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Diets containing shrimp protein hydrolysates provided protection to European sea bass (*Dicentrarchus labrax*) affected by a *Vibrio pelagius* natural infection outbreak

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21

22 **Abstract**

23 This study was conducted to investigate the effects of the dietary supplementation of
24 shrimp protein hydrolysate (SPH) on somatic growth performance, innate immune
25 response in juvenile European sea bass (*Dicentrarchus labrax*) and their differential
26 cumulative mortality when affected by a *Vibrio pelagius* natural infection outbreak. A
27 control diet containing 20% fish meal (FM) was used as a control, whereas three other diets
28 differing in the level of FM inclusion (75 and 25% FM replacement by plant protein sources)
29 and the inclusion of the additive (5% FM, 5% FM + 5% SPH and 15% FM + 5% SPH) were
30 tested. After 110 days, there were no statistically significant differences in somatic growth
31 parameters nor proximate composition in fish fed different experimental diets ($P > 0.05$),
32 while the humoral non-specific immune responses (lysozyme, bacteriolytic and
33 complement activities) were significantly enhanced by the inclusion of SPH in diets ($P <$
34 0.05). Additionally, an outbreak of the pathogenic bacteria *V. pelagius*, a bacterial species
35 previously described as producer of the virulence factor hemolysin, occurred in all
36 experimental tanks (4 replicates per diet) due to crowding and repeated handling stress for
37 fish sorting. Survival rates among different experimental groups ten days after the bacterial
38 epizootic differed depending on the diets, with groups containing SPH showing the best
39 results ($P < 0.05$). In particular, fish fed the 15% FM + 5% SPH diet showed the highest
40 survival rate (96.4 ± 5.0%), followed by those fed the 5% FM5 + 5% SPH5 (61.8 ± 16.3%). In
41 contrast, survival rates in fish fed diets deprived of the additive (20% FM and 5% FM5 diets)
42 were the lowest ones (32.0 ± 6.7% and 38.2 ± 13.5%, respectively). The present study
43 showed that SPH can be incorporated in aquafeeds with high levels of FM substitution by

44 PP sources without detrimental impact on the somatic growth performance of fish. In
45 addition, the non-specific humoral immunity in seabass and their survival when affected by
46 an epizootic outbreak of *V. pelagius* were positively affected, which showed the
47 immunomodulatory benefits of shrimp hydrolysate to promote health and prevent
48 diseases in fish.

49

50 *Keywords*: feed additive, fishmeal substitution, shrimp protein hydrolysate, non-specific
51 serological immune parameters, natural infection outbreak, functional feed.

52

53 1. Introduction

54 During the last decade, there has been an increasing interest in the role of bioactive
55 peptides in animal nutrition. Addition of animal by-products or plant-source feedstuffs,
56 modified through chemical, enzymatic, or microbial hydrolysis of proteins, prior to feeding
57 is an attractive means of generating high-quality small or large peptides that have both
58 nutritional and physiological or regulatory functions in livestock, poultry and fish (Hou et
59 al., 2017). Peptides of plant or animal sources have shown antimicrobial, antioxidant,
60 antihypertensive, and immunomodulatory activities beyond their nutritional value
61 (Martínez-Alvarez et al., 2015; Hou et al., 2017). Within this context, by-products from both
62 the fishery and aquaculture industries are considered as a potential source of raw materials
63 to produce sustainable fishmeal (Hardy et al., 2005; Hsu, 2010; Lee et al., 2010) and protein
64 hydrolysates (Robert et al., 2015; Hou et al., 2017). The performance of protein
65 hydrolysates could be highly dependent on the way the raw materials were hydrolyzed

66 resulting from the methods and the equipment used for their production. These
67 procedures may alter factors such as the nutritional and functional properties of protein
68 hydrolysates that are closely related to their specifications, which are represented by the
69 abundance and diversity of different oligo-peptides, as well as the peptides molecular
70 weight (Liaset et al., 2000; Robert et al., 2015). In particular, protein hydrolysates in
71 aquafeeds have been reported to increase feed intake, feed utilization and somatic growth
72 (Refstie et al., 2004; Aksnes et al., 2006; Zheng et al., 2011, 2013; Khosravi et al., 2015), as
73 well as to promote the immune system (Kotzamanis et al., 2007; Ovissipour et al., 2014;
74 Khosravi et al., 2015 among others), and enhance the harmonious development of the
75 skeleton and digestive systems in fish larvae (Cahu et al., 1999; Gisbert et al., 2012; Delcroix
76 et al., 2014; Johannsdottir et al., 2014).

77 In this study, authors described the effects of diets containing different levels of
78 fishmeal and shrimp protein hydrolysate (SPH) on growth performance, feed efficiency,
79 non-specific serological immune parameters and their response to an outbreak of the
80 pathogenic bacteria *Vibrio pelagius*, a bacterial species previously described as producer of
81 the virulence factor haemolysin (Zhang and Austin, 2005).

82

83 **2. Material and Methods**

84 *2.1 Fish and experimental diets*

85 European sea bass fingerlings were obtained from a fish farm (Piscicultura Marina
86 Mediterránea SL, Spain), transported by road to the IRTA-SCR facilities and acclimated for
87 14 days to new husbandry and water conditions in a 2 m^3 circular fiberglass tank. During

88 this period, fish were fed twice a day with EFICO YM (BIOMAR, Spain) at 2% of the stocked
89 biomass. Before the onset of the trial, all fish were anaesthetized (tricaine
90 methanesulfonate, MS-222, 150 mg/L), individually weighed for body weight (BW) and
91 measured for standard length (SL) to the nearest 0.1 g and 1 mm, respectively; and then
92 distributed into sixteen fiberglass cylindrical tanks of 450 liters (sixty fish per tank, BW_i =
93 19.8 ± 1.3 g, mean ± standard deviation).

94 Four isonitrogenous (crude protein: 45%) and isolipidic (crude fat: 16%) diets were
95 formulated in order to obtain graded levels of fishmeal (FM) inclusion (FM: 5, 15 and 20%)
96 and supplemented with SPH (Actipal HP1, Diana Aqua, Symrise AG, Elven, France).
97 Experimental diets were as follows: FM20 (positive control), FM5 (no SPH added),
98 FM5+SPH5 (5% SPH) and FM15+SPH5 (5% SPH). All the diets were balanced for deficient
99 amino acids according to the requirements determined for European sea bass (Wilson,
100 2002). The SPH was produced from the cephalothorax of white shrimp (*Litopenaeus*
101 *vannamei*) (Table 1). Diet manufacturing was contracted to a technical centre (Tech Centre
102 Biomar, Brande, Denmark). Information about feed ingredients and diet proximate
103 composition is shown in Table 2.

104

105 *2.2 Rearing conditions, growth performance and non-specific hematological immune*
106 *parameters*

107 During the trial, water temperature and pH (pH meter 507; Crison Instruments), salinity
108 (MASTER-20T; ATAGO Co. Ltd) and dissolved O_2 (OXI330; Crison Instruments) were 20.2 ±
109 0.2 °C, 7.7 ± 0.2, 36 mg/L and 6.9 ± 0.2 mg/L, respectively. Water flow rate in experimental

110 tanks was kept at *ca*. 9.0 L/min via a recirculation system (IRTAmar®) that maintained
111 adequate water quality (total ammonia and nitrite were ≤0.15 and 0.6 mg/L, respectively)
112 through UV, biological and mechanical filtration. Photoperiod followed natural changes
113 according to the season of the year (January-March; latitude 40°37'41'N). Each diet was
114 tested with four replicates for 110 days. Diets were distributed eight times per day by
115 automatic feeders (ARVO-TEC T Drum 2000; Arvotec, Finland) at the daily rate of 3.0 % of
116 the stocked biomass, which approached apparent satiation.

117 Sampling to monitor fish growth took place monthly from the onset of the feeding
118 period in order to adjust feeding rate and evaluate somatic growth performance. For that
119 purpose, all fish in each tank were netted, anaesthetized and their wet BW and standard
120 length (SL, cm) determined. Fish growth and feed utilization from different experimental
121 groups were evaluated by means of the following indices: Fulton's condition factor (K) =
122 $(BW/SL^3) \times 100$; specific growth rate in BW (SGR_{BW}, %) = $((\ln BWf - \ln BWi) \times 100)/time$ (d);
123 feed conversion ratio (FCR, g/g) = FI/(Bf −Bi) and where FI was the total feed intake (g)
124 during the experimental period considered and, Bi and Bf were the initial and final biomass
125 (g). Feed intake was calculated by collecting the uneaten feed from the bottom of the tank
126 two times per day, drying it in an oven (80-90 °C) for 24 h, and its weight subtracted from
127 the daily FI.

128 After fish were measured, blood (500-700 µL) was taken from anesthetized fish (n
129 = 6 fish per tank) by caudal puncture with lithium-heparinized syringes and immediately
130 centrifuged (2,000 × *g* for 20 min at 4 °C) to separate serum. The lysozyme activity in serum
131 was measured according to the method of Ellis (1990). Briefly, a sample of 0.05 mL serum
132 was added to 1.4 mL of a suspension of *Micrococcus lysodeikticus* (0.2 mg/mL) in a 0.1 M

133 sodium phosphate buffer (pH 6.8). The reaction was conducted at 25 °C and absorbance
134 was measured at 530 nm after 0.5 and 4.5 min in a spectrophotometer, using egg-white
135 lysozyme as standard. Each unit (kU/mL) is defined as the amount of sample causing a
136 decrease in absorbance of 0.001 per min. The hemolytic assay for alternative complement
137 pathway (ACP) was determined as described by Sunyer et al. (1995) with minor
138 modifications for ELISA plates. The results are expressed in alternative complement units
139 per mL, which are defined as the titer at which 50% hemolysis is produced. For the
140 bacteriolytic test, bacteria (*Escherichia coli*) were grown for 20 h in 20 mL of lysogeny broth
141 at 37°C in an orbital incubator at 200 rpm. A 1:100 bacterial suspension was chosen to give
142 an optical reading of 0.5 to 0.6 at a wavelength of 540 nm then added to the serum dilution
143 (1:1, bacterial suspension:serum), after setting the zero value using sterile Luria-Bertani
144 medium. The mixture was placed for 1 h at 37 °C on an orbital-shaking incubator (200 rpm).
145 To study the bactericidal kinetics of fish serum, a 0.5-mL aliquot was taken at intervals of
146 30 min and read at $\lambda = 540$ nm with a microplate reader (Tecan Infinite M200; Tecan Group
147 Ltd., Barcelona, Spain). Results are given as fold increase of the absorbance. All chemicals
148 and reagents used for evaluating different hematological immune parameters were
149 purchased from Sigma-Aldrich (Madrid, Spain).

150 For determining the body proximate composition of fish and feed, samples were
151 homogenized (Ultra-Turrax T25 basic, IKA©; Werke), and small aliquots were dried (120°C
152 for 24 h) to estimate water content. The total fat content in samples was quantified
153 gravimetrically after extraction in chloroform–methanol (2:1) and evaporation of the
154 solvent under a stream of N_2 followed by vacuum desiccation overnight (Folch et al., 1957).
155 Crude protein content was determined according to Lowry et al. (1951). Ash contents were

156 determined by keeping the sample at 500–600°C for 24 h in a muffle furnace (AOAC, 1990).

157 All chemical analyses were performed in triplicate per fish and feed samples.

158

159 *2.3 Fish handling and tank redistribution*

160 After fish growth parameters measurement at the end of the trial (110 days), all fish from
161 the same dietary treatment (n = 240) were sorted and pooled together in a 500 L tank
162 (stocking density: 28.2 – 29.6 kg/m^3, water temperature: 21-22 °C, oxygen levels at
163 saturation) for 1-1.5 h, and redistributed by hand-netting again into 4 tanks that were
164 already used during the nutritional experimental period. After redistribution of the fish,
165 water quality conditions were similar to those of the nutritional trial, and fish were fed the
166 same diets that they were offered during the nutritional trial.

167

168 *2.4 Pathogen identification*

169 Prior to opening the abdominal cavity, mucus from the skin and was collected using sterile
170 cotton swabs. After external samples were collected, the entire surface of the fish was
171 rinsed with 70% ethanol to remove viable bacterial contaminants. The fish abdominal
172 cavity was opened to expose the internal organs using sterile scissors and the viscera was
173 displaced using sterile forceps to expose the kidney. Sterile cotton swabs were used to
174 recover material of their head kidney from both healthy and moribund specimens (n = 5
175 per group). These microbiological samples were streaked onto TSA + 5% sheep´s blood agar
176 plates (Ref #770103, Difco™, Fisher Scientific, Spain) and TCBS media (Ref #413817.1210,
177 PANREAC Applichem, Spain). Additionally, 100 µL of water from the expansion tank of the

178 RAS was collected and spread onto the same culture media. Colonies that appeared after
179 48 hours at 23 °C were collected from the agar using sterile toothpicks and placed into 200
180 µL of DNA extraction lysis buffer containing proteinase K, and extractions performed
181 following the manufacturer's protocol (DNeasy Blood and Tissue Kit, Ref #69506, Qiagen,
182 Spain). Extracted DNA was evaluated by spectrophotometry to determine purity and
183 concentration prior to PCR analysis. Specific primers for the detection of *Vibrio anguillarum*
184 (Hong et al., 2007) were used in an attempt to identify the bacteria responsible for the
185 infectious outbreak (n = 10). Amplification was performed in 20 µL reactions containing Taq
186 polymerase buffer (1X), 0.5 U of Taq polymerase, $MgCl_2$ (2mM), dNTP´s (900 µM), and 1µM
187 of each primer specific for *V. anguillarum* (Hong et al., 2007). The conditions for
188 amplification were as follows: Initial denaturation of template DNA at 95 °C for 10 min,
189 followed by 25 cycles of 30 sec at 92 °C, 30 sec at 56 °C, and 30 sec at 72 °C with a final
190 extension step of 7 min at 72 °C.

191 Additionally, universal eubacterial primers (Suzuki et al., 1996) were used to identify
192 the source of DNA from colonies, which tested negative by the species-specific primers. For
193 these primers, amplification was performed in 20 µL reactions containing Taq polymerase
194 buffer (1X), 0.5 U of Taq polymerase, $MgCl_2$ (2mM), dNTP´s (900 µM), and 1µM of each
195 primer. The amplification conditions included 5 min at 95ºC followed by 30 sec at 94 °C, 45
196 sec at 48 °C, and 1.5 min at 72 °C for 35 cycles, and terminating with a final extension cycle
197 of 7 min at 72 °C. Following amplification, and prior to sequence analysis, amplified DNA
198 was purified using standard spin-column protocols described for the QIAquick PCR
199 Purification Kit (Ref# 28104, Qiagen, Spain). All sequences were determined by bi-
200 directional sequencing using the same primers as those in the original amplification.

201 Sequencing was performed by Sistemas Genómicos (Valencia, Spain). The final sequences
202 obtained were compared to the GenBank database using BLAST to see what species,
203 previously identified, may be taxonomically related to the pathogen cultured from
204 moribund fish. Determination of aetiology using phylogenetic methods were especially
205 relevant in this instance as the genus *Vibrio* contains a great diversity of sometimes very
206 closely related species. Thus, phylogenetic analysis was performed with 28 taxa and a total
207 of 786 nucleotide positions using Maximum Likelihood and Neighbor-Joining methods in
208 MEGA 5. In selecting taxa from GenBank for these analyses, sequences shorter than 800
209 bp, or sequences with numerous inconclusively determined nucleotides were excluded,
210 whereas all positions containing gaps and missing data were eliminated.

211

212 *2.5 Ethics statement*

213 All animal experimental procedures were conducted in compliance with the experimental
214 research protocol approved by the Committee of Ethics and Animal Experimentation of the
215 Institut de Recerca i Tecnologia Agroalimentàries (IRTA), the Departament d'Agricultura,
216 Ramaderia, Pesca, Alimentació i Medi Natural (permit number 7962) and in accordance
217 with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory
218 animals.

219

220 *2.6 Statistical analyses*

221 Data were expressed as the mean ± standard error of the mean (SEM) (n = 4), with the
222 exception of cumulative mortality rates that were expressed as mean ± standard deviation

223 (n = 5). Arcsine transformations were conducted on all the data expressed as percentages.
224 Results were compared by means of One-way ANOVA (data normally distributed and
225 homogeneity of variances checked) and, when significant differences were detected ($P < 0.05$),
226 the Tukey multiple-comparison test was used to detect differences among
227 experimental groups. All statistical analyses were performed using SigmaPlot version 12.0
228 (Systat Software Inc., Chicago, IL).

229

230 **3. Results**

231 At the end of the trial (110 days), no differences in survival, somatic growth in BW and SL,
232 SGR_{BW}, and Fulton's condition factor were observed among European sea bass fed diets
233 containing different FM levels and supplemented with SPH ($P > 0.05$; Table 3). In addition,
234 the inclusion of SPH into experimental diets (FM5+SPH5 and FM15+SPH5) improved the
235 FCR values in comparison to the control (FM20) and FM5 diets ($P < 0.05$). In this sense, fish
236 fed the diet with 75% FM substitution by PP (FM5) showed the worst FCR values; by
237 contrast, the group with the best FCR was that fed with the FM15+SPH5 diet (Table 3).
238 There were no differences in the proximate composition of the fillet in European sea bass
239 juveniles fed different experimental diets with different levels of FM and SPH ($P > 0.05$,
240 Table 4).

241 The non-specific serological immune response in European sea bass juveniles fed
242 experimental diets varied depending on the dietary levels of SPH ($P < 0.05$; Table 5). In
243 particular, fish fed FM5+SPH5 and FM15+SPH5 diets showed higher values of lysozyme,

244 complement and bactericidal activity than fish fed the control diet (FM20) and those fed
245 the FM5 diet ($P < 0.05$).

246 Three days after the end of the feeding trial and after having redistributed fish fed
247 the same experimental diets into tanks of the same RAS unit, mortalities started to occur
248 in all tanks (Fig. 1). Moribund fish (specimens displaying erratic and torpid swimming, as
249 well as altered respiration; Supplementary file 1) had damaged dorsal, pectoral, ventral and
250 anal fins with redness about their base, as well as in the mouth and vent areas, whereas in
251 some cases erosion of the skin was evident (Fig. 2a-c). Internally, fish displayed enlarged
252 spleen and, petechial hemorrhagic liver and intestine (Fig. 2d, e). Samples for
253 microbiological culture analysis were collected from tank water, and from skin and head
254 kidney of healthy and moribund specimens from all experimental groups that were plated
255 onto TSA, TCBS, and blood agar media for microbiological analyses. From apparently
256 healthy fish, bacterial growth was obtained from skin smears of two of the five fish tested,
257 but the kidney smears were negative. All moribund fish had the same colony morphologies
258 recovered externally (skin smears) as well as internally (head kidney smears). On TCBS
259 media incubated 24 hrs at 23 °C, the culture presented as regular, convex, glistening yellow
260 colonies approximately 2 mm in diameter. On blood agar plates, alpha hemolysis was
261 evident. The pattern of positive culture samples obtained from moribund and healthy
262 individuals suggested that a bacterial pathogen present in the water had penetrated the
263 skin and developed into septicemia in the moribund fish (Supplementary file 2). All samples
264 (n = 10) tested by PCR using specific primers for *Vibrio anguillarum* were negative (Fig. 3);
265 thereafter, non-specific 16S rDNA amplification was performed on DNA from isolated
266 colonies grown on TCBS and Blood agar plates. DNA sequencing and BLAST analysis of the

267 16S amplicon gave a putative identification as *V. pelagius* in all samples analyzed (Fig. 4; n
268 = 5). Maximum Likelihood analysis gave a confirmation of this identification as *V. pelagius*
269 for all the isolates that were sequenced from this work, both from fish and expansion tank
270 water (Fig. 2).

271 Cumulative survival among different experimental groups ten days after the
272 bacterial epizootic outbreak differed depending on the diet, with groups containing SPH
273 showing the best results (Fig. 1; $P < 0.05$). In particular, fish fed the FM15+SPH5 diet showed
274 the highest cumulative survival rate (96.4 ± 5.0%), followed by those fed the FM5+SPH5
275 (61.8 ± 16.3%). In contrast, cumulative survival rates in fish fed FM20 and FM5 diets were
276 the lowest ones (32.0 ± 6.7% and 38.2 ± 13.5%, respectively).

277

278 **4. Discussion**

279 The level of FM inclusion within compound diets for marine finfish has steadily declined
280 during recent years due to the incorporation of proteins derived from plants (PP) (Tacon
281 and Metian, 2008) or heterotrophic bacteria (Ganuza et al., 2008; Garcia-Ortega et al.,
282 2016). As Sitjà-Bobadilla et al. (2005) indicated, the suitability of this replacement in terms
283 of growth performance has resulted in considerable variability among different fish species
284 and experimental conditions; thus, specific trials have to be performed for each species
285 and each of the alternative raw-materials being considered. Under present experimental
286 conditions, FM substitution by PP sources did not negatively affect the somatic growth
287 performance in European sea bass juveniles fed diets containing 20 or 5% FM (75% of FM
288 substitution in the FM5 diet). These results were in agreement with those already reported

289 by several authors in different marine fish species (Robaina et al., 1995; Kaushik et al., 2004;
290 Salze et al., 2010; Moxley et al., 2014; Ribeiro et al., 2015; Minjarez-Osorio et al., 2016
291 among many others); but they differed from those reported in salmonids, which seem to
292 be more sensitive to plant ingredients than marine species. In particular, reduced growth
293 performance in salmonids fed diets with high levels of FM replacement by PP sources is
294 postulated to be due to reduced digestibility, the presence of growth inhibitors and/or
295 antinutritional factors in plant ingredients (Barrows et al., 2007; Collins et al., 2013;
296 Lazzarotto et al., 2018).

297 Protein hydrolysates derived from fish, shrimp, krill, soybean, yeast and pig blood
298 have been tested in different nutritional trials, even though results differed between
299 studies depending on the type and dietary level of protein hydrolysates, as well as on the
300 fish species. For instance, FM was successfully replaced by fish protein hydrolysate (FPH)
301 at low levels in diets for Atlantic salmon (*Salmo salar*) (Espe et al., 1999; Refstie et al., 2004;
302 Hevrøy et al., 2005), red seabream (*Pagrus major*) (Bui et al., 2013), turbot (*Scophthalmus*
303 *maximus*) (Zheng et al., 2013) and Japanese flounder (*Paralichthys olivaceus*) (Zheng et al.,
304 2011; Khosravi et al., 2015), although when exceeding a specific level, fish showed reduced
305 growth and feed efficiency parameters (Refstie et al., 2004; Hevrøy et al., 2005). In addition,
306 FPH was able to successfully replace FM in diets for turbot (*Scophthalmus maximus*) (Oliva-
307 Teles et al., 1999) and coho salmon (*Oncorhynchus kisutch*) (Murray et al., 2003), as well as
308 in Atlantic cod (*Gadus morhua*) and turbot fed diets with high levels of PP sources (Askness
309 et al., 2006; Xu et al., 2017), although in the above-mentioned studies growth performance
310 results were never higher than fish fed the control diet. In the current study, growth
311 performance parameters (BW, SL, SGR_{BW}) and body condition (K) in sea bass fed diets low

312 and intermediate levels of FM containing SPH (FM5+SPH5 and FM15+SPH5, respectively)
313 were similar to those of the control group. These results were in agreement with those
314 reported in juveniles of olive flounder (Khosravi et al., 2015) fed diets containing high levels
315 of PP sources and supplemented with SPH.

316 Fish and crustacean protein hydrolysates have been reported to have varied
317 biological benefits such as antimicrobial (Sila et al., 2014; Robert et al., 2015), antioxidant
318 (García-Moreno et al., 2014) or antihypertensive activities (Ktari et al., 2014). The above-
319 mentioned functions may be attributed to the presence of biologically active peptides with
320 immunostimulating and antibacterial properties due to the enzymatic hydrolysis of
321 proteins (Martínez-Alvarez et al., 2015; Hou et al., 2017). Additionally, if dietary protein is
322 presented in a more appropriate form for physiological requirements such as di- and
323 tripeptides and other oligopeptides, it can be more completely assimilated (Arredondo-
324 Figueroa et al., 2013), which would benefit all physiological functions of the fish and other
325 aspects of performance may also be improved. *In vivo* studies in different fish species have
326 demonstrated that FPH and SPH have immunostimulatory properties (Bøgwald et al. 1996;
327 Kotzamanis et al. 2007; Liang et al., 2006; Bui et al., 2013; Khosravi et al., 2105), whereas
328 others have reported no immunostimulatory effect (Murray et al., 2003; Zheng et al.,
329 2013). Under current experimental conditions, the incorporation of SPH in diets with 25
330 and 75% of FM replacement by PP sources (Diets FM5+SPH5 and FM15+SPH5, respectively)
331 resulted in an enhancement of the humoral non-specific immune response in sea bass.
332 Thus, fish fed those diets incorporating the SPH showed higher levels of lysozyme,
333 complement and bacteriolytic activities in serum than those observed in the control group
334 (Diet FM20) and those with 25 and 75% of FM replacement by PP sources (Diets FM5 and

335 FM15, respectively). These results are of special importance as they showed that the
336 inclusion of SPH in aquafeeds may improve health and disease resistance in fish, although
337 it is generally accepted that the definitive evaluation of a dietary ingredient as an
338 immunomodulator generally requires a challenge with an active pathogen (Vallejos-Vidal
339 et al., 2016). However, the relevance of the present findings is suggested by the fact that
340 this improvement of humoral non-specific immune response provided protection of sea
341 bass in front of an outbreak of *V. pelagius,* a species known for the production of
342 hemolysins, a significant class of virulence factors (Zhang et al. 2005) often required for
343 penetration of the physical immune barrier of the skin as well as disruption of cellular
344 immune response elements. The identification of the bacterial strains isolated from this
345 epizootic episode by phylogenetic methods is of significance for this work, since there are
346 misidentified strains in culture collections reported as *V. pelagius* (Macian et al., 2000;
347 Thompson et al., 2003) that confound a simple BLAST analysis. Herein, it has been
348 demonstrated the pathogen isolates collected were *V. pelagius*, although confirmation of
349 the findings of Macian et al. (2000) can be seen, as one misidentified strain from a culture
350 collection (ATCC 25916T) lies outside the *V. pelagius* clade (Fig. 2). As *V. pelagius* is
351 commonly isolated from marine ecosystems, it likely entered the experimental system as
352 an opportunistic pathogen and/or a part of the normal microbiota of the skin. Specific
353 changes with the experimental conditions (*i.e.* crowding and repeated handling stress)
354 likely changed the synergy of some characteristic of the host-environment-pathogen triad
355 leading to an unexpected disease outbreak. The precise mechanism that provided
356 protection against Vibrio hemolysin-like virulence factors is suggested by a study in
357 *Caenorhabditis elegans* where using microarray analysis demonstrated that the expression

358 of hemolysin by *Vibrio cholerae* specifically upregulates C-type lectin genes (among others)
359 in the host (Sahu et al. 2012); mechanisms that are well-conserved also among vertebrates.
360 In addition, some peptides may directly damage the bacterial membranes of different
361 species/strains of *Vibrio* as it was shown for lactoferrin-derived peptides (Acosta-Smith et
362 al., 2018). Whether these specific immune pathways are specifically upregulated by the
363 diet used in this trial remains to be determined. However, the benefits of the different diet
364 formulations was evident, as survival of sea bass fed diets FM5+SPH5 and FM15+SPH5 was
365 higher than in fish fed the control diet (FM20) and those with 25 and 75% of FM
366 replacement by PP sources (Diets FM5 and FM15, respectively). The dietary inclusion of
367 krill hydrolysate and FPH from tilapia at 5% significantly reduced cumulative mortality of
368 red sea bream against *Edwardsiella tarda* exposed to a laboratory bacterial challenge,
369 whereas no effect of SPH was found regardless of the impact of this PH on non-specific
370 serological immune parameters (Bui et al., 2013). Similar results were reported by Khosravi
371 et al. (2015) in olive flounder, where different protein hydrolysates increased non-specific
372 immune parameters without successfully improving survival rates in olive flounder
373 challenged with *V. anguillarum*. The varying degrees of success in different studies may be
374 due to the variation of bioactive peptide profiles of hydrolyzed protein produced using
375 different raw material, enzyme source and hydrolysis conditions (Klompong et al., 2009).
376 Thus, it is critical to standardize the above-mentioned production parameters when
377 manufacturing protein hydrolysates for aquafeeds in order to guarantee not only their
378 quality and safety, but also their immunomodulatory and health protective properties.

379 In conclusion, the present study showed that SPH can be incorporated in aquafeeds
380 with high levels of FM substitution by PP sources with no detrimental impact on the somatic

381 growth performance of fish. In addition, the non-specific humoral immunity of the fish was
382 enhanced, which may explain the higher cumulative survival of fish fed with SPH-
383 supplemented diets when affected by an epizootic outbreak of *V. pelagius*.
384
385
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390 and immunological analyses.
391
392
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593

594 **Figure captions**

595

596 Figure 1. Cumulative survival rates (CS, %) of European sea bass (*Dicentrarchus labrax*) ten days
597 after the bacterial epizootic with the pathogenic bacteria *Vibrio pelagius*. Data are expressed as
598 mean ± SD (n = 5 replicate tanks). Different letters denote statistically significant differences in
599 final cumulative survival rates ($P < 0.05$).

600

601 Figure 2. External (a, b, c) and internal (d, e) signs of *Vibrio pelagius* infected on affected European
602 sea bass (*Dicentrarchus labrax*) juveniles. Redness and inflammation of the head, operculum (a)
603 and ventral area comprissed between pectoral and anal fins (c). Erosion of caudal fin and redness
604 and swelling of the base of the pectoral and pelvic fins (a, c). Skin erosion evident laterally and
605 abdominally (b, c). Petechial hemorrhaging of the liver and intestine (d, e) and enlargement of the
606 spleen (e).

607

608 Figure 3. PCR analysis for the identification of *Vibrio pelagius* isolates growth in TCBS and blood
609 agar plates. The first approach conducted for identifying the pathogen was with *Vibrio*
610 *anguillarum* primers (Hong et al., 2007), but all tested samples were negative for this species.
611 Thus, non-specific 16S rDNA amplification on DNA was performed and the 16S amplicon sent for
612 sequencing, BLAST and phylogenetic analysis (see results in Figure 4). Lanes are labeled as follows:
613 the first letter represents either moribund (M) or healthy (S), the second letter represents either
614 head kidney (K) or skin (S) tissue, and the last letter represents the type of media used for
615 isolation, being either TCBS (T) or blood agar (B). Additional samples were collected from the
616 source water for the challenge room (Ch) or the expansion tank water of the RAS module (V3). *V.*
617 *anguillarum* genomic DNA isolated was included as positive PCR amplification control.

618

619 Figure 4. Phylogenetic analysis of the *Vibrio* isolates. The evolutionary history of the *Vibrio* isolates
620 were inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. The
621 tree with the highest log likelihood (-1894.3268) is shown. Bootstrap confidence values are shown
622 next to the branch nodes. A discrete Gamma distribution was used to model evolutionary rate
623 differences among sites [5 categories (+G, parameter = 0.0723)]. The tree is drawn to scale, with
624 branch lengths measured in the number of substitutions per site. Isolates of *V. pelagius* from this
625 study are shown in bold. ■ = isolates from moribund fish; ● = isolates from "healthy" fish.

626

627

628 Supplementary file 1. Video of European sea bass (Dicentrarchus labrax) showing signs of an
629 epizootic infection of *Vibrio pelagius*. Note the presence of hemorrhagic pectoral and dorsal fins,
630 as well as the darkening of body pigmentation in some specimens with a more advanced stage of
631 the infection.

632

633 Supplementary file 2. Swabs of the head kidney from healthy and moribund specimens (n = 5 per
634 group) of *European sea bass* (*Dicentrarchus labrax*) affected by an epizootic infection of *Vibrio*
635 *pelagius*, as well as water samples streaked onto TCBS agar plates (Ref #413817.1210,
636 PANREAC Applichem, Spain) . Note the change of colour of the microbiological medium from
637 green to yellow where there is growth of *Vibrio* bacteria.

638

639 Table 1. Specifications of the shrimp hydrolysate (SPH) tested in the present study. Data

640 provided by the manufacturer.

Specifications	**% of product**
Dry Matter	95.7
Crude Protein	68.7
Crude Fat	7.9
Ash	11.9
Energie (kcal/g)	5.0
Soluble protein	58.2
Peptide profile (% of soluble protein)	
20,000 < MW (Da)	0.0
10,000 < MW (Da) < 20,000	0.2
5,000 < MW (Da) < 10,000	1.0
1,000 < MW (Da) < 5,000	10.3
500 < MW (Da) < 1,000	10.1
MW (Da) < 500	78.4

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643

644 Table 2. Ingredient list (%) and proximate composition (%) in a dry basis of experimental

645 diets.

	Experimental diets			
Ingredient	**FM5**	**FM20**	**FM5+SPH5**	**FM15+SPH5**
Fish meal LT	5.00	20.00	5.00	15.00
Shrimp protein hydrolysate (SPH)	0.00	0.00	5.00	5.00
Corn gluten	17.00	11.00	15.00	11.00
Wheat gluten	19.71	14.00	18.92	14.00
Rapeseed	11.15	7.00	9.00	7.00
HP soy 48	17.00	14.78	15.00	14.78
Wheat	11.25	17.05	13.80	16.95
Methionine	0.57	0.44	0.53	0.44
Lysine	1.58	1.04	1.52	1.14
Monocalcium phosphate	2.48	1.18	2.08	1.18
Fish oil into the mix	3.00	2.25	2.89	2.25
Fish oil top-coating	10.00	10.00	10.00	10.00
Premix DK4	0.75	0.75	0.75	0.75
Stay-C 35	0.46	0.46	0.46	0.46
Vit E-50 Adsorbate	0.025	0.03	0.03	0.03
Barox	0.025	0.03	0.03	0.03
Proximate composition				
Crude protein (%)	45.8 ± 0.2	45.5 ± 0.3	45.8 ± 0.1	45.2 ± 0.3
Crude fat (%)	15.8 ± 0.3	16.0 ± 0.2	15.9 ± 0.3	16.1 ± 0.1
Ash (%)	8.3 ± 0.1	8.4 ± 0.1	7.8 ± 0.1	7.5 ± 0.1
Gross energy (Kcal/g)[a]	5.03 ± 0.05	5.04 ± 0.05	4.99 ± 0.05	5.00 ± 0.05

646 [a] Gross energy content was estimated by NIR spectrophotometry (AQUATIV, Diana Aqua, France).

647

648 Table 3. Somatic growth performance and feed efficiency parameters (mean ± SEM; n = 58-
649 60 per tank; n = 4 per dietary group) of European sea bass (*Dicentrarchus labrax*) fed
650 experimental diets containing different levels of fishmeal (FM) and shrimp protein
651 hydrolysates (SPH). Values with different superscripts are statistically significant different
652 ($P < 0.05$).

	FM20	FM5	FM5+SPH5	FM15+SPH5
Survival (%)	95.0 ± 2.5	90.0 ± 5.0	95.0 ± 2.5	95.5 ± 2.5
BW (g)	59.5 ± 1.3	58.6 ± 2.0	61.0 ± 2.6	61.8 ± 1.5
SL (cm)	14.4 ± 0.1	14.4 ± 0.2	14.6 ± 0.1	14.7 ± 0.1
K	1.98 ± 0.01	1.96 ± 0.05	1.95 ± 0.05	1.94 ± 0.01
SGR_{BW} (%/day)	1.10 ± 0.05	1.08 ± 0.05	1.12 ±0.03	1.13 ± 0.04
FCR	1.28 ± 0.06 b	1.41 ± 0.04 c	1.19 ± 0.05 ab	1.15 ± 0.02 a

653

654

655

656 Table 4. Proximate composition (mean ± SEM; n = 4 per dietary group) in dry weight of

657 European sea bass (*Dicentrarchus labrax*) fed experimental diets containing different levels

658 of fishmeal (FM) and shrimp protein hydrolysates (SPH).

	FM20	FM5	FM5+SPH5	FM15+SPH5
Protein (%)	42.5 ± 0.5	41.6 ± 1.1	41.5 ± 1.0	41.6 ± 0.6
Lipid (%)	34.7 ± 1.2	35.9 ± 1.6	35.3 ± 1.2	35.0 ± 1.1
Carbohydrates (%)	1.8 ± 0.1	1.7 ± 0.1	1.7 ± 0.2	1.9 ± 0.1
Ash (%)	4.3 ± 0.3	4.1 ± 0.1	3.9 ± 0.3	3.7 ± 0.2

659

660

661 Table 5. Non-specific immune responses in serum (lysozyme, hemolytic assay for
662 alternative complement pathway, and bacteriolytic activity) (mean ± SEM; n = 6 per tank;
663 n = 4 per dietary group) from European sea bass (*Dicentrarchus labrax*) fed experimental
664 diets containing different levels of fishmeal (FM) and shrimp protein hydrolysates (SPH).
665 Values with different superscripts are statistically significant different ($P < 0.05$).

	Lysozyme (KU/mL)	Complement (ACH_{50}U/mL)	Bacteriolytic activity (%Abs)
FM20	205.4 ± 21.3 b	211.4 ± 37.2 b	56.3 ± 3.1 b
FM5	201.3 ± 12.3 b	197.7 ± 8.5 b	47.1 ± 4.5 b
FM5+SPH5	414.6 ± 98.3 a	467.3 ± 33.2 a	83.8 ± 4.7 a
FM15+SPH5	456.9 ± 37.8 a	474.1 ± 35.2 a	85.1 ± 3.1 a

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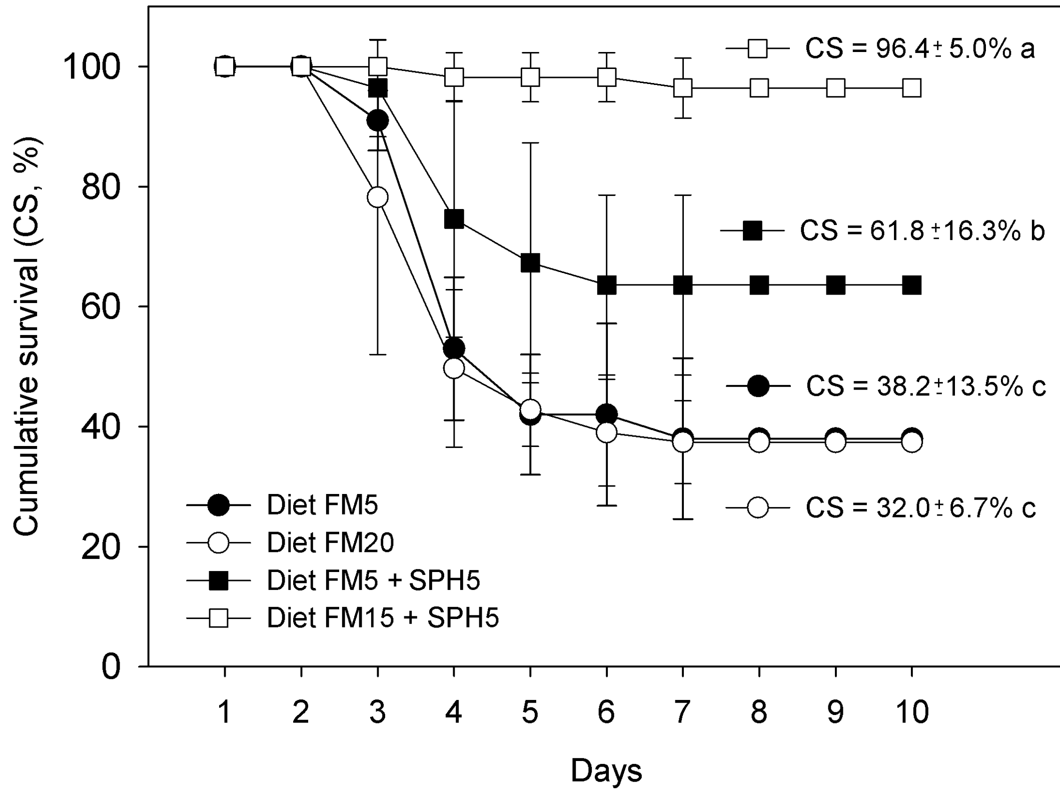
682 **Figure 1**



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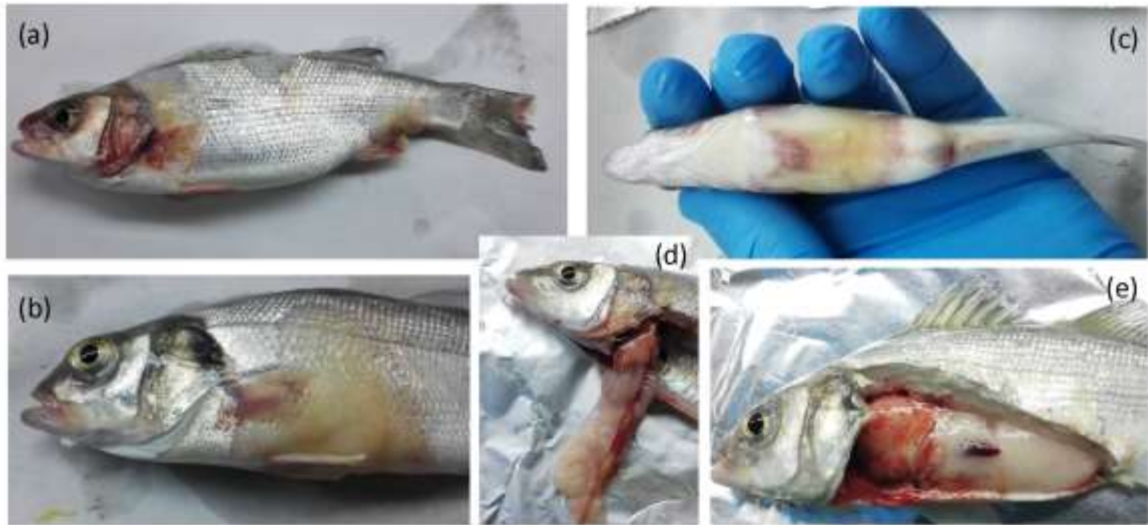
693 **Figure 2**



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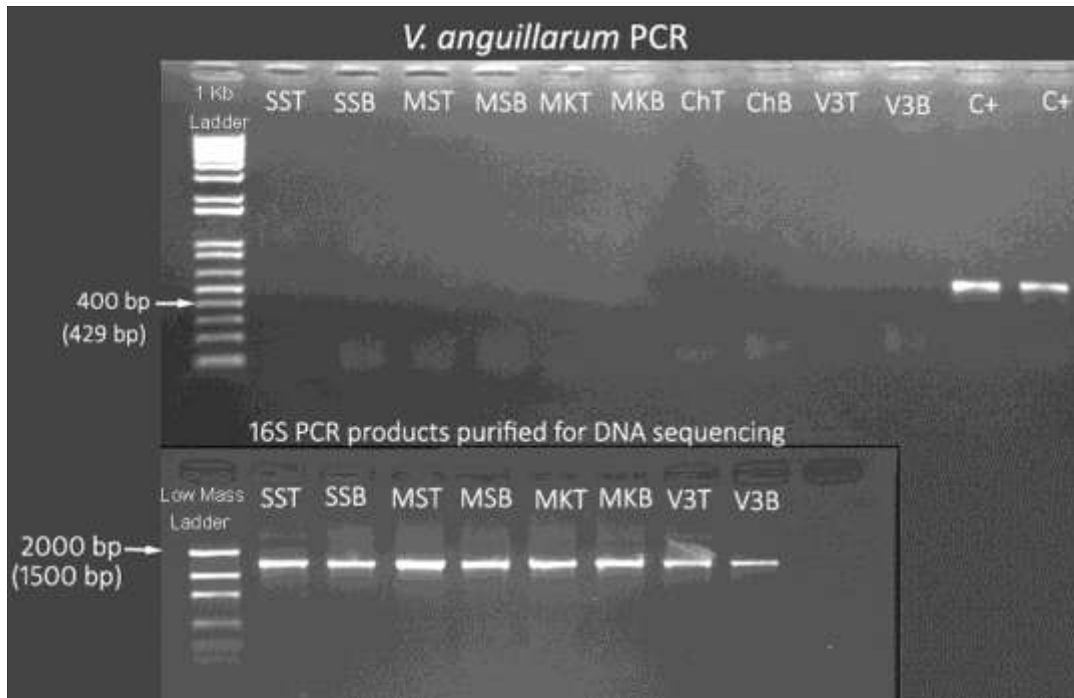
707 **Figure 3**



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710 **Supplementary file**

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722 **Figure 4**

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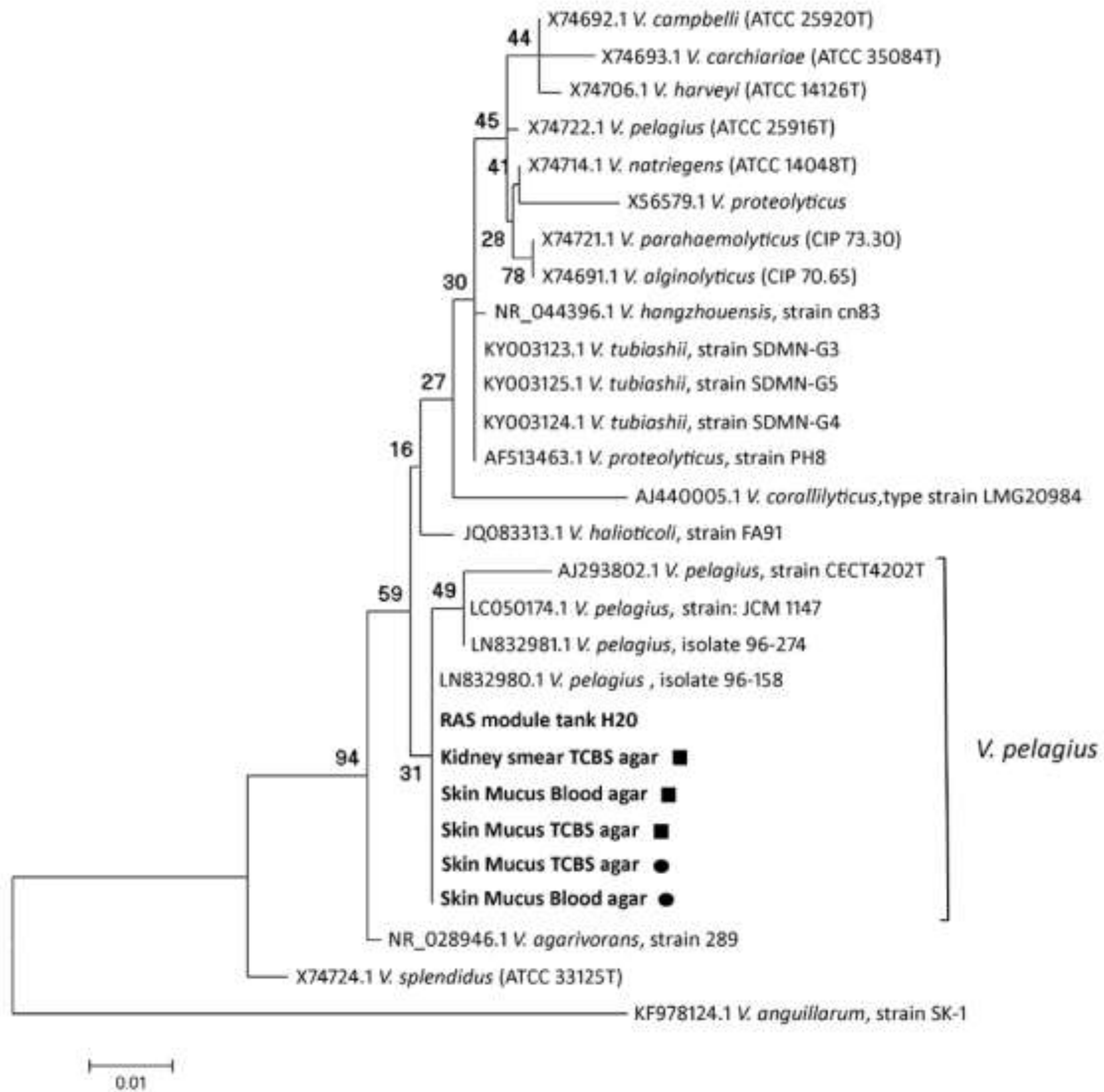

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