 40 M^{-1}
255 cm^{-1}), using 1 mM 1-chloro-2,4-dinitrobenzene, and 1 mM glutathione as substrates [47]. Glutathione
256 reductase (GR) activity was determined by measuring the oxidation of nicotinamide adenine dinucleotide
257 phosphate reduced (NADPH) at $\lambda = 340$ nm (e = 6.22 mM^{-1} cm^{-1}), using 20 mM glutathione disulphide
258 and 2 mM NADPH as substrates [48]. Total glutathione peroxidase (GPX) was determined by measuring
259 the consumption of NADPH at $\lambda = 340$ nm (e = 6.22 mM^{-1} cm^{-1}), using 75 mM glutathione and 8.75 mM
260 NADPH as substrates [49]. Oxidative stress enzyme activities were expressed as specific enzyme activities
261 (nmol min $^{-1}$ mg protein^{-1}). Soluble protein of crude enzyme extracts was quantified by means of the
262 Bradford's method [32] using bovine serum albumin as standard. All the assays for evaluating the activity
263 of digestive and antioxidative stress enzymes were made in triplicate (methodological replicates) for each
264 tank and the absorbance was read using a spectrophotometer (Tecan™ Infinite M200, Männedorf,
265 Switzerland).

266 *2.7. Statistical analysis*

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

273

274 **3. Results**

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

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

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

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

279

280 Furthermore, the inclusion of SDPP or PPH in meagre diets slightly increased the protein content (1.5%
281 and 1.7%, respectively) and reduced the lipid levels (2.4 and 2.2%, respectively) in muscle (Table 3; $P <$
282 0.05).

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

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

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

283

284 Experimental diets did not alter the specific activity of gastric (pepsin) and pancreatic (trypsin, total alkaline
285 proteases, bile salt-activated lipase and α-amylase) digestive enzymes, nor that of alkaline phosphatase from
286 the intestine (Table 4; $P > 0.05$).
287

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

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

Values are mean ± standard error of mean.

288 No statistically significant differences were found regarding the levels of lipid peroxidation (TBARS) in
289 the liver nor in the activity of selected (SOD, CAT, GR, GST and GPX) oxidative stress enzymes (Table
290 5; $P > 0.05$).

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

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

Values are mean ± standard error of mean.

291

292 Results from the main skin mucus biomarkers and ratios as well as the mucus antioxidant power values are
293 shown in Table 6. The replacement of 5% of FM and fish product hydrolysed by porcine by-products SDPP
294 or PPH did not modify skin mucus composition in terms of glucose (ranging 15-20 µg mL^{-1}), lactate (6-8
295 µg mL^{-1}), soluble protein (2-4 mg mL^{-1}), cortisol (0.1-0.3 ng mL^{-1}) and antioxidant power (190-230 µmols
296 mL^{-1}) contents ($P > 0.05$). Furthermore, the calculated ratios of these parameters with respect to protein
297 values are also provided to avoid possible mucus dilution or concentration effects due to mucus collection.
298 As no significant changes in the amount of soluble protein content was detected between treatments,

299 different ratios of glucose/protein, lactate/protein or cortisol/protein led to the same results than the
300 respective levels of these biomarkers (*P* > 0.05). Finally, the metabolic aerobic response measured in mucus
301 as glucose/lactate ratio was not modified regardless of the dietary condition (*P* > 0.05).

302

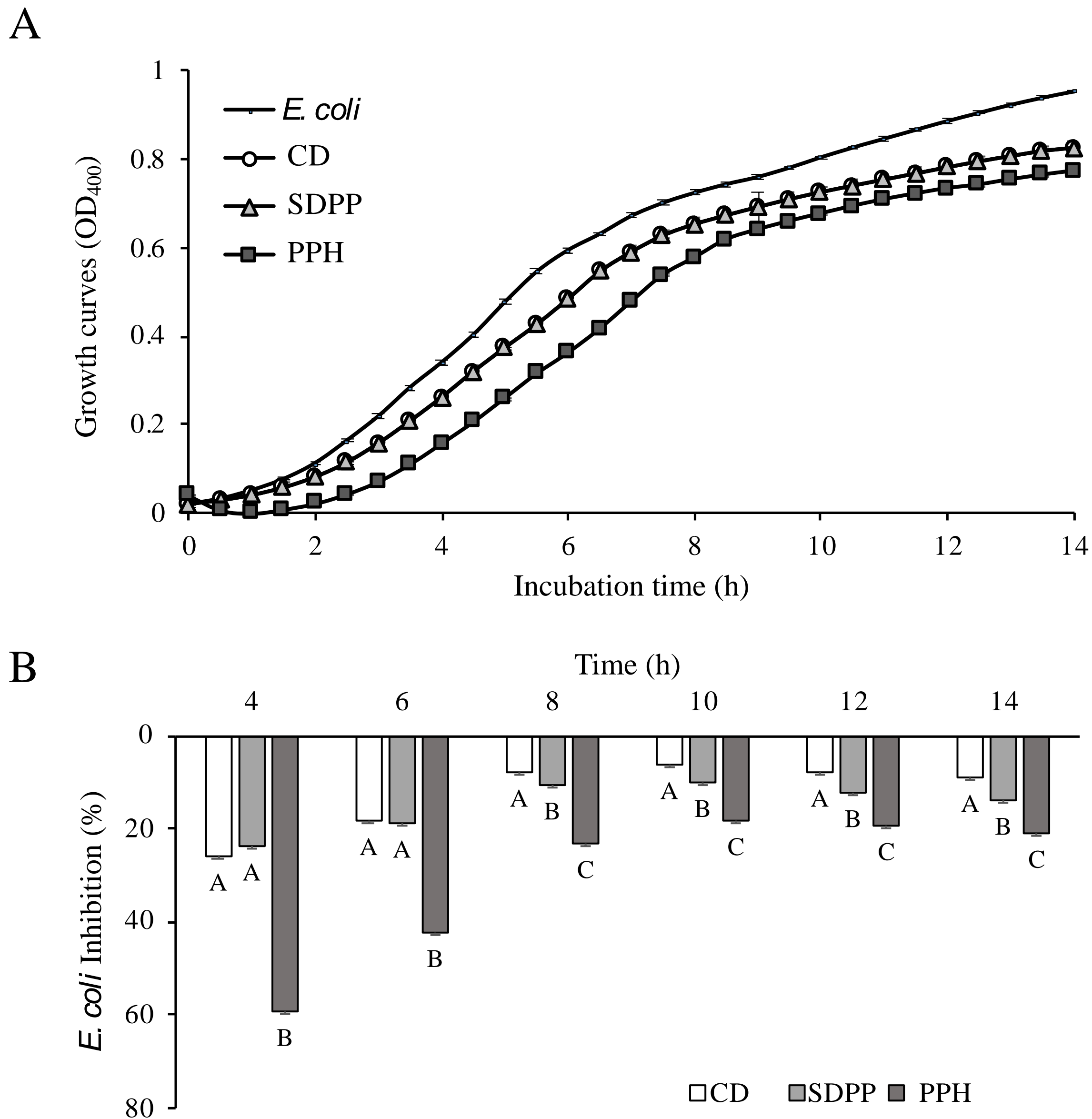
Table 6. Main biomarkers and ratios of skin mucus of meagre fed two functional diets, containing 5% porcine spray-dried plasma (SDPP) and pig protein hydrolysate (PPH), over nine weeks.

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

Values are mean ± standard error of mean.

303

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

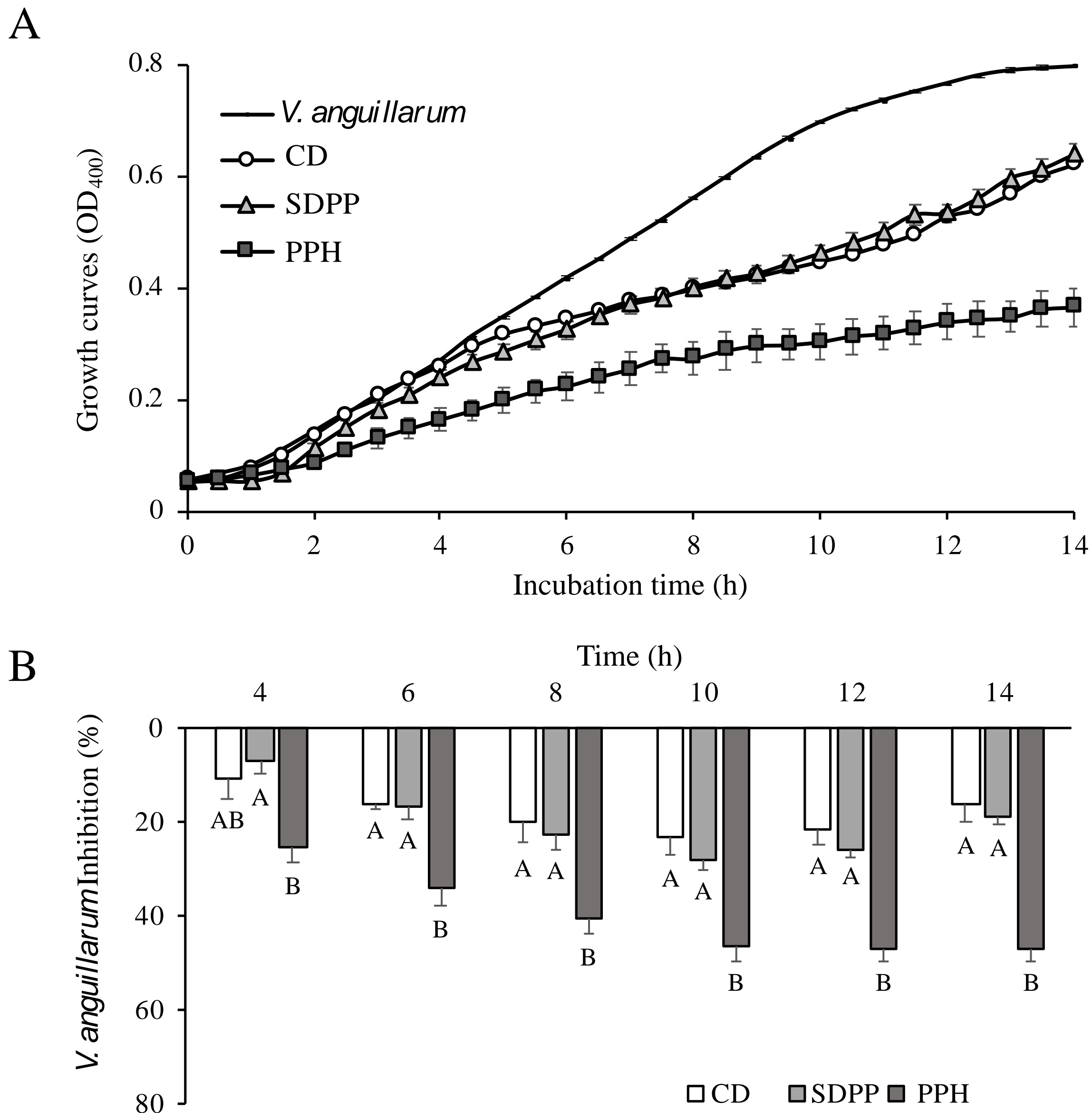


317

318 **Figure 1**. Bacterial growth (A) and inhibition rate (B) against *Escherichia coli* of meagre skin mucus fed
319 with the indicated diets. Different letters indicate significant differences among diets within each time ($P <$
320 0.05, one-way ANOVA).

321 The antibacterial activity of skin mucus against marine pathogenic bacteria *V. anguillarum* and *P.*
322 *anguilliseptica* is presented in Figures 2 and 3, respectively. Contrary to *E. coli* co-culture, meagre skin
323 mucus co-cultured with *V. anguillarum* showed the minimum inhibition capacity the first 6 h and maximum
324 inhibitory growth values between the 8-12 h period (from 15% at 4 h to 25% at 10 h). The antibacterial

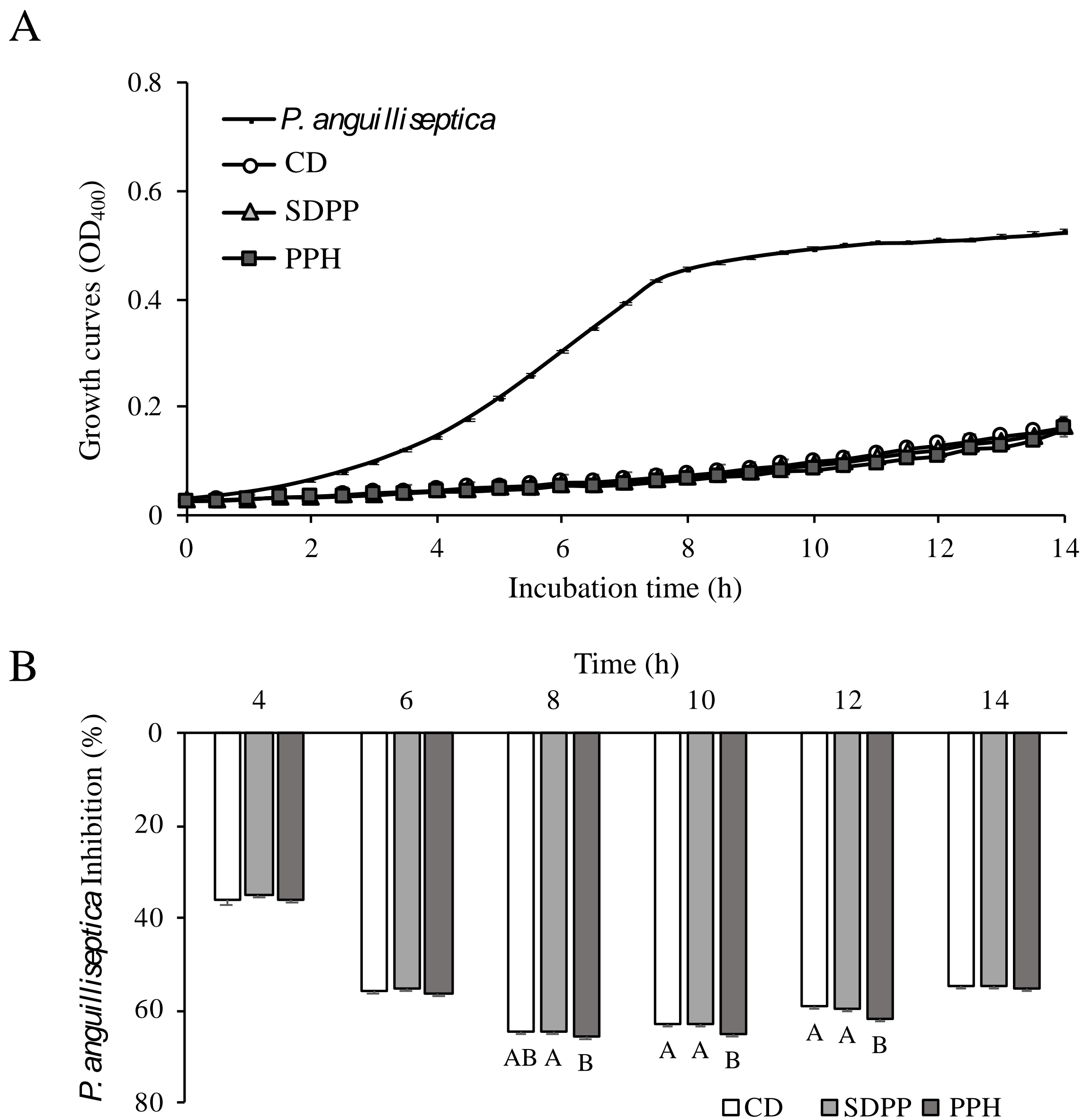
325 activity of skin mucus from meagre juveniles fed the 5% SDPP experimental diet did not significantly differ
326 from that of the control diet (Figs. 2A and 2B, $P > 0.05$ for all time values). However, the inclusion of PPH
327 in the diet significantly enhanced the inhibitory activity of skin mucus against *V. anguillarum* (Fig. 2A,
328 2B). The inhibitory dynamics of this co-culture evidenced a two-fold higher inhibitory capacity than both
329 control and SDPP diets through all the time-course. The maximal inhibitory capacity of meagre skin mucus
330 fed the PPH diet against *V. anguillarum* was recorded during the 10-14 h interval.



331

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

335 With regard to *P. anguilliseptica,* the co-cultures with skin mucus from the different experimental
336 diets showed a great capacity to inhibit bacterial growth, already at initial period (over 30%) and increased
337 during the rest of co-culture (Fig. 3A and Fig. 3B). Even considering the great inhibitory potential of the
338 fish mucus against *P. anguilliseptica*, PPH diet exhibited slight but significant higher inhibitory capacity
339 than the other two diets for the interval of 10-12 h ($P < 0.05$).



340

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

344 **4. Discussion**

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

357 In the current experiment, functional diets were formulated by replacing 5% of FM and fish product
358 hydrolysed by two functional ingredients obtained from the rendering industry: the porcine spray-dried
359 plasma, SDPP, and the pig protein hydrolysed, PPH. These hemoderivates are abattoir by-products obtained
360 from animal blood, which have been reported to promote feed intake, somatic growth, improve FCR and/or
361 reduce stress in several livestock [8,52] and aquaculture species [6,7,9]. Surprisingly, under present
362 experimental conditions the replacement of low levels of FM (2%) and the absence of fish protein
363 hydrolysate (CPSP90) in meagre experimental diets resulted in the reduction of 8.8 and 7.1% in somatic
364 growth in fish fed SDPP and PPH diets, respectively. This reduction in BW was also coupled with a slight
365 reduction in daily weight gain, a slight increase in FCR and decrease in feed intake values. Additionally,
366 the above-mentioned changes in growth and feed efficiency parameters were accompanied by changes in
367 muscle composition with increased protein content and decreased lipid levels. The results from the current
368 study in meagre were not in agreement with previous studies which reported that SDPP inclusion in diet
369 enhanced growth performance in several fish species, such as rainbow trout [53], gilthead sea bream [6,9]
370 and Nile tilapia [7]. Such findings in terms of somatic growth and muscle proximate composition may be
371 attributed to a reduction in the content of FM and CPSP90 in isoproteic experimental diets, which has
372 traditionally been used as the main protein source in aquafeeds. In particular, FM is not only nutritionally

373 reputed by its excellent amino acid profile, palatability and digestibility, but also because it is a source of
374 nucleotides, essential fatty acids, phospholipids, minerals and lipid- and water-soluble vitamins [54].
375 Furthermore, reductions in FI may also be attributed to changes in diet palatability and changes in dietary
376 amino acids known as feed attractants (alanine, glycine, isoleucine, histidine, leucine, and proline) [55].
377 Considering these unexpected results further research is needed to provide insights on how a small reduction
378 in FM resulted in a significant reduction of the performance indicators. A limited knowledge on the
379 nutritional requirements of the meagre exists [56] and further studies should be focused on growth
380 parameters, for instance studying amino acid profile requirements or the key growth signalling pathways.

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

390 In order to evaluate the tested functional diets, we proposed to study meagre's skin mucus biomarkers
391 and its antibacterial activity as a tool to monitor in a preliminary and minimally invasive way the welfare
392 of fish. Recently, different plasma metabolites considered as biomarkers of the fish physiological condition
393 (i.e., glucose, lactate, cortisol, and total protein levels) have been correlated with their contents in the skin
394 mucus levels in different freshwater [27] and marine species [16,17,20,29]. These classic biomarkers are
395 rapid and easy to be measured, especially in skin mucus, providing valuable information on the fish
396 response to different biotic and abiotic stressors [15]. Under present experimental conditions, meagre skin
397 mucus content on glucose, lactate or soluble protein from different dietary groups were within the range of
398 values previously reported for this species [15,16,21] and it was not affected by the porcine-derived
399 ingredient included in the diet. Although data describing the effects of the nutritional status on mucus
400 metabolites are scarce, the maintenance of these soluble components in the mucus seems to be important
401 for the adequate functionality of the skin mucus layer [15,18,20,26]. Beside the main mucus metabolites,
402 stress markers like lactate and cortisol levels are also of major interest in cultured fishes. Cortisol is secreted
403 and released in responses to stress, although the levels strongly vary within species according to the duration
404 or severity of the stressor [58]. Skin mucus cortisol have been detected in several fish species [29,59], and
405 the correlation between mucus cortisol and plasma cortisol has been recently confirmed in meagre [16].

406 However, the mechanisms involved in cortisol exudation in skin mucus are still unknown in fish. From our
407 best knowledge, only one study evaluated mucus cortisol levels in meagre under a sustained dietary
408 modified condition [21]. The former authors related lower levels of mucus cortisol to stress-attenuated role
409 of tryptophan addition to diet. As it could be expected, in the current study, experimental diets assayed did
410 not modify the skin mucus cortisol levels or the lactate levels, indicating that the dietary modifications did
411 not impair the stress condition of animals. Skin mucus antioxidant capacity was also measured using FRAP
412 analysis [19,27,60]. In fish plasma and tissues, FRAP levels have been considered as a key mechanism in
413 the epidermis response to oxidative stress [61,62]. The FRAP levels obtained were within the range of
414 values reported for this species [19], although no changes of the mucus antioxidant power were observed
415 for both tested experimental diets. Dietary plasma porcine products were reported to enhance the
416 antioxidant capacity in the intestine of gilthead sea bream [6]. However, this characteristic was not
417 evidenced from our mucus FRAP's analyses in the mucus, similarly to other studies testing phytogenic in
418 this species [60]. In addition, under current experimental conditions no differences in the levels of lipid
419 peroxidation and activity of antioxidative stress enzymes in the liver were found between experimental
420 diets. The divergence between current results in meagre and those previously reported from the gut of
421 gilthead seabream [6] need to be further explored to get insights into the potentially different mode of action
422 of the tested functional ingredients on different species as well as in different mucosal tissues that may
423 differently respond to dietary ingredients.

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

439 Depending on the ingredient considered, the inclusion of SDPP or PPH in meagre diets resulted in
440 different results in terms of the antibacterial activity of skin mucus. Interestingly, the antibacterial activity
441 of the meagre skin mucus also differed depending on the pathogenic bacteria considered. In particular, skin
442 mucus from PPH-fed fish caused a greater inhibition of *V. anguillarum* in comparison to control and SDPP
443 dietary groups. Vibriosis generally affects cultured fish recurrently, and *V. anguillarum* has been
444 demonstrated to be a responsible agent for chronic pathogenic outbreaks in meagre among other farmed
445 species. Moreover, high incidences of vibriosis can occur in hatchery and pre-on-growing facilities, as
446 juveniles are more sensitive to this infectious disease; thus, the benefits of the application of a preventive
447 nutritional strategy targeting for the improvement of the mucus antibacterial activity may be advisable and
448 alienated with the strategy of reducing antimicrobial agents use. Our results seemed to indicate that the
449 hydrolysed form of porcine hemoderivates seemed to be more effective than the atomized form of plasma,
450 which may be attributed to their different composition of bioactive compounds. In particular, SDPP is an
451 ingredient with highly digestible proteins and amino acids, and significant concentrations of functional
452 bioactive components including immunoglobulins, transferrin, growth factors, peptides, and other
453 biologically active components [65]. Protein hydrolysates also includes biologically active peptides with
454 immune-stimulating and antibacterial properties produced during the hydrolysing procedure, regardless of
455 the aquatic [10] or terrestrial [12] origin considered. In this sense, it has been reported that the
456 hydrophobicity of peptides has been related to their antimicrobial activity, as higher hydrophobicity is
457 useful in the binding of lipopolysaccharides on the outer cell membrane of the bacteria [12]. Furthermore,
458 the characterization of the antibacterial activity of meagre skin mucus against *P. anguilliseptica* was
459 previously described in Sanahuja et al. (2019) [19]. In the present study, we showed that meagre skin mucus
460 was able to strongly inhibit the bacterial growth of *P. anguilliseptica* regardless of the experimental group
461 considered, although this antibacterial activity was slightly enhanced by the administration of the PPH diet.
462 *P. anguilliseptica* is considered an opportunistic pathogen whose infections occur when fish immune
463 system is depressed, mainly at low water temperatures [70]. Different results on the antibacterial capacity
464 of meagre skin mucus against *V. anguillarum* and *P. anguilliseptica* may be also attributed to different
465 bacteria virulence, differences in chemotaxis to skin mucus and their adherence capacity [26]. As fish
466 condition during the current experiment was optimal, the real benefits of both porcine by-products against
467 *P. anguilliseptica* could be masked by the unaltered mucus composition from external or internal stressors.
468 Finally, the non-pathogenic fish bacterium *E. coli* was also used as an indicator of the potential antibacterial
469 capacity of the skin mucus, neglecting a potentially acquired immunization against fish-specific pathogens
470 [19,23]. Similarly, to *V. anguillarum*, the PPH diet enhanced the inhibitory capacity of meagre skin mucus
471 against *E. coli* growth when compared to SDPP. It is also interestingly to mention that the evaluation of
472 mucus antibacterial activity using growth curves was time-dependent [19]. Thus, the maximum inhibitory

473 activity was detected at different intervals depending on the bacteria considered. In particular, this
474 maximum inhibitory activity was found between 4 and 8h of co-culture for *E. coli*, at the 10-14h interval
475 for *V. anguillarum* and at the 8-12h interval for *P. anguilliseptica*, irrespective of the diet administered.
476 These results revealed the relevance of skin mucus renewal process and the importance of the mucus
477 components exudation processes to guarantee the antibacterial mucus capacity over time [24]. Regarding
478 the observed differences in the antibacterial capacities of skin mucus between meagre fed diets containing
479 PPH and SDPP, it could be speculated that the tested hemoderivates have a different way of acting on the
480 skin-associated lymphoid tissue, as they have a different content in immunoglobulins (SDPP = 270 g/kg;
481 data provided by the manufacturer). Further studies are necessary to elucidate how the antibacterial capacity
482 is improved beyond the study of the current mucus biomarkers such as cortisol, glucose, lactate or soluble
483 proteins. Skin mucus is biochemically complex [71,72], integrating a wide variety of biological processes
484 of relevance to fish health. Thus, the activities of these pig hemo-compounds as innate humoral parameters
485 inductors in skin mucus should be deeper studied to better understand the bactericidal enhancement of the
486 skin mucosa and even its extrapolation to other fish mucosas.

487 **5. Conclusions**

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

506

507

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512 **Author statement**

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

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