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Dietary administration of the probiotic *Shewanella putrefaciens* Pdp11 promotes transcriptional changes of genes involved in growth and immunity in *Solea senegalensis* larvae

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1 **Dietary administration of the probiotic *Shewanella putrefaciens*
2 Pdp11 promotes transcriptional changes of genes involved in
3 growth and immunity in *Solea senegalensis* larvae**

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20 **Abstract**

21 Senegalese sole (*Solea senegalensis*) has been proposed as a high-potential species
22 for aquaculture diversification in Southern Europe. It has been demonstrated that a
23 proper feeding regimen during the first life stages influences larval growth and survival,
24 as well as fry and juvenile quality. The bacterial strain *Shewanella putrefaciens* Pdp11
25 (SpPdp11) has shown very good probiotic properties in Senegalese sole, but
26 information is scarce about its effect in the earliest stages of sole development. Thus,
27 the aim of this study was to investigate the effect of SpPdp11, bioencapsulated in live
28 diet, administered during metamorphosis (10-21 dph) or from the first exogenous
29 feeding of Senegalese sole (2-21 dph). To evaluate the persistence of the probiotic
30 effect, we sampled sole specimens from metamorphosis until the end of weaning (from
31 23 to 73 dph). This study demonstrated that probiotic administration from the first
32 exogenous feeding produced beneficial effects on Senegalese sole larval development,
33 given that specimens fed this diet exhibited higher and less dispersed weight, as well as
34 increases in both total protein concentration and alkaline phosphatase activity, and in
35 non-specific immune response. Moreover, real-time PCR documented changes in the
36 expression of a set of genes involved in central metabolic functions including genes
37 related to growth, genes coding for proteases (including several digestive enzymes), and
38 genes implicated in the response to stress and in immunity. Overall, these results
39 support the application of SpPdp11 in the first life stages of *S. senegalensis* as an
40 effective tool with the clear potential to benefit sole aquaculture.

41

42 **Keywords**

43 Senegalese sole *(Solea senegalensis)*; probiotics; *Shewanella putrefaciens* Pdp11 (SpPdp11);

44 larval development; absolute transcription profiles.

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46 **1. Introduction**

47 In the past five decades, aquaculture has become one of the fastest-growing food
48 industries. It is estimated that, currently, more than half of the total food of aquatic
49 origin consumed by the world population comes from this sector [1]. Senegalese sole
50 (*Solea senegalensis*) is a flatfish considered one of the most interesting species for
51 European aquaculture diversification owing to its nutritional properties, high
52 commercial value and growing market demand [2]. Although the Senegalese sole
53 production cycle can be successfully completed in fish farms, the consolidation of its
54 industrial production is hampered by several factors including high larval mortality rates
55 related to nutrition, growth dispersion, difficulty in establishing optimal weaning
56 conditions, elevated incidence of skeletal anomalies, and high vulnerability to infectious
57 diseases [2]. As in all flatfish, sole metamorphosis is characterized by a dramatic
58 anatomical transformation that involves a remodelling of the head and a change to an
59 asymmetric shape. Such transformation is associated with a change from a pelagic to a
60 benthic habitat that implies important changes in food habits and digestive physiology.
61 As a result, the early stages of larval development and particularly metamorphosis are
62 critical periods of sole rearing, determining further growth features in later production
63 steps [2]. Along these lines, it is worth noting that bacterial colonization of the fish gut
64 occurs during the larval stage, when the gastrointestinal tract is not yet fully developed,
65 and that the composition of the larval microbiota resembles the microflora of the first
66 ingested live feed rather than that of the surrounding environment [3]. In fish, the
67 intestinal microbiota plays a pivotal role, being involved in the stimulation of epithelial
68 proliferation, in nutrition and digestion through the production of vitamins and
69 enzymes, and in the immune system development of the host (reviewed in [4]).

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70 Therefore, it is of utmost importance to provide a proper feeding regimen during the
71 first life stages to produce juvenile sole of high quality.

72 In the search for increased productivity and economic benefits, aquaculture practices
73 can sometimes produce a degree of stress in fish, decreasing their immune competence
74 and making animals more susceptible to disease, especially to infections ([5] and
75 references within). Control of pathogenic bacteria in aquaculture has traditionally been
76 carried out by administration of antibiotics or chemotherapeutic agents. However, the
77 massive use of antimicrobials has a negative environmental impact and promotes
78 increased resistance, jeopardizing the viability of the sector and affecting animal health
79 and welfare, food safety and environmental protection [6]. Therefore, viable alternatives
80 to stimulate the natural mechanisms of animal immune defence are essential. In this
81 context, probiotics constitute a promising ecologically and economically sustainable
82 option to improve the health status of cultured fish while reducing the use of antibiotics
83 and other chemotherapeutic agents [7]. In fish aquaculture, probiotic-supplemented
84 diets favourably affect growth performance and feed efficiency in addition to improving
85 animal welfare, stress tolerance and resistance to diseases, particularly through the
86 modulation of the intestinal microbiota and the immune system [7]. *Shewanella*
87 *putrefaciens* Pdp11 (SpPdp11) is a bacterial strain initially isolated from the skin mucus
88 of healthy gilthead seabream (*Sparus aurata*) that exerts remarkable probiotic
89 characteristics in gilthead seabream and Senegalese sole farming [8]. Several studies
90 have demonstrated that juvenile specimens of *S. senegalensis* that received SpPdp11 in
91 the diet displayed a modulation of the intestinal microbiota and liver fatty acid
92 composition, enhanced growth, and heightened pathogen resistance [8, 9]. Moreover,
93 the dietary administration of this probiotic to juvenile sole can improve stress tolerance
94 to high stocking densities by modulating the expression of important immune genes and
95 the intestinal conditions [5]. Furthermore, when administered jointly with the antibiotic

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96 oxytetracycline, SpPdp11 compensates the apoptotic effect of this drug [10]. More
97 recently, it has been established, also in juvenile sole, that the administration of
98 SpPdp11 in combination with the prebiotic alginate resulted in an increase of the
99 transcription of genes related to antioxidative defences, and, more importantly, these
100 effects were maintained after the cessation of the probiotic treatment [11]. To our
101 knowledge, only two studies have investigated the effects of this probiotic when
102 administered during the larval development of *S. senegalensis* [4, 12]. These studies
103 have employed probiotic pulses of different durations but always beginning at
104 metamorphosis (10 days post-hatching, dph) and they have focussed on the modulation
105 of the gut microbiota composition. Therefore, there is no information concerning the
106 effect of SpPdp11 when administered to *S. senegalensis* larvae from the first exogenous
107 feeding, and little is known about the molecular mechanisms underlying the effect of
108 this probiotic in the early life stages of the Senegalese sole.
109 In an attempt to answer these questions, we herein compared the effects of two
110 different probiotic administration protocols during Senegalese sole larval development,
111 one from the first exogenous feeding until the completion of metamorphosis (2-21 dph)
112 and the other during metamorphosis (10-21 dph). We examined the transcriptional
113 expression of 18 genes encoding proteases (including digestive enzymes) or coding for
114 relevant proteins involved in major biological functions such as growth, stress response
115 and immunity. To assess the persistence of the stimulatory effects of the probiotic
116 during sole larval development and after the suspension of the treatment, we evaluated
117 early and late responses (at 23 and 73 dph). Effects on weight, total protein content,
118 alkaline phosphatase activity, intestinal microbiota composition and innate immune
119 parameters were also investigated to support the biological interpretation of the gene
120 expression results.

121 # 2. Materials and Methods

122 *2.1. Fish rearing conditions and experimental design.*

123 Animals were cultured according to the European Union Guidelines (2010/63/EU)
124 and Spanish legislation (RD 1201/2005 and law 32/2007) at the Spanish Institute of
125 Oceanography (Santander, Spain), and all experiments were approved by the Ethics and
126 Animal Welfare Committee of this institution. Embryos were obtained from wild,
127 naturally spawning captive broodstock held under natural condition. Eggs were
128 incubated at 19.0 ± 0.5 ºC in 70 l cylinder-conical incubating tanks with gentle aeration
129 and a continuous water flow of 0.5 l min^{-1}. After hatching, larvae were distributed into
130 280 l circular polyester resin tanks (40 larvae l^{-1}), with constant aeration and water
131 renewal. Temperature was maintained at 18.3 ± 0.8 ºC and salinity at 35.4 g l^{-1}
132 throughout the experiment. Continuous illumination of 1,000 lux at the surface of the
133 water was provided until 10 dph, and then a 12:12 L:D cycle was established until day
134 21, whereas a 0:24 L:D cycle was used after the pelagic stage [13]. Continuous water
135 inflow was maintained at 5–80 % exchange (2–21 dph), increasing from weaning until
136 400 %, to supply appropriate oxygen and nitrite levels for sole larval and post-larval
137 culture [14, 15]. The feeding regimen was as described by Cañavate and Fernández-
138 Díaz [13]. From 2 to 10 dph, larvae were fed twice a day with rotifers enriched with the
139 marine microalgae *Nannochloropsis gaditana* and *Isochrysis galbana*. From 10 to 60
140 dph, a co-feeding consisting of *Artemia* and the commercial pellet diet Gemma Micro
141 (total lipids 15 %, crude protein 55 %, Skretting, Burgos, Spain) was introduced.
142 *Artemia* nauplii (AF strain INVE Aquaculture, Ghent, Belgium) were supplied from 10
143 to 12 dph and *Artemia* metanauplii (EG strain, INVE Aquaculture, Ghent, Belgium)
144 thereafter. Both *Artemia* stages were previously enriched with a commercial emulsion
145 (DHA Super Selco, INVE Aquaculture, Ghent Belgium) and then added to the tanks

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146 four times a day, whereas dry feed was supplied eight times a day (four of them at
147 night). *Artemia* doses were increased (from 2 to 14 metanauplii ml^{-1}) with larval age.
148 Weaning started at 48 dph and finished at 66 dph, when larvae were fed with dry feed
149 only (total lipids 17 %, crude protein 58 %, GemmaWean, Skretting, Burgos, Spain).
150 During this period, the amount of dry feed was gradually increased (from 11.2 g m^{-2},
151 45.5 % of total feed to 39.2 g m^{-2}), while *Artemia* doses were progressively reduced
152 (from 14 metanauplii ml^{-1}). At the end of the experiment, the fry were fed 7 % of total
153 tank biomass.

154 The experimental design of this study is shown in Supplementary Fig. 1. Three
155 groups on feeding regimens were compared: two groups receiving the SpPdp11
156 probiotic bacterial strain (CP and PP) and the control group (CC). The group CP group
157 received the probiotic through the living vector of *Artemia*, throughout metamorphosis,
158 from 10 to 21 dph. On the other hand, the PP group was given the probiotic,
159 bioencapsulated in rotifers or *Artemia*, from the first exogenous feeding to the end of
160 metamorphosis (2 to 21 dph). The control group, CC, was fed a standard diet without
161 probiotics. Each diet was evaluated in triplicate.

162 Sampling times were selected on the basis of changes in morphogenesis in order to
163 cover metamorphosis, weaning and post-weaning, given that those are the most
164 important stages in Senegalese sole development. Thus, at least ten specimens from
165 each group were collected once metamorphosis was completed (23 dph), at the
166 beginning and at the end of weaning (48 and 66 dph), and at 73 dph. Fish were
167 anaesthetized with tricaine methanesulphonate (MS-222), rinsed with distilled water,
168 weighed, immediately frozen in liquid nitrogen and stored at -80 ºC for later use.
169

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170 *2.2. Total protein content and alkaline phosphatase activity*

171 Total protein content and alkaline phosphatase activity were determined in cytosolic
172 extracts. At least three larvae from each batch, collected on 48, 66 and 73 dph, were
173 pooled and lysed in buffer containing 20 mM Tris-HCl, pH 7.6, 0.5 sucrose, 0.15 M
174 KCl, 20 mM DTT, 1 mM PMSF and protease inhibitors (Sigma, P2714) at a ratio of 3
175 ml/g. The mixture was manually homogenized with a plastic pestle, and cell debris was
176 cleared by centrifugation (14000 x *g*, 10 min, 4 ºC). The supernatant was treated with
177 Benzonase and ultracentrifuged (100000 x *g*, 60 min, 4 ºC) as described [16]. Protein
178 content was determined by the Bradford method [17] using Coomassie Brilliant Blue R-
179 250 staining (Bio-Rad Laboratories, Hercules, CA). Following a spectrophotometric
180 assay, alkaline phosphatase activity was determined using a commercial kit
181 (Biosystems, Barcelona, Spain) according to the manufacturer's instructions. In brief,
182 the kit employs a 2-amino-2- methyl-1-propanol (AMP) buffer. Alkaline phosphatase
183 catalyses the hydrolysis of the colourless organic phosphate ester substrate, p-
184 nitrophenylphosphate, to give p-nitrophenol and inorganic phosphate, which is
185 transferred to AMP. At the pH of the assay (alkaline), the p-nitrophenol is in the yellow
186 phenoxide form. The rate of absorbance increase at 404 nm is directly proportional to
187 the alkaline phosphatase activity in the sample [18].
188

189 *2.3. Intestinal microbiota analysis*

190 Six larvae from each batch were collected on days 48, 66 and 73 dph, and the
191 digestive microbiota was studied. The whole intestines were aseptically removed and
192 stored at –20 °C until further analysis. The intestinal contents were homogenized in 1
193 ml of PBS (pH 7.2), and a 1 ml aliquot was centrifuged at 1000 × *g* for 5 min. Total
194 DNA was extracted from the samples according to Martínez et al. [19], with some

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195 modifications as described by Tapia-Paniagua et al. [20]. Agarose gel (1.5 % [wt/vol])
196 electrophoresis in the presence of ethidium bromide was used to visually check for
197 DNA quality and yield. DNA from an axenic culture of SpPdp11 in TSB was used as a
198 positive control for the presence of the probiotics in intestinal samples.
199 DNA was amplified using the 16S rDNA bacterial domain-specific primers 677-GC-R
200 (5´-CGGGGCGGGGCACGGGGGGATMTCTACGCATTTCACCGCTAC-3´) and
201 309-F (5´-ATCCCTACGGGAGGCWGCAG-3´). Primer 677-CG-F carries a 35-bp GC
202 clamp. Both primers amplify the V6-V8 regions of 16S rDNA and yield 470-bp
203 amplicons. The PCR mixes and conditions were as previously described [20]. The
204 amplicons obtained were separated by denaturing gradient gel electrophoresis (DGGE)
205 according to the specifications of Muyzer et al. [21] using a Dcode^{TM} system (Bio-Rad
206 Laboratories, Hercules, CA). The gels were subsequently stained with $AgNO_3$ [22]. A
207 DGGE analysis of all samples was performed twice. The structural diversity of the
208 microbial community was determined on the basis of DGGE patterns, which were
209 analysed using the Software FPQuest version 4.0 (Applied Maths BVBA, Sint-Martens-
210 Latem, Belgium). A similarity matrix using the Bray–Curtis dissimilarity index was
211 calculated for densitometric curves. Clustering of DGGE patterns was achieved by
212 construction of dendrograms using the unweighted pair groups method with arithmetic
213 averages (UPGMA). To be able to compare intestinal microbial communities, we
214 evaluated several parameters: (1) species richness (R), based on the total number of
215 bands; (2) Shannon diversity index (H´), according to the function $H' = -\Sigma \, Pi \log Pi$,
216 where Pi is defined as (ni/N), ni is the peak surface of each band, and N is the sum of
217 the peak surfaces of all bands; and (3) range-weighted richness (Rr) [23], calculated as
218 the total number of bands multiplied by the percentage of denaturing gradient needed to
219 describe the total diversity of the sample analysed, following the formula: $Rr = (N^2 \times$

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220 Dg), where N represents the total number of bands in the pattern, and Dg the denaturing
221 gradient between the first and the last band of the pattern.
222

223 *2.4. Immune parameters*

224 The lysozyme activity of samples was measured following a turbidimetric assay
225 based on the ability of lysozyme to lyse the bacteria *Micrococcus lysodeikticus* [24]
226 using the *Lysozyme Detection Kit* (Sigma, LY0100), according to the manufacturer's
227 instructions. Briefly, serial dilutions of samples in potassium phosphate buffer (66 mM;
228 pH 6,24) were mixed with a solution of *Micrococcus lysodeikticus* in the same buffer
229 (0.1 mg/ml). The reaction was carried out a 25ºC and the absorbance at 450 nm
230 recorded for 5 min. One unit of lysozyme activity was defined as the reduction in
231 absorbance of 0.001 min^{-1}. The amount of lysozyme in the samples was calculated form
232 a standard curve made with chicken egg white lysozyme serially diluted in potassium
233 phosphate buffer (66 mM; pH 6,24) and the results were expressed as U/mg of protein.
234 Alternative complement pathway (ACH50) activity was measured using rabbit red
235 blood cells (RaRBC) as target cells as described by Sunyer and Tort [25]. ACH50 units
236 were defined as the concentration of larval extract giving 50% lysis of RaRBC. Disc-
237 assay method [26] was employed to test bactericidal activity. One marine pathogenic
238 (*Vibrio harveyi*) and one non-pathogenic bacteria (*Escherichia coli*) were used. Sterile
239 filter paper discs (Whatman®, diam. 6 mm) were impregnated with extracts from 73
240 dph specimens and then placed on tryptic soy (TSB, Sigma) or Luria (LB, Sigma) agar
241 plates inoculated with *V. harveyi* or *E. coli*, respectively. Plates were incubated for 48 h
242 at 22 ºC and the presence of inhibition zones (haloes) was registered and measured.
243

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244 *2.5. Absolute transcript quantification by real-time qRT-PCR*

245 In the transcriptional expression studies, larvae were sampled at 23 and 73 dph. RNA
246 preparation, cDNA synthesis and real-time qRT-PCR were performed as previously
247 described [27-29]. Total RNA was extracted from pooled specimens (≥ 3
248 individuals/pool, 3 pools/condition), using an AllPrep DNA/RNA/Protein Mini Kit
249 (Qiagen Inc., CA, USA) according to the manufacturer's instructions. Genomic DNA
250 contamination was avoided by subsequent cleanup with RNeasy reagents (Qiagen Inc.).
251 RNA integrity was checked using an Agilent 2100 Bioanalyzer and the concentrations
252 were determined by spectrophotometry. Reverse transcription was carried out with 2 μg
253 of total RNA using the M-MLV reverse transcriptase and random hexamers (Invitrogen,
254 Spain). Real-time PCR reactions were performed in a volume of 25 μl with 50 ng/well
255 of cDNA. In addition, the reaction mixture contained 0.3 μM of each primer, 3 mM
256 $MgCl_2$, 250 μM of each DNT, 0.75 units of Platinum Taq DNA polymerase and
257 1:100000 SYBR Green I dye/fluorescein (Roche, Spain). Reactions were analysed in
258 quadruplicate on a CFX96 Real-Time PCR System (Bio-Rad Laboratories), and the
259 cycling conditions were as follows: an initial denaturation cycle of 95ºC for 2 min for
260 Platinum Taq activation, followed by 40 cycles of 95 ºC for 15 s (melting) and 70 ºC for
261 30 s (annealing/extension). After denaturation at 95 ºC for 15 s and cooling to 60 ºC for
262 30 s, a melt curve (Tm) was generated by heating the samples from 60 ºC to 95 ºC with
263 0.5 ºC increments and 10 s dwell time, and fluorescence was read at each temperature to
264 confirm the specificity of the reactions for the amplified products. The absolute
265 quantification of a set of 18 transcripts was carried out (Supplementary Table 1). These
266 transcripts are involved in growth (GH, SMTL, IGF-I and IGF-IR), encode proteases
267 (CPA1, TRYP1, CTSZ and PMSD3), or play key roles in stress response (CEBPB,
268 HSP70, HSP90A and HSP90B) and immunity (C7, HAMP1, HP, LECT2, NCCRP1 and

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269 TNFAIP9). The primers used in this work are listed in Table 1. Some of these primer
270 pairs were described and used in previous studies [5, 28, 30], but seven of them were
271 designed especially for this work with the software Oligo 7 (Molecular Biology Insights
272 Inc., Colorado Springs, CO) according to known restrictions (high Tm ≥ 80 ºC, optimal
273 3-ΔG value ≥ 3 kcal mol^{-1}, and no hairpins or duplexes) to obtain high specificity and
274 performance. Primers designed with these guidelines yielded PCR products of the
275 predicted size that were further verified by nucleotide sequencing. No primer dimers
276 were detected. The absolute quantification relates the Ct value in real-time PCR to the
277 input copy number using a calibration curve ($y = -3.326x + 39.693$; $r^2 = 1.00$; 100 %
278 efficiency) that was constructed with an *in vitro*-synthesized RNA as previously
279 described [29]. The reliability of an absolute quantification depends on identical
280 amplification efficiencies for both the target and the calibrator. All primers amplified
281 with the same optimal PCR efficiency (~100 %) in the range of 20 pg to 2×10^5 pg of
282 total RNA input with high linearity ($r \geq 0.98$) (Supplementary Table 2 and [5, 28, 30]).
283 The number of transcript molecules was calculated from the linear regression of the
284 calibration curve as described [27-29].
285

286 *2.6. Statistics*

287 Comparisons were made using Student's *t*-test. The assumption of populations with
288 equal SDs, an underlying assumption of the *t*-test, was assessed with an F-test. When
289 the difference between the two SDs was significant, the means were compared with an
290 alternative non-parametric test (the Mann-Whitney test). Significant differences were
291 defined at $p < 0.05$.

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292 ## 3. Results and Discussion

293 Recent studies have demonstrated the beneficial properties of the probiotic *S.*
294 *putrefaciens* Pdp11 (SpPdp11) administered during Senegalese sole larviculture, but
295 although the durations of the probiotic pulses in these studies are variable, they were
296 always centred around metamorphosis and post-metamorphic stages [4, 12]. In addition,
297 little is known about the molecular mechanisms that support these positive effects.
298 Thus, this work focusses on clarifying the molecular mechanisms underlying the effects
299 of short pulses of the probiotic SpPdp11 administered during early stages of *S.*
300 *senegalensis* larval development with special emphasis on the persistence of these
301 effects along the development of this flatfish.
302

303 ### *3.1. Effect of the probiotic SpPdp11 on growth and protein content*

304 The effect of the probiotic diets on weight along different stages of post-
305 metamorphic development (23, 48, 66 and 73 dph) was evaluated (Fig. 1). The CP
306 group (larvae fed the probiotic during metamorphosis, 10-21 dph) weighed slightly
307 more than the control group, although the differences were not statistically significant at
308 any of the tested times. However, the probiotic administered from the first exogenous
309 feeding (to the PP group) promoted a statistically significant growth enhancement from
310 48 dph, and the differences increased with time. Additionally, these specimens showed
311 less weight dispersion even at the earliest timepoint of 23 dph, when the differences
312 from the control group were not statistically significant (Supplementary Table 3). These
313 data agree with a previous study [12] that described a similar effect, increasing the
314 growth and decreasing the size heterogeneity of *S. senegalensis*, when SpPdp11 was
315 administered in the diet for a longer period of time (10 to 86 dph). These results were
316 related to superior nutrient utilization and digestion. In this sense, it is well known that

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317 weaning is a critical period in sole cultivation that usually causes a reduction in growth
318 rates because of post-larval digestive adaptation to an inert diet [31, 32]. Nevertheless,
319 in the above-referenced paper, Lobo et al. [12] demonstrated that growth was
320 significantly enhanced in sole fed with SpPdp11 during this period. Here, we have
321 corroborated these results and established that the effect of probiotic administration in
322 early phases of larval development persists even beyond weaning, continuing 52 days
323 after the end of the treatment, in agreement with a similar persistent effect of probiotic
324 feeding on the modulation of larval intestinal microbiota [4]. The durability of the
325 probiotic effects both on growth (Fig. 1) and on growth dispersion (Supplementary
326 Table 3) can be very valuable, on one hand, for the next culture steps based on an inert
327 diet and, on the other hand, for preventing the stress caused by manipulation during the
328 size classification of the animals in the aquaculture farms [12].

329 Table 2 shows the total protein content at 48, 66 and 73 dph in soles receiving the
330 probiotic (CP and PP) or the standard control diet (CC). The CP group showed no
331 statistically significant difference from the CC group. By contrast, the animals that
332 received the probiotic from the first exogenous feeding (PP group) presented a
333 significant increase in total protein content at every sampling time, although the
334 statistical significance was greater at the two later timepoints (66 and 73 dph). These
335 results agree with other works in sole larvae that received SpPdp11 during development
336 [12], as well as in juveniles fed with the probiotic for 60 days [33]. The elevated protein
337 content in the PP animals (Table 2) could be the cause of the significantly enhanced
338 growth of these specimens, and it constitutes an important nutritional advantage that can
339 increase the commercial value of these fish.

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341 *3.2. Effect of the probiotic SpPdp11 on alkaline phosphatase activity and*
342 *intestinal microbiota composition*

343 Alkaline phosphatase (ALP) (EC 3.1.3.1) catalyses the hydrolysis of the phosphoric
344 ester bond between a phosphate group and an organic radical at an alkaline pH for
345 various types of organic molecules, such as nucleotides and proteins. ALP is involved in
346 the defence and integrity of the intestinal mucosa, preventing inflammation that could
347 provoke the establishment of intestinal microflora [34, 35]. Moreover, the levels of ALP
348 are related to the functionality of the membranes of the enterocytes and to the processes
349 of absorption through the intestinal epithelium [33]. Therefore, it seemed of interest to
350 explore whether the administration of the probiotic SpPdp11 to *S. senegalensis* larvae
351 altered the levels of ALP activity. Table 3 shows the levels of ALP activity detected in
352 post-larvae at 48, 66 and 73 dph in the experimental groups CC, CP and PP. First, it is
353 remarkable that the levels of ALP activity in control animals were very similar at all
354 three timepoints examined, indicating that, in standard conditions, intestinal
355 functionality was similar in this age range. Regarding the effect of the probiotic, the CP
356 group (receiving the shortest probiotic pulse) did not show statistically significant
357 differences from the CC group (control) at any time. However, the PP group (receiving
358 the largest probiotic pulse) showed a significant increase in ALP activity at 48 and 73
359 dph in comparison to their corresponding controls. At 66 dph, this increase in activity
360 was not statistically significant, probably owing to the greater variability in the control
361 group. The enhancing effect of SpPdp11 on ALP activity would not be in agreement
362 with the results described by Sáez de Rodrigáñez et al. [33] who did not report
363 significant changes in the activity of this enzyme in juvenile Senegalese sole specimens
364 fed this probiotic during 60 days. Therefore, the administration of the probiotic
365 SpPdp11 in early stages of *S. senegalensis* larval development leads to an increase in

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366 alkaline phosphatase activity in post-larvae, which could be indicative of an increase in
367 enterocyte functionality and intestinal absorption and, consequently, of a more efficient
368 nutritive utilization of feeds. The increase in ALP activity would be in line with the
369 effect of the dietary administration of this probiotic on weight and total protein content
370 discussed above.

371 Taken together, these results could be associated with the ability showed by *S.*
372 *putrefaciens* Pdp11 to colonize the intestine of larvae and juveniles of *S. senegalensis*
373 [4, 12, 20], as well as in other fish species such as gilthead seabream (*S. aurata*) [8]. In
374 agreement with this suggestion, the analysis based on the Bray-Curtis similarity of
375 DGGE patterns from intestines of fish showed clearly defined clusters at 73 dph (Fig.
376 2), showing significant differences ($p < 0.002$) among the CC, CP and PP groups. These
377 differences were concordant with significant variations in the ecological parameters
378 analysed at 73 dph (Table 4). In this way, the CP group showed significant reductions in
379 the values of Shannon's diversity index (H´) and range-weighted richness (Rr)
380 compared with the CC group. By contrast, fish in the PP group showed significant
381 increases regard to all ecological parameters and, in addition, also showed significant
382 increases in these parameters when compared with fish of the CP group. The number,
383 evenness and genetic variability of species of an ecosystem are important for its
384 functionality and stability, because they protect against alterations of its functionality
385 [36, 37], allowing adaptation to changing conditions [38]. According to this idea, the
386 administration of the probiotic SpPdp11 to larvae of Senegalese sole from 2 to 21 dph
387 induced shifts in the diversity and genetic variability of the intestinal bacterial
388 microbiota, which could be beneficial. In previous studies similar results were obtained
389 enhancing the development of the gastrointestinal tract and histological changes in the
390 enterocytes producing a greater functionality [8, 20]. In this context, in this study an

391 increase of the activity of ALP has been observed at 73 dph, and it could be also
392 associated with the changes observed in the intestinal microbiota.

393 Significant decreases of the intestinal alkaline phosphatase have been associated
394 with factors causing dysbiosis [39, 40], whereas in other studies it has been
395 demonstrated that this enzyme contributed to the maintenance of the intestinal
396 microbiota homeostasis, because the oral administration of intestinal alkaline
397 phosphatase to mice under antibiotic treatment enhanced the restoration of the gut
398 microbiota [41]. On the other hand, significant increases of intestinal and serum alkaline
399 phosphatase have been reported in fish fed diets supplemented with probiotic strains
400 and showing changes in their intestinal microbiota [42, 43]. In addition, the results of
401 this study contrast with those previously obtained and which reported that APL activity
402 was not affected when the probiotic SpPdp11 was orally administered to larvae and
403 juvenile specimens of *S. senegalensis* from 10 to 30 dph [33, 44].

404 Therefore, the results of this work suggest that the administration of SpPdp11 during
405 the early life stages of farmed Senegalese sole would have a high impact on the
406 gastrointestinal tract, favouring greater intestinal function, improving the digestive
407 processes and producing an increase in nutrient absorption, which is retained even after
408 the discontinuation of the treatment and would result in a higher weight and protein
409 content in the specimens fed with the probiotic from the first exogenous feeding.
410

411 *3.3. Effect of the probiotic SpPdp11 on immune parameters*

412 During early development stages fish only possess their innate immune response to
413 respond against stress situations and infections. To evaluate the effect of the probiotic
414 on the Senegalese sole innate immunity, lysozyme, alternative complement pathway
415 and antibacterial activities were measured in extracts from sole post-larvae receiving the

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416 two probiotic (CP and PP) or the control diet (CC) (Tables 5 to 7). Lysozyme activity is
417 frequently used as an indicator of innate immunity of fish, and its levels can vary
418 depending on age and size, nutritional status and infections, among other factors [45]. In
419 fact, several studies showed enhanced lyzozyme activity in several species of fish fed
420 with live and dead probiotics (e.g. [46]). In the current work, at the three ages studied
421 (48, 66 and 73 dph), fish in the PP group showed higher lysozyme levels when
422 compared with fish in the CC group, but statistically significant differences were only
423 found at 73 dph (Table 5). On the contrary, the alternative complement pathway activity
424 was significantly enhanced in all the groups that received the probiotic (Table 6). The
425 complement is a major part of innate immunity in teleost fish and is well known for
426 being responsible for various functions including elimination of invading pathogens,
427 inflammatory responses and clearance of cell debris. In addition, complement system
428 activation contributed significantly to fish immunity by enhancing the adaptive immune
429 response [47]. Hence, the administration of the probiotic SpPdp11 to sole larvae may
430 protect fish against future exposures to pathogens or even produce better responses to
431 immunization with vaccines. We have also tested the antibacterial activity of whole sole
432 extracts against both pathogenic and non-pathogenic bacteria (Table 7). The pathogenic
433 species tested in this study, *Vibrio harveyi*, can cause vibriosis one of the most
434 economically important diseases in sole larviculture and aquaculture [48]. Fish fed the
435 probiotic diets pointed to an inhibitory effect on the growth of *V. harveyi* compared with
436 the results obtained from the control fish, however *E. coli* viability was unaffected.
437 Despite the absence of significant differences, probably due to the high variability
438 exhibited by the control soles, the inhibitory effect against *V. harveyi* in the PP
439 treatment was about twice higher than in the control CC group.
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441 *3.4. Absolute transcriptional expression profiles of selected genes*

442 Although proteins are the final gene products, gene transcription is a highly regulated
443 critical step; therefore, analysis of transcriptional expression patterns provides
444 information of great interest about the coordinated functioning of genes in response to
445 different environmental, physiological and pathological variables [49]. For this reason,
446 the absolute quantification of a set of 18 transcripts was carried out. The transcripts
447 were selected for the relevance of the encoded proteins to different biological functions
448 related to probiotic administration effects or by their role in general fish welfare and
449 health. Although according to their primary function, these transcripts can be classified
450 as related to growth, encoding proteases (including several digestive enzymes), and
451 implicated in or immune response, most of them are indirectly involved in immunity or
452 have potential immune roles. These analyses investigated the effect of probiotic pulses
453 at 23 dph, that is, immediately after its administration, and at the more distant time
454 period of 73 dph, to investigate the persistence of probiotic effects on transcriptional
455 expression profiles.
456

457 *3.4.1. Transcriptional expression kinetics along sole development*

458 The absolute transcript quantification (determination of the molecule number / pg of
459 total RNA of each transcript in each sample) carried out in the present study allows us
460 not only to estimate the variation in transcript levels relative to the control, but also to
461 obtain information about the basal levels and about the kinetics of the expression
462 patterns during sole development (Fig. 3 to 6). Supplementary Table 4 compares the
463 steady-state levels of transcripts examined at 23 dph (immediately after the end of
464 metamorphosis) and at 73 dph (one week after the end of weaning). Given the
465 biological relevance of the selected transcripts, several of them have been previously

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466 investigated (e.g., [50-52]) in order to characterize their expression kinetics during
467 Senegalese sole larval development, but most of these studies have focussed on the
468 temporal interval concerning metamorphosis. Consequently, this is the first study that
469 compares the levels of these transcripts in post-metamorphic larvae with specimens in
470 more advanced development stages. As expected, significant differences in abundance
471 were found depending on the transcript. Thus, very abundant (> 500 mol / pg) to
472 infrequent (<1 mol/pg) mRNAs were quantified (Supplementary Table 4). Considering
473 the functional categories, all transcripts related to growth should be considered
474 infrequent, while in the other functional groups there are large case by case differences.
475 Regarding expression patterns over time, 13 of the 18 transcripts analysed showed
476 statistically significant changes in their levels depending on the sampling times, and
477 noticeably, all these changes involved an increase in the oldest animals, although the
478 magnitude of these changes were variable. It is worth mentioning the case of HP, which
479 increased its levels 86.01 times, although the statistical significance of this change was
480 lower than that obtained for other less pronounced increases, probably because of the
481 great interindividual variability observed in this transcript (Supplementary Table 4). By
482 functional categories, regarding genes involved in growth, only SMTL (somatolactin)
483 showed a statistically significant increase in the number of transcripts at 73 dph
484 compared with 23 dph (Fig. 3 and Supplementary Table 4), in agreement with the rise
485 of plasma somatolactin levels associated with the increase of fish size (advancement of
486 age) described in gilthead seabream [53]. The four genes encoding proteases showed a
487 statistically significant increase in their mRNA levels at 73 dph (Fig. 4 and
488 Supplementary Table 4). However, the changes in CPA1 and TRYP1 expression were
489 greater and more significant than those in the other two proteases (CTSZ and PSMD3).
490 CPA1 and TRYP1 encode digestive enzymes; therefore, this increase might lead to
491 further development and functionality of the gastrointestinal tract in older animals. In

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492 the group of genes related to stress response, the levels of CEBPB and HSP90AB
493 transcripts remained unchanged, while HSP70 and HSP90AA showed a significant
494 increase in their mRNA abundance at 73 dph (Fig. 5 and Supplementary Table 4). The
495 different behaviour of transcripts encoding the two cytosolic HSP90 proteins described
496 in sole is consistent with a previous work analysing their transcript levels in sole larvae
497 before and during metamorphosis [52]. In that study and in concordance with our
498 results, HSP90AB mRNA levels hardly varied through-out pre-metamorphosis and
499 metamorphosis, while HSP90AA increased before metamorphosis and dropped
500 progressively until 15-16 dph, and this was followed by mRNA increasing until the end
501 of metamorphosis. Along these lines, the authors also demonstrated that, in fish, the
502 expression of both HSP90 genes is tightly and differentially regulated under diverse
503 stressors and in response to different hormonal factors [52]. Finally, all transcripts
504 related to immune response showed a significant increase in their levels in animals from
505 73 dph compared with 23 dph, with C7 and LECT2 presenting the highest significance
506 (Fig. 6 and Supplementary Table 4). This statistically significant increase in the levels
507 of the immune response-related transcripts at the latest time would indicate a general
508 and nonspecific maturation and improvement of the immune system along the
509 development of Senegalese sole.
510
511 *3.4.2. Effect of the probiotic SpPdp11 on transcriptional expression*

512 The effects of the dietary administration of the probiotic SpPdp11 during early
513 development stages in *S. senegalensis* on the transcriptional expression profiles of the
514 selected transcripts are presented in Fig. 3 to 6, where the quantified transcripts are
515 shown grouped according to their primary biological functions. In all cases, the
516 specimens from the two modalities of the probiotic administration, CP (short pulse from

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517 10-21 dph) and PP (long pulse from 2-21 dph) were compared with control animals
518 (CC) receiving the standard diet without probiotic. In addition, the effect immediately
519 after finishing the administration of the probiotic (23 dph) and its prevalence over time
520 (73 dph) were studied. Overall, it should be noted that the inter-individual variation of
521 most analysed transcripts was in the same range in control and in probiotic treated
522 animals, indicating an equivalent susceptibility of individuals to the treatments.
523 Here, the expression of four genes involved in the endocrine growth axis of sole has
524 been analysed. These genes are GH (growth hormone), SMTL (somatolactin), IGF-I
525 (insulin-like growth factor-I) and IGF-IR (insulin-like growth factor-I receptor) (Fig. 3).
526 The GH / IGF axis regulates several biological functions in fish, including growth,
527 osmoregulation, development and reproduction. IGFs are key factors in the
528 somatotrophic axis, acting as mediators of the action of GH on somatic growth
529 regulation [54]. Moreover, GH is considered a stimulator of innate immune parameters
530 in teleost fish, and numerous studies point to an important role of GH/IGF-I,
531 particularly during immune organ development (reviewed in [55]). To exert their action,
532 the IGFs bind to a membrane receptor, IGF-IR. Thus, the effect of IGF-I can be
533 modulated by altering its own production and release, as well as by adjusting the
534 expression of IGF-IR [56]. Somatolactin is a teleost-specific hormone belonging to the
535 GH / prolactin family. Although its physiological role is not fully understood, both *in*
536 *vivo* and *in vitro* studies have revealed that somatolactin may be involved in acid-base
537 regulation, calcium regulation, stress response, reproduction, control of lipolysis and
538 energy mobilization, and immune response [53, 57, 58]. Despite the functional
539 relationships of the proteins coded for these four genes, each transcript presented a
540 characteristic expression profile (Fig. 3). Hence, GH significantly increased the number
541 of transcripts, at both 23 and 73 dph, but only in PP soles compared with their
542 respective controls. However, the increase was greater in the 73-day-old animals (3.8-

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543 fold increase at 73 dph vs. 1.6-fold at 23 dph). In fact, the increase observed at 73 dph
544 in the CP (a 2.26-fold increase with respect to its control group) group was probably not
545 significant because of the larger interindividual variability in expression observed in the
546 three experimental groups at 73 dph. Regarding IGF-I and IGF-IR, although the
547 expression of the former remains unchanged in all studied conditions, the expression of
548 IGF-IR does not appear to be affected immediately after the treatment (23 dph), but in
549 the long term (73 dph), a significant increase occurs in both CP and PP animals, and of
550 a very similar magnitude (Fig. 3). According to these results, IGF-I showed no variation
551 in mRNA levels during Senegalese sole larval development [50] and neither among
552 zebrafish larvae showing growth rate variability [59] but IGF-IR mRNA level was
553 positively correlated with growth rates in fish [59]. Therefore, we can conclude that the
554 administration of the probiotic SpPdp11 to *S. senegalensis* larvae causes an increase in
555 the number of transcripts of the GH and IGF-IR genes, and, consequently, an increase in
556 their growth. Less obvious is the case of SMTL, since its expression remains unchanged
557 at 23 dph, whereas its quantity statistically significantly decreased at 73 dph, by which
558 time basal levels had substantially increased because of age. This decrease affects the
559 two groups that received the probiotic, but the reduction was more pronounced in the PP
560 soles, that almost recovered the 23 dph control expression levels (Fig. 3). Recently, it
561 has been described that the administration of SpPdp11 to *S. senegalensis* larvae causes
562 an alteration in the lipid metabolism of the fish, increasing the ratio of proteins to lipids
563 and altering the fatty acid content profiles [12]. This could be related to the role of
564 somatolactin in fish fatty metabolism as an "anti-obesity hormone" [53], and it would be
565 consistent with the highest protein content of probiotic-treated soles (Table 2).
566 The transcripts encoding proteases were CPA1 (carboxypeptidase A1), TRYP1
567 (trypsinogen 1), CTSZ (cathepsin Z) and PMSD3 (proteasome 26S non-ATPase subunit
568 3) (Fig. 4). As stated above, CPA1 and TRYP1 encode digestive enzymes and presented

569 similar transcriptional profiles, whereas the expression patterns of the other two
570 proteases, which are involved in diverse biological functions, were different. Therefore,
571 the levels of the CPA1 and TRYP1 transcripts underwent a statistically significantly
572 increase with the probiotic under both administration protocols (the CP and PP groups)
573 and at both sampling times except for CPA1 expression in the CP group at 73 dph,
574 where the trend was similar, but the increase did not reach statistical significance.
575 Analysis of digestive enzyme levels provides valuable information on nutrient
576 utilization; particularly, the quantification of their mRNA levels has been postulated as
577 a biomarker for evaluation of digestive capacity and growth in the early life stages of
578 Senegalese sole under different dietary sources [60]. Our results thus indicate a positive
579 effect of the probiotic on the utilization and incorporation of nutrients, and therefore, on
580 the general nutritional status of the animals. CTSZ encodes a lysosomal cysteine
581 protease belonging to the cathepsin family. Although the primary role of cathepsins
582 appeared to be intracellular protein degradation and turnover, several studies suggest
583 that these enzymes may also play more specific functions related to polypeptide chain
584 processing, bone modelling, proenzyme activation, etc. ([61] and references within).
585 CSTZ levels were not affected by probiotic administration at 23 dph. However, at 73
586 dph, the sole larvae that received the long probiotic pulse showed a significant increase
587 in the CSTZ mRNA expression (Fig. 4). Accordingly, changes in CSTZ expression
588 level have been observed in trout as an adaptation to specific plant-based diets [62].
589 PSMD3 codes for an essential non-ATPase regulatory subunit at the proteasome lid.
590 The proteasome is a large cytosolic protease complex that degrades unnecessary or
591 damaged proteins. The PMSD3 gene had significantly increased transcriptional
592 expression at the two sampling times (23 and 73 dph), but only in the PP group (Fig. 4).
593 So far, in fish, only one paper exists describing that PMSD3 transcript level was
594 increased in response to metal-induced stress or against LPS (a mimetic of bacterial

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595 infections) [30]. Nonetheless, these data are in line with a recent paper in gilthead
596 seabream (*S. aurata*) suggesting that cathepsins and the ubiquitin-proteasome proteins
597 genes are co-ordinately regulated during ontogeny and indicating that feeding regimens
598 can modulate their expression [63].

599 The genes related to the stress response studied in this work were CEBPB, HSP70,
600 HSP90AA, HSP90AB (Fig. 5). CEBPB codes for a transcription factor (CCAAT
601 enhancer binding protein beta) that regulates essential biological functions such as cell
602 cycle, cell proliferation, haematopoiesis, differentiation, adipogenesis, inflammation and
603 immune response [64, 65]. In fish, CEBPB exhibits high transcriptional diversity and
604 plasticity, and consequently its expression is induced by different stimuli including
605 hyperosmotic stress [66], metal exposition and infections [28]. In agreement with these
606 previous results, CEBPB increased its levels when the probiotic was administered to the
607 larvae, both in the CP group and in the PP group, at 73 dph. However, at the earliest
608 sampling time of 23 dph, the long probiotic pulse (PP group) was necessary to obtain a
609 statistically significant up-regulation (Fig. 5). The other transcripts analysed in the
610 category of stress response encoded heat shock protein 70 (HSP70) and heat shock
611 protein 90 (HSP90), which are multifunction chaperons mainly involved in protein
612 folding that prevent protein aggregation by directing poorly folded proteins to specific
613 degradation pathways and regulating the activity of other cytosolic proteins. Thus, in
614 fish, as in other organisms, heat shock proteins (HSPs) play a pivotal role in protein
615 homeostasis and in the cellular stress response, being induced in response to a wide
616 variety of stressors [67]. In aquatic animals, HSPs also play and important role in
617 health, mainly through their role in relation to the function of the immune system [68].
618 Furthermore, HSP70 and HSP90 protein expression are considered markers to assess
619 nutritional stress in the early life stages of fish [69]. The expression of HSP70 remained
620 unaffected in the probiotic-treated sole of 23 dph in comparison to the control (Fig. 5),

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621 but at the sampling time of 73 dph, both probiotic-fed groups (CP and PP) showed up-
622 regulation of HSP70 mRNA expression, and the increases were of similar magnitude in
623 both probiotic administration modalities. On the other hand, the transcripts encoding the
624 two cytosolic forms of HSP90 (HSP90AA and HSP90AB) were also induced by the
625 probiotic, but with different expression profiles, in agreement with the differential
626 regulation of the two genes referenced above. HSP90AA showed a significant increase
627 in response to both the short and the long probiotic pulse at 23 dph, while at 73 dph we
628 observed a statistically nonsignificant decreasing trend in HSP90AA expression. By
629 contrast, HSP90AB increased its transcript levels in all tested conditions, although
630 statistical significance was only reached in the PP group, at both sampling times (23 and
631 73 dph). These results agree with previous studies proposing that HSP90AA and
632 HSP90AB are involved in cell proliferation and differentiation, but with different tissue
633 patterns of expression and different response to stress profiles because of a severe
634 differential regulation of their expression [52]. In short, all genes related to stress
635 response increase their transcriptional expression with the administration of the
636 probiotic SpPdp11; this increase lead to greater tolerance of sole against stress
637 situations resulting from many procedures of fish aquaculture [5] and, consequently, to
638 a rise in productivity.
639 The immunity-related genes studies in this work were C7, HP, HAMP1, LECT2,
640 NCCRP1 and TNFAIP9 (Fig. 6). C7 is a component of the complement system and
641 belong to the category of acute-phase proteins (APPs), whose synthesis increases
642 immediately upon inflammatory stimuli [70, 71]. In mammals, TNFAIP9 (tumour
643 necrosis factor-alpha-induced protein 9) acts as a protective anti-inflammatory factor
644 whose expression is induced by TNF-α [72] but as a metalloreductase it can facilitate
645 the cellular uptake of iron [73]. Although the biological functions of fish TNFAIP9 are
646 not well understood yet, previous works in Senegalese sole suggest that it would be

647 regulated more by the inflammatory response than through metal homeostasis [5, 30,
648 74]. Here, C7 and TNFAIP9 transcripts showed similar expression profiles (Fig. 6),
649 with no differential expression in 23 dph samples, while a highly significant increase in
650 the number of transcripts was observed in the two probiotic-fed sole groups at 73 dph.
651 By contrast, HP (haptoglobin) and NCCRP1 (non-specific cytotoxic cell receptor
652 protein 1) could be considered early response genes, given that their transcripts were up-
653 regulated in the experimentally treated specimens at 23 dph independently of the
654 duration of the probiotic pulse, whereas the expression levels of treated and control
655 specimens were similar at 73 dph (Fig. 6). HP is one of the APPs and could contribute
656 to the uptake of iron to hamper microbial growth in defence against bacterial infections
657 in fish. NCCRP1 is a receptor present in non-specific cytotoxic cells (NCC), the teleost
658 equivalent to mammalian NK cells, and the most important cytotoxic cell effectors in
659 the innate immune response of teleost fish [75]. It is a membrane protein with antigen-
660 binding, signalling and transcriptional-activation domains able to recognize the NK
661 antigen on a broad range of target cells [76, 77]. In accordance with these functions, the
662 levels of HP and NCCRP1 transcripts are up-regulated in *S. senegalensis* infected with
663 bacterial pathogens and in response to LPS [5, 28, 78]. HAMP1 (hepcidin 1) is an
664 antimicrobial peptide, a component of the innate immune system, which plays an
665 important role in fish defence against invasive pathogens [79]. The expression of this
666 gene was up-regulated in all tested experimental conditions (both sampling times and
667 both probiotic administration protocols) with respect to the values recorded for control
668 fish, although the increase in the PP group at 23 dph was not statistically significant,
669 probably owing to interindividual variability (Fig. 6). In agreement with our results,
670 HAMP1 expression is influenced by the ration size in juvenile Senegalese sole [80].
671 LECT2 (leucocyte cell-derived chemotaxin 2) encodes a multifunctional protein
672 involved in growth, differentiation, and autoimmune response [81]. Interestingly, this

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673 gene presented a singular expression profile. At the two timepoints analysed, LECT2
674 transcript levels were increased in the two fish groups that received the probiotic (CP
675 and PP). However, this is the only one of the 18 analysed transcripts that showed a
676 greater response in the group that received the probiotic short pulse (CP) than in the
677 group receiving the probiotic for a longer period (Fig. 6). The up-regulation of LECT2
678 mRNA by the probiotic agrees with previous observations in rainbow trout fed different
679 functional diets supplemented with the probiotic *Saccharomyces cerevisiae* or with
680 prebiotic mannan-oligosaccharides [82].

681 On the basis of the results obtained, the probiotic SpPdp11 modulates the expression
682 of most of the immunity-related genes, depending on the duration of the probiotic pulse
683 and on the elapsed time since the treatment. These data are in full agreement with the
684 stimulation of several innate immune parameters that was caused by the dietary
685 administration of the SpPdp11 probiotic along the first life steps of sole development
686 (Tables 5-7). In line with our results and given that a diet supplemented with SpPdp11
687 enhanced disease resistance in Senegalese sole specimens farmed at high stocking
688 densities [5], the administration of SpPdp11 during early stages of larval development
689 of *S. senegalensis* would lead to fish immunostimulation beyond the period of
690 treatment, thus reducing the impact of infections, one of the main causes of economic
691 losses in sole farming.

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693 **4. Conclusions**

694 In short, previous studies showed the ability of the probiotic SpPdp11 to modulate gene
695 expression of juvenile *S. senegalensis* specimens. In this study, it has been demonstrated
696 that the administration of this probiotic during early stages of sole larval development
697 modulates the levels of several transcripts implicated in key cellular functions, notably
698 related to growth, stress response and immunity. The increased expression levels of
699 transcripts involved in stress and immune responses suggest that larval specimens fed
700 the probiotic could be more resistant to stress situations inherent to the aquaculture
701 management, as well as to infectious diseases, in accord with the beneficial effects
702 reported here and in other studies regarding the use of this probiotic in the culture of *S.*
703 *senegalensis*. Overall, the results described in this work reveal that the administration of
704 SpPdp11 to *S. senegalensis* from the first exogenous feeding is a highly promising tool
705 to improve the larval development and the aquaculture of this species and, therefore, to
706 increase its productivity.

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976 **Figure captions**

977

978 **Fig. 1.** Total weight (mg) of Senegalese sole specimens fed the control diet (CC) or a
979 diet supplemented with the probiotic SpPdp11 from 10 to 21 dph (CP) or from 2 to 21
980 dph (PP). Data are means ± SEM (n = 10). Comparisons were made using Student's *t*-
981 test. Statistical significance is expressed as $^{**}p<0.01$.

982

983 **Fig. 2**. Non-metric multidimensional scaling (NMDS) plots using the Bray-Curtis
984 index of the denaturing gradient gel electrophoresis (DGGE) patterns associated with
985 the intestinal microbiota of Senegalese sole larvae at 48 dph (A), 66 dph (B) and 73 dph
986 (C). Larvae received the probiotic SpPdp11 from 10 to 21 dph (CP) and from 2 to 21
987 dph (PP). The control group (CC) was fed a standard diet without probiotics. Each diet
988 was evaluated in triplicate.

989

990 **Fig. 3.** Absolute quantification of transcripts related to growth (GH, SMTL, IGF-I
991 and IGF-IR) in 23 and 73 dph Senegalese sole specimens fed the control diet (CC) or
992 the diets supplemented with the probiotic SpPdp11 from 10 to 21 dph (CP) or from 2 to
993 21 dph (PP). Data are means ± SEM (n = 3 pooled samples of at least 3 specimens per
994 pool). Comparisons were made using Student's *t*-test. Statistical significance is
995 expressed as $^{*}p<0.05$ and $^{**}p<0.01$ compared with the corresponding CC values.

996

997 **Fig. 4.** Absolute quantification of transcripts coding for proteases (CPA1, TRYP1,
998 CTSZ and PMSD3) in 23 and 73 dph Senegalese sole specimens fed the control diet
999 (CC) or a diet supplemented with the probiotic SpPdp11 from 10 to 21 dph (CP) or
1000 from 2 to 21 dph (PP). Data are means ± SEM (n = 3 pooled samples of at least 3
1001 specimens per pool). Comparisons were made using Student's *t*-test. Statistical

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1002 significance is expressed as *p<0.05, **p<0.01 and ***p<0.001 compared with the
1003 corresponding CC values.
1004

1005 **Fig. 5.** Absolute quantification of transcripts related to the stress response (CEBPB,
1006 HSP70, HSP90A and HSP90B) in 23 and 73 dph Senegalese sole specimens fed the
1007 control diet (CC) or a diet supplemented with the probiotic SpPdp11 from 10 to 21 dph
1008 (CP) or from 2 to 21 dph (PP). Data are means ± SEM (n = 3 pooled samples of at least
1009 3 specimens per pool). Comparisons were made using Student's *t*-test. Statistical
1010 significance is expressed as *p<0.05 and **p<0.01 compared with the corresponding
1011 CC values.
1012

1013 **Fig. 6.** Absolute quantification of transcripts related to the immune response (C7,
1014 HAMP1, HP, LECT2, NCCRP1 and TNFAIP9) in 23 and 73 dph Senegalese sole
1015 specimens fed the control diet (CC) or a diet supplemented with the probiotic SpPdp11
1016 from 10 to 21 dph (CP) or from 2 to 21 dph (PP). Data are means ± SEM (n = 3 pooled
1017 samples of at least 3 specimens per pool). Comparisons were made using Student's *t*-
1018 test. Statistical significance is expressed as *p<0.05, **p<0.01 and ***p<0.001
1019 compared with the corresponding CC values.

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Table 1
Primers used for absolute mRNA quantifications by real-time qRT-PCR in *S. senegalensis*.

Gene[a]	GenBank accession No.	Size (bp)[b]	Sequence 5´-3´[c]	Reference
C7	FF682388	78	F: 5´-GGCACACACTATCTGTCGCAGGGCTC-3´ R: 5´-GGCGAACGCCTGATGGTTTAACTCCAG-3´	[25]
CEBPB	FF682571	192	F: 5´-GTGAGGAAGAGCAGGGACAAAGCCAAG-3´ R: 5´-CGCAGGTCACGCTGAGCTGGAG-3´	[25]
CPA1	FF291493	150	F: 5´-CGTCAGAGTTCCCCTCGACAGTCTGGAG-3´ R: 5´-CTGGGCTGAGCGAAGCGAGCAG-3´	This work
CTSZ	FF682475	107	F: 5´-CTGTGGCTCCTGCTGGGCTCAC-3´ R: 5´-GCACATGTTGGACAGACAGATACGCAGAC-3´	[25]
GH	U01143	177	F: 5´-GAGTTGTCATCGTGCTGTCTGTCCTGTCTG-3´ R: 5´-TTTTGTTGACCTGACGTTGATCCTCAGTCTG-3´	This work
HAMP1	FJ263548	173	F: 5´-ACACTCGTGCTCGCCTTTGTTTGCTTC-3´ R: 5´-TGACTCCAGCGTCTGTGTCTGACATGATTC-3´	[27]
HP	FF682335	105	F: 5´-GAAATGTAGACCATGACCTGCCAGGGGAC-3´ R: 5´-TGAAACATCTGTCGCAGCCTCCAGGTTAG-3´	[25]
HSP70	AB513855	157	F: 5´-CCGTGATCTCAACAAGAGCATCAACCCTG-3´ R: 5´-GCCTCCAGCGGTTTCAATTCCCAG-3´	[5]
HSP90AA	AB367526	98	F: 5´-ACCAAGCACAACGATGATGAGCAGTACA-3´ R: 5´-GTGCCTCTGCCAATAGACTCTCCAGTATCAG-3´	This work
HSP90AB	AB367526	148	F: 5´-TCAGTTTGGTGTGGGTTTCTACTCGGCTTAC-3´ R: 5´-GCCAAGGGGCTCACCTGTGTCG-3´	[5]
IGF-I	AB248825	143	F: 5´-GTGCGTCCTCACCCTGACTCCGAC-3´ R: 5´-GCCGTGTGTTGTAGCCATAGCCTGGT-3´	This work
IGF-IR	FJ515914	101	F: 5´-CCTACGCTCCCTCCGACCTAAAGAGCAAC-3´ R: 5´-CCATGCCATCAGCGATCTGTCCTG-3´	This work
LECT2	FF283810	127	F: 5´-GGGTCAGAGGTAGGGTTAGAAGTAGGGCGTG-3´ R: 5´-GAAGGGTCAGAGGATCGGCGTCATG-3´	This work
NCCRP1	FF682594	121	F: 5´-ATGCCCGACAGCCTGGACTGG-3´ R: 5´-CCAACTCAGGTTCAGGTGGAGGGATG-3´	[25]
PSMD3	FF682487	100	F: 5´-GATCGTGGTGGAGCTGTTGTTGGGAG-3´ R: 5´-TTGTGTGAGCAGGAAGTAGGGCATCAGTG-3´	[27]
SMTL	U06753	114	F: 5´-TGTCTCGCTGTCCCTTCATCTCCCAAG-3´ R: 5´-CATGCGTAGCCGACCGTGTTTCTCTG-3´	This work
TNFAIP9	FJ263550	160	F: 5´-CTGGGACTGCTGGCACTTGGATTTG-3´ R: 5´-CAGTTCTCCACGCTGACGTACTGTCGAAC-3´	[27]
TRYP1	AB359189	160	F: 5´-GACAAGATCGTCGGAGGGTATGAGTGCCAG-3´ R: 5´-CGCCCAGACGCACCTCCACAC-3´	This work

[a] Gene symbols are according to the NCBI Gene database.
[b] PCR product size (bp).
[c] Sequences of forward (F) and reverse (R) primers.

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Table 2
Total protein content in Senegalese sole specimens treated with the probiotic SpPdp11.

	Total protein (μg) / weight (mg)[a]		
	48 dph	66 dph	73 dph
CC[b]	19.18 ± 1.03	20.98 ± 0.96	22.06 ± 1.31
CP	20.23 ± 0.65	21.34 ± 0.73	21.25 ± 0.81
PP	23.33 ± 0.44*	27.58 ± 1.40**	32.22 ± 1.48**

[a] Data are means ± SEM (n =3 pooled samples of at least 3 specimens per pool). Comparisons were made using Student's *t*-test. Statistical significance is expressed as *p<0.05 and **p<0.01 compared with the corresponding CC values.

[b] *Solea senegalensis* specimens were fed the control diet (CC) or a diet supplemented with the probiotics from 10 to 21 dph (CP) or from 2 to 21 dph (PP) and were sampled at 48, 66 and 73 dph.

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Table 3
Alkaline phosphatase activity in Senegalese sole specimens treated with the probiotic SpPdp11.

	Alkaline phosphatase activity (mU/mg of protein)[a]		
	48 dph	66 dph	73 dph
CC[b]	13.59 ± 0.61	12.30 ± 1.14	14.14 ± 0.62
CP	14.85 ± 0.69	12.27 ± 0.96	16.90 ± 0.38
PP	19.65 ± 0.89**	15.50 ± 0.35	21.16 ± 1.34**

[a] Data are means ± SEM (n =3 pooled samples of at least 3 specimens per pool). Comparisons were made using Student's *t*-test. Statistical significance is expressed as *p<0.05 and **p<0.01 compared with the corresponding CC values.

[b] *Solea senegalensis* specimens were fed the control diet (CC) or a diet supplemented with the probiotics from 10 to 21 dph (CP) or from 2 to 21 dph (PP) and were sampled at 48, 66 and 73 dph.

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Table 4
Species richness (R), range-weighted richness (Rr) and Shannon diversity index (H´) values of intestinal microbiota DGGE patterns of Senegalese sole larvae[a].

Treatment[b]	dph[b]	R	Rr	H´
CC	48	16.75 ± 5.75	81.25 ± 51.00	2.28 ± 0.05
CP	48	17.62 ± 2.99	78.50 ± 42.50	2.18 ± 0.10
PP	48	15.00 ± 2.10	58.50 ± 51.00	2.35 ± 0.07
CC	66	16.00 ± 2.44	66.25 ± 24.00	2.29 ± 0.08
CP	66	10.00 ± 4.33[d]	31.25 ± 25.00[d]	1.87 ± 0.10[c]
PP	66	23.00 ± 2.70	107.60 ± 27.60[c, d]	2.21 ± 0.14
CC	73	16.00 ± 2.60	61.24 ± 21.00[c]	2.19 ± 0.10
CP	73	11.00 ± 2.13[d]	29.15 ± 15.00[c, d]	1.81 ± 0.06[c, d]
PP	73	25.00 ± 1.80[c]	119.50 ± 16.80[c]	2.25 ± 0.14[c]

[a] Data are means ± standard deviation.

[b] *Solea senegalensis* specimens were fed the control diet (CC) or a diet supplemented with the probiotics from 10 to 21 dph (CP) or from 2 to 21 dph (PP) and were sampled at 48, 66 and 73 dph.

[c] Significant differences compared with the control CC.

[d] Significant differences between the CP and PP groups.

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Table 5
Lysozyme activity in Senegalese sole specimens treated with the probiotic SpPdp11.

	Lysozyme activity (U/mg of protein)[a]		
	48 dph	66 dph	73 dph
CC[b]	37.31 ± 3.89	40.68 ± 4.94	51.18 ± 5.50
CP	39.54 ± 5.82	44.03 ± 6.60	68.97 ± 7.51
PP	42.69 ± 6.15	58.46 ± 7.32	78.97 ± 8.83*

[a] Data are means ± SEM (n =3 pooled samples of at least 3 specimens per pool). Comparisons were made using Student's *t*-test. Statistical significance is expressed as $*p<0.05$ compared with the corresponding CC values.
[b] *Solea senegalensis* specimens were fed the control diet (CC) or a diet supplemented with the probiotics from 10 to 21 dph (CP) or from 2 to 21 dph (PP) and were sampled at 48, 66 and 73 dph.

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Table 6
Alternative complement pathway (ACH50) activity in Senegalese sole specimens treated with the probiotic SpPdp11.

	ACH50 activity (U/mg of protein)[a]		
	48 dph	66 dph	73 dph
CC[b]	30.23 ± 2.25	33.71 ± 4.58	50.32 ± 6.11
CP	52.32 ± 4.08**	47.62 ± 9.72	81.25 ± 7.65*
PP	51.39 ± 5.69*	61.24 ± 7.51*	92.85 ± 8.42*

[a] Data are means ± SEM (n =3 pooled samples of at least 3 specimens per pool). Comparisons were made using Student's *t*-test. Statistical significance is expressed as $*p<0.05$ and $**p<0.01$ compared with the corresponding CC values.

[b] *Solea senegalensis* specimens were fed the control diet (CC) or a diet supplemented with the probiotics from 10 to 21 dph (CP) or from 2 to 21 dph (PP) and were sampled at 48, 66 and 73 dph.

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Table 7
Bactericidal activity in Senegalese sole specimens treated with the probiotic SpPdp11 against pathogenic or non-pathogenic bacteria for sole.

	Disc assay method (mm)[a]		
	CC[b]	CP	PP
V. harveyi	7.2 ± 2.45	10.9 ± 1.01	14.57 ± 1.80*nqs*
E. coli	6.6 ± 0.78	7.3 ± 1.62	7.6 ± 2.31

[a] Data are means ± SEM (n =3 pooled samples of at least 3 specimens per pool).
[b] *Solea senegalensis* specimens were fed the control diet (CC) or a diet supplemented with the probiotics from 10 to 21 dph (CP) or from 2 to 21 dph (PP) and were sampled at 73 dph. Comparisons were made using Student's *t*-test with the corresponding CC values. *nqs*: not quite significant.

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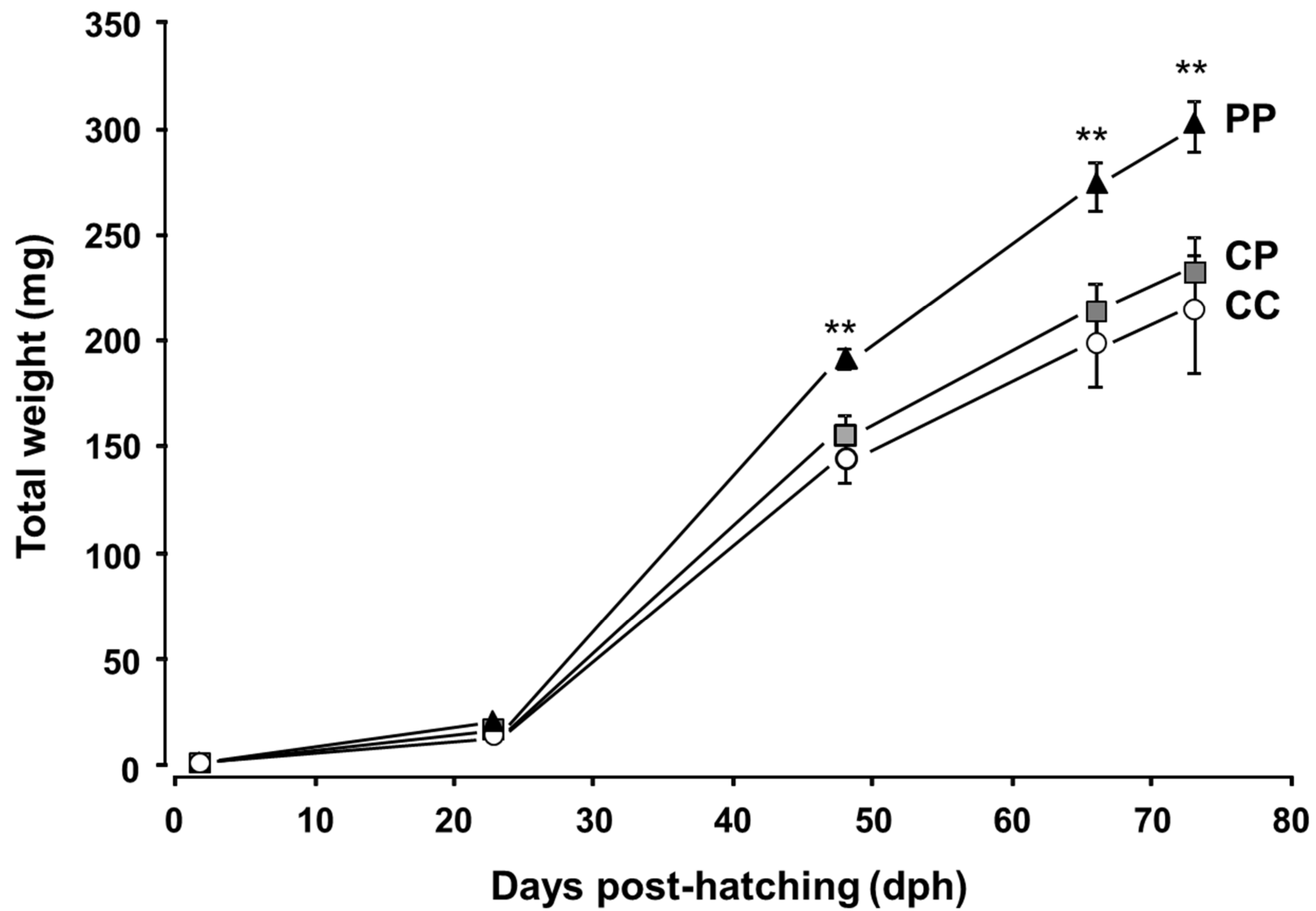

Figure 1

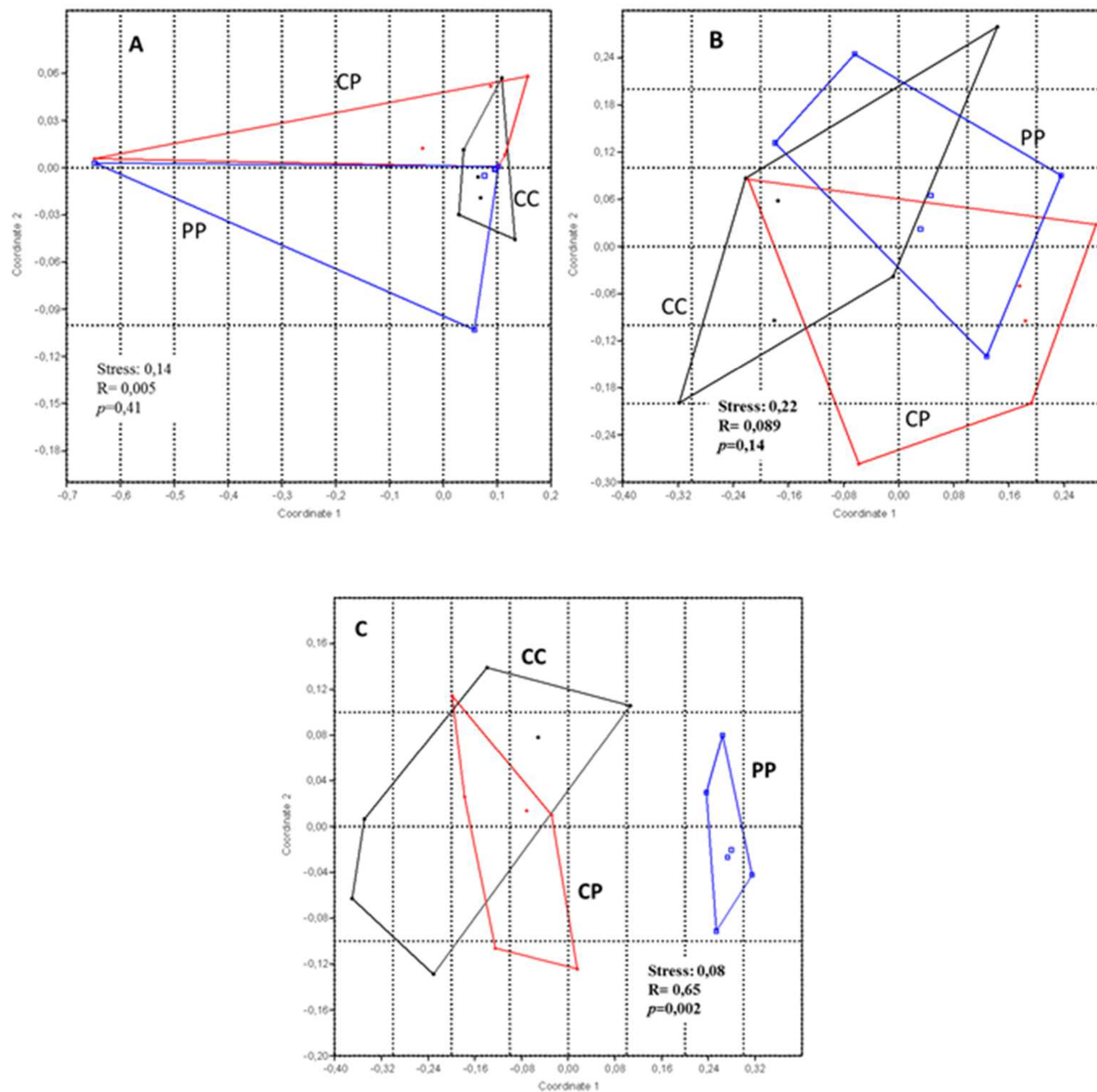

Figure 2

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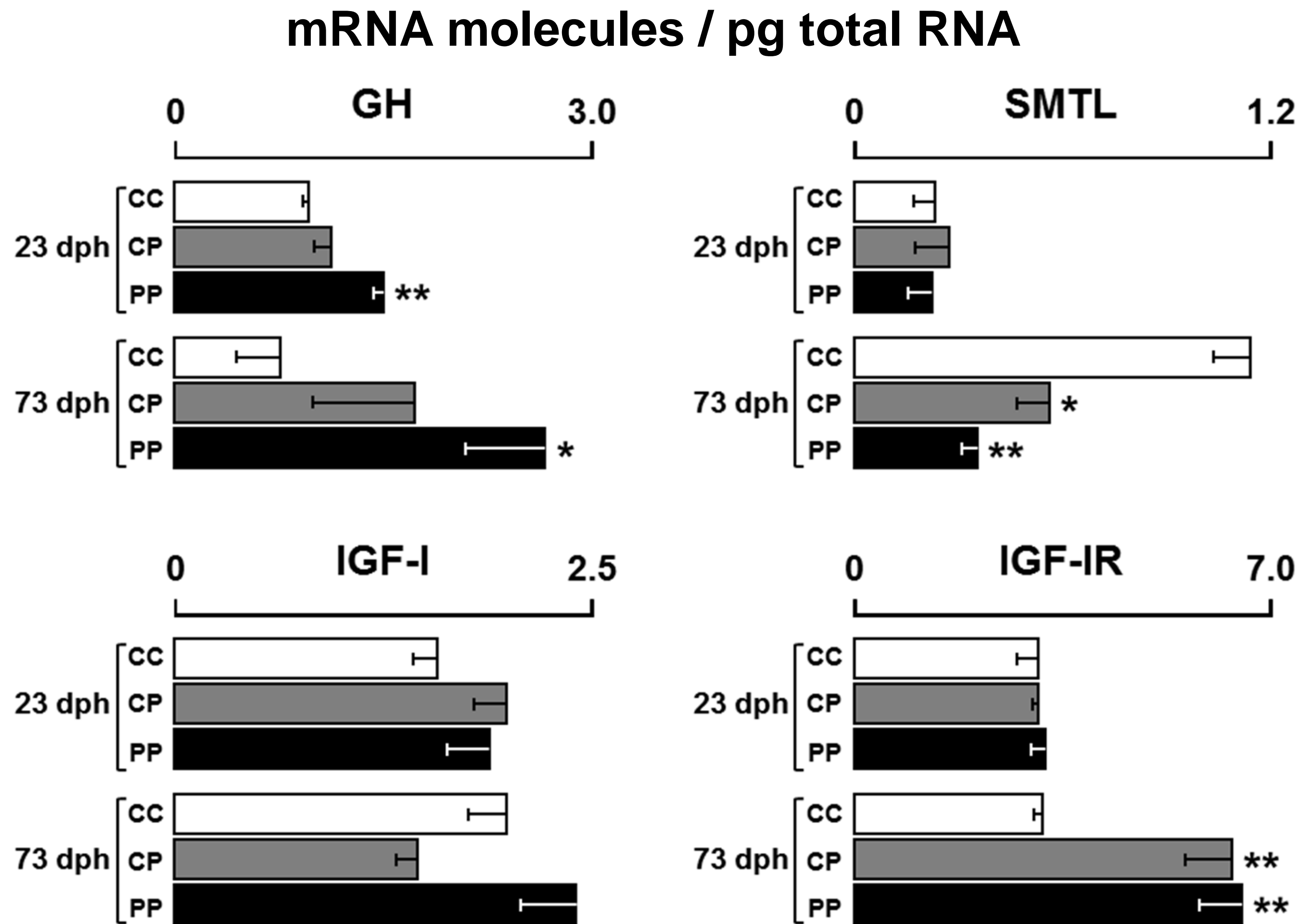

Figure 3

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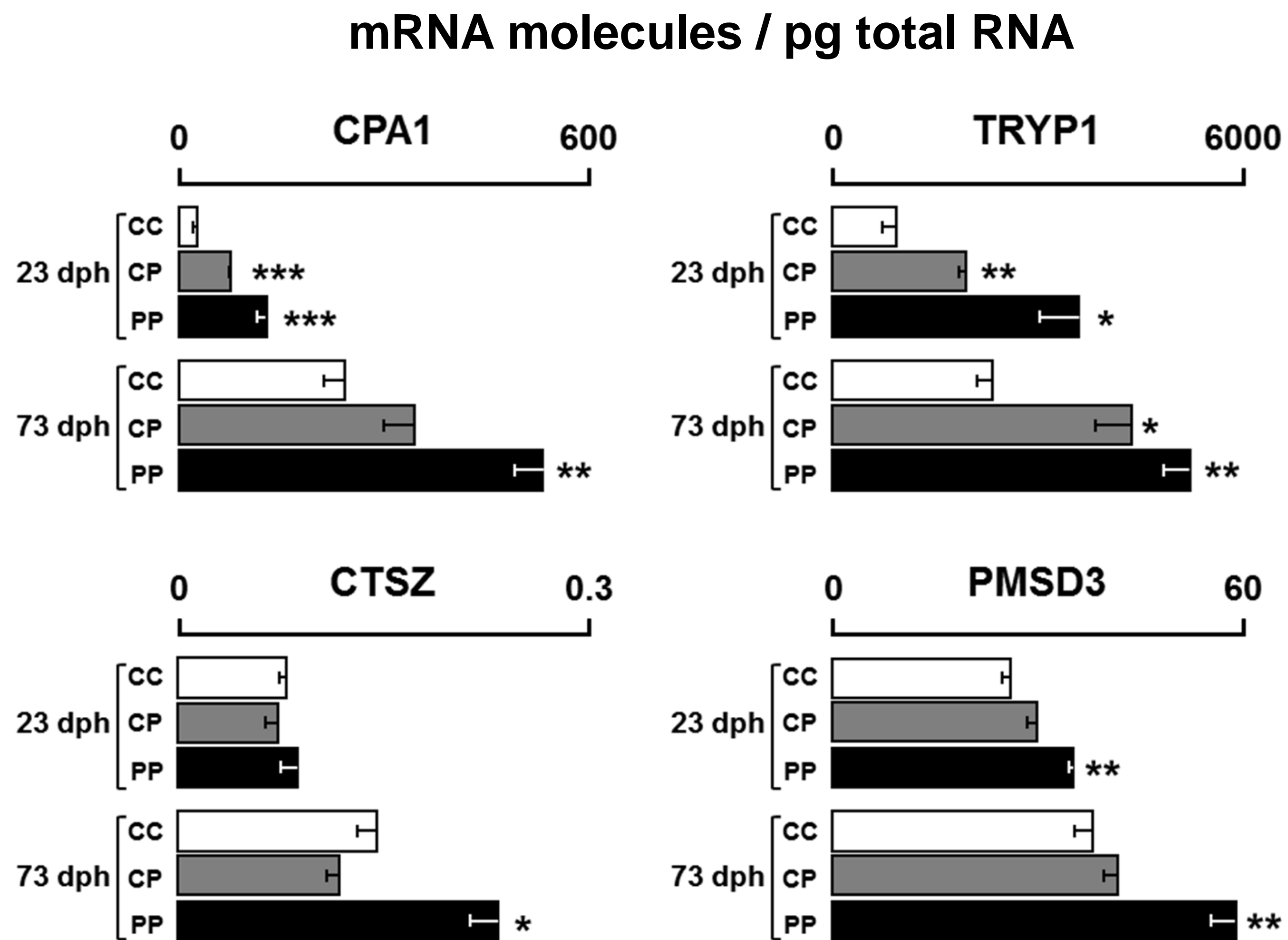

Figure 4

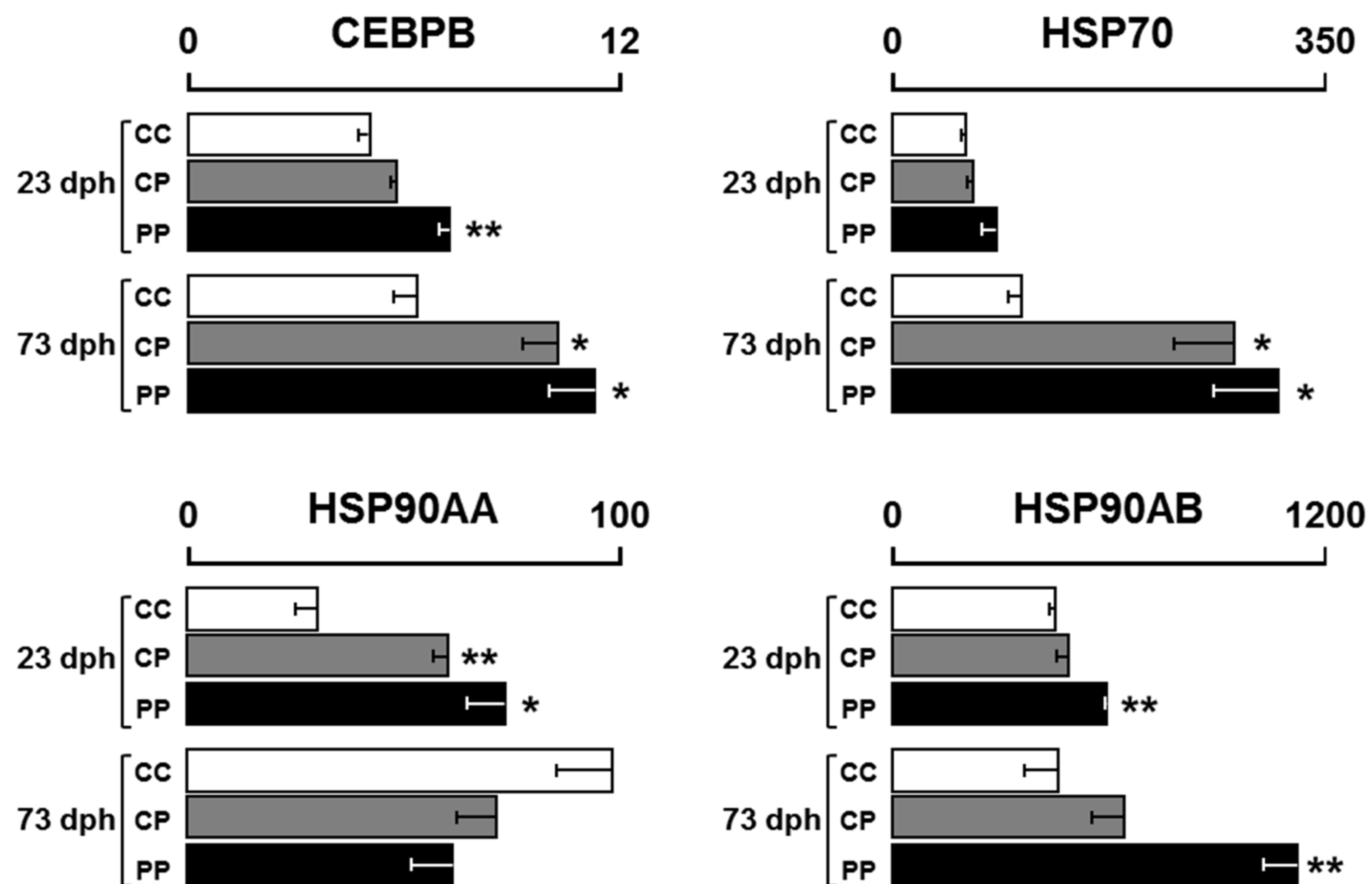

Figure 5

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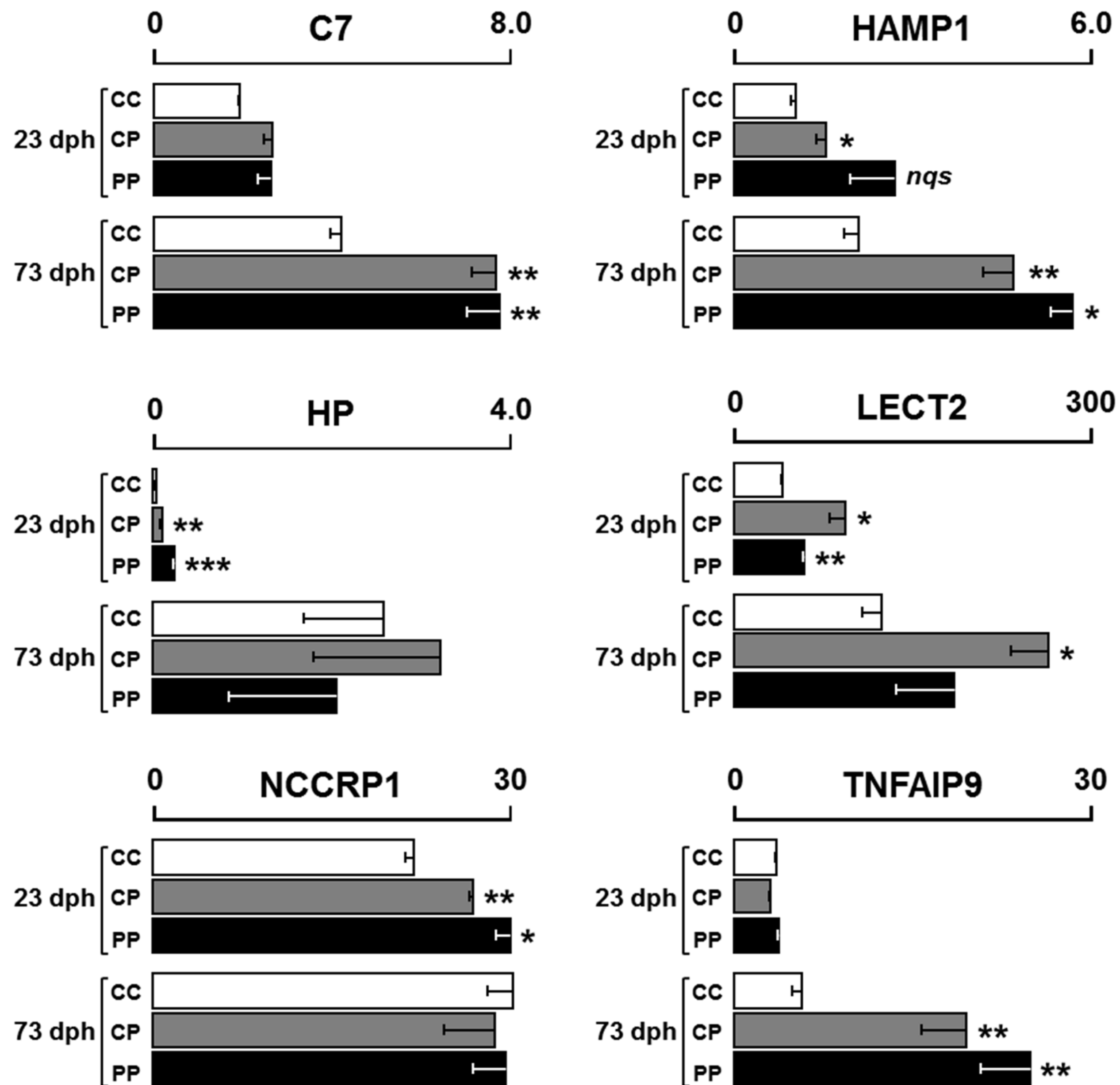

Figure 6

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Highlights

- *S. senegalensis* larvae were fed the probiotic SpPdp11 during early life stages.
- SpPdp11 increased growth and decreased size dispersion in post-larvae.
- SpPdp11 increased ALP activity and affected intestinal microbiota composition.
- SpPdp11 modulated expression of genes involved in growth, stress response and immunity.

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