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Vitamin A supplementation enhances Senegalese sole (*Solea senegalensis*) early juvenile's immunocompetence: new insights on potential underlying pathways

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Abstract :

Senegalese sole (*Solea senegalensis*) has been considered since the 1990´s to be a promising flatfish species for diversifying European marine aquaculture. However, pathogen outbreaks leading to high mortality rates can impair Senegalese sole commercial production at the weaning phase. Different approaches have been shown to improve fish immunocompetence; with this in mind the objective of the work described herein was to determine whether increased levels of dietary vitamin A (VA) improve the immune response in early juveniles of Senegalese sole. For this purpose, Senegalese sole were reared and fed with *Artemia* metanauplii containing increased levels of VA (37,000; 44,666; 82,666 and 203,000 total VA IU Kg^{-1}) from 6 to 60 days post-hatch (early juvenile stage). After an induced bacterial infection with a 50 % lethal dose of *Photobacterium damselae* subsp. *damselae*, survival rate, as well as underlying gene expression of specific immune markers (*C1inh, C3, C9, Lgals1, Hamp, LysC, Prdx1, Steap4* and *Transf*) were evaluated. Results showed that fish fed higher doses of dietary VA were more resistant to the bacterial challenge. The lower mortality was found to be related with differential expression of genes involved in the complement system and iron availability. We suggest that feeding metamorphosed Senegalese sole with 203,000 total VA IU Kg^{-1} might be an effective, inexpensive and environmentally friendly method to improve Senegalese sole immunocompetence, thereby improving survival of juveniles and reducing economic losses.

Highlights

► Dietary vitamin A (VA) enhances immune system in *Solea senegalensis* juveniles. ► Sole fed 203,000 VA IU Kg^{-1} had higher resistance to an induced bacterial infection. ► Complement system and iron homeostasis genes were differentially expressed. ► Dietary VA levels impact on the fish osteoimmunological response.

Keywords : vitamin, retinoic acid, immune system, gene expression, Senegalese sole *Solea senegalensis*, Photobacterium *damselae* subp. *damselae*

1. Introduction

Diversification of marine aquaculture is a major issue, with Senegalese sole (*Solea senegalensis*) being one of the most promising species for aquaculture diversification in the Iberian Peninsula [1]. As recently reviewed [1], an increased understanding has been obtained on the manner in which husbandry practices, environmental conditions (such as rearing temperature, salinity, light spectrum and intensity), genetic background and/or nutrition influences fish physiology. However, despite this research effort, multiple bottlenecks like resistance to pathogen infection, outbreaks of infectious disease, incidence of skeletal deformities, and impaired reproductive performance in captivity still exist [1].

In Senegalese sole farms, a wide array of pathogens has been detected [2], which are commonly treated with antibiotics and/or chemotherapeutics. In particular, *Photobacterium damselae* subsp. *piscicida* (*Phdp*), the aetiologic agent of fish pasteurellosis, induces economic losses [3] and thus, has received significant attention. In contrast, less is known regarding *P. damselae* subsp. *damselae* (*Phdd*), although it is an emerging pathogen in marine aquaculture [4].

Nowadays, more environmentally friendly aquaculture strategies to fight bacterial infections are being sought. In this context, probiotics as well as other immunomodulatory substances like vitamins, minerals and amino acids seem to offer an attractive alternative [5, 6] to chemotherapies. Vitamin A (VA), and its derivatives (retinoids), have pleiotropic roles. Since fish are not able to synthezise VA compounds *de novo*, they have to acquire them from the diet. Their excess or deficiency cause abnormal development/homeostasis in different tissues, organs and/or systems [7]. In particular, VA is widely known to promote the mammalian immune response [8], although the mechanism by which VA effects thisis still not clearly understood. In different fish species, several VA dose-response studies have been conducted to establish the minimum and optimal requirements for the development of some specific tissues/organs [9-17], and suggesting that those requirements seem to be specific for different cell types, tissues, developmental stages and fish species [12]. In contrast, limited work has

been carried out for deciphering the roles and requirements of different VA metabolites in fish immunocompetence [18-23]. Thus, the present study aimed at investigating the effects of different levels of dietary VA content on Senegalese sole immunocompetence against an induced bacterial infection. Furthermore, an expression analysis of representative immune-related genes by relative quantitative PCR (qPCR) has been performed in order to gain some insights on how dietary VA content could affect Senegalese sole resistance to bacterial infection.

2. Materials and methods

2.1 Ethics statement

Present work was carried out in accordance with EU Directive 2010/63/EU for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm), following approved protocols by the institutional ethics committee.

2.2 Fish rearing and experimental diets

Newly hatched larvae of Senegalese sole were obtained from Stolt Sea Farm SA (Cambre, La Coruña, Spain) and shipped to IRTA facilities. After their acclimatization, larvae were distributed in cylindrical tanks (100 L) connected to a recirculation unit (IRTAMar™). Senegalese sole larvae were reared and fed enriched rotifers (Easy Selco^{TM}) first from 3 to 10 days post hatch (dph), after which from 6 dph onwards, larvae were fed with *Artemia* metanauplii containing increased levels of VA (37,000, 44,666, 82,666 and 203,000 total VA IU kg^{-1}; labeled “Control”, VA1, VA2 and VA3, respectively) by adding different amounts of retinyl palmitate (1,600,000 IU g^{-1}, Sigma-Aldrich, Spain) to a commercial enrichment emulsion (Easy Selco^{TM}), as previously described [15]. From 33 dph onwards early juveniles were fed with frozen *Artemia* metanauplii enriched according to the procedures mentioned above.

2.3 Bacterial challenges

Inoculum of *Photobacterium damselae* subsp. *damselae* (ATCC® 33539) (*Phdd*) was grown at 22 ℃ in tryptic soy broth (TSB) or tryptic soy agar (TSA) supplemented with NaCl to a final concentration of 2% (w/v), then stored at -80 ℃ in TSB supplemented with 15% (v/v) glycerol. To prepare the inocula for intramuscular injection, frozen stock of bacteria were inoculated into 10 mL of TSB medium with 2% NaCl and cultured for 24 h at 22 ℃ with continuous shaking (90 rpm). Then, bacteria were inoculated onto TSA medium with 2% NaCl overnight at the same temperature. Colonies from exponentially growing bacteria were resuspended in sterile sea water (SSW) with 2.5% NaCl and the density of the culture was adjusted to McFarland standard of 0.5. From this suspension, 10-fold dilutions ranging from $2*10^1$ to $2*10^6$ CFU mL^{-1} were prepared. Aliquots of the serial dilutions were plated onto TSA 2% NaCl and the number of CFU were counted following incubation at 22 ℃ in order to confirm CFU concentrations. A standard curve for this bacterium was developed by plotting optical density at 610 nm against bacterial counts (CFU mL^{-1}).

Three different challenge trials were performed in this study. In the first trial, the determination of the LD_{50} for *Phdd* in Senegalese sole early juveniles (LD_{50} – *challenge*) was conducted in 45 dph fish fed with 37,000 total VA IU kg^{-1} (Control group). Juveniles were inoculated intramuscularly (10 fish per 1 L aquarium) with 10 µL containing 0 (C), $6*10^1$, $6*10^2$, $6*10^3$, $6*10^4$ and $6*10^5$ CFU mL^{-1} suspended in SSW. Each dose was done in quadruplicate. A second bacterial challenge trial was conducted to determine the resistance to an induced bacterial infection of juveniles (60 dph; 15.4 ± 1.2 mm in standard length) when fed with increasing dietary VA levels (*VA* – *challenge*). Thus, fish from each dietary VA group (n = 7) were intramuscularly injected (10 µL) with the LD_{50} previously calculated ($6*10^2$ CFU mL^{-1}) in quadruplicate. In addition, a Control group was also injected with 10 µL of SSW (Control - Control group). A third bacterial challenge trial was also implemented to get some insights on how VA supplementation could modulate the immunocompetence of Senegalese sole juveniles (75 dph). For this purpose 30 fish from the Control and VA3 groups were inoculated as described in the *VA - challenge* and 5 fish were sampled at 4, 8, 12 and 24 h post-injection

(*Underlying pathways – challenge*). In all trials, fish were fed daily with frozen *Artemia* previously enriched with their respective VA level, while water temperature was maintained at 18.0 ± 1.0 ℃ and with a 50 % water renewal in aquaria. Mortality was recorded on a daily basis up to 18 days post injection. Survival rate (%) was calculated as the final number of inoculated fish / initial number of inoculated fish * 100. All sampled fish were euthanized with an overdose of anaesthetic (Tricaine methanesulfonate, MS-222, Sigma). Fish sampled during the *Underlying pathways – challenge* were individually frozen in RNA later (Ambion®) and stored at -80 ℃ until gene expression analysis. Additionally, to confirm *Phdd* presence/absence, several moribund *Phdd* infected fish, as well as healthy SSW injected specimens were sampled, washed in SSW, directly frozen and stored at -20 ºC until DNA extraction.

2.3 DNA extraction and confirmation of presence of P. damselae subsp. damselae by PCR

Total DNA isolation from fish specimens (infected or not) was performed following a proteinase K lysis buffer protocol. *Phdd* presence/absence were then confirmed by PCR (initial denaturation at 95 ºC for 8 min, followed by 40 cycles of a 1 min denaturation at 94 ºC, annealing at 58 ºC for 1 min, elongation at 72 ºC for 1 min and a final extension of 10 min at 72 ºC) with 16S-like ssrDNA degenerate primers (Supplementary Table S1) and subsequent sequencing of the resulting amplicon.

2.4 RNA extraction, reverse transcription and gene expression analysis

From 3 injected fish collected at each sampling time and from both dietary groups (Control and VA3) total RNA was extracted using TRIzol reagent (Invitrogen®) following manufacturer specifications. The quantity of RNA isolated was determined using a Gene-Quant spectrophotometer (Amersham Biosciences), purity was established by the absorbance ratio 260/280 nm (ranging from 1.7 to 2.0 in all samples). The quality of the RNA was examined using 1.2% TAE agarose gel electrophoresis. A reverse transcription reaction was carried out

using equal quantities of total RNA (1 µg) from each sample and Quanti Tect Reverse Transcription Kit (Qiagen®). Electrophoresis using a 1.2% TAE agarose gel was run to assess the RT-PCR product. Real-time qPCR was performed using an ABI PRISM 7300 (Applied Biosystems). For each gene, species and gene-specific primers were designed according to sequences available in GenBank (NCBI, www.ncbi.nlm.nih.gov) and the SoleaDB databases (www.aquagenet.eu; Supplementary Table S1). The efficiencies of all qPCR reactions were close to 100%. qPCR reactions were performed in triplicate in 96-well plates containing: 10 µL of SYBR® Green PCR Master Mix (Life Technologies), 0.5 µL of forward and reverse primers (10 µM), 7 µL of molecular biology grade water (Sigma), and 2 µL of a 1:10 dilution of cDNA template. Standard amplification parameters were: 95 ºC for 5 min followed by 45 amplification cycles (95 ºC for 45 s, 59 ºC for 1 min and 70 ºC for 1min). A final dissociation reaction (melting curve) was performed (95 ºC for 15 s, 60 ºC for 20 s, 95 ºC for 15 s and 60 ºC for 15 s) to evaluate the specificity of the amplicons. A calibrator sample was included in each qPCR plate [24].

2.5 Data Processing

Relative gene expression was determined from the ratio between PCR efficiency and threshold cycle of a sample compared with the control, and expressed in comparison to the reference gene, according to Pfaffl's mathematical model [25]. Relative gene expression was normalized using ubiquitin (*Ubq*), a previously reported reference gene for accurate normalization in qPCR studies with Senegalese sole [26], and using the gene expression level of the Control group as reference sample.

2.6 Statistical analysis

Results are given as mean ± standard deviation. All data were checked for normality (Kolmogorov–Smirnov test) and homoscedasticity of variance (Bartlett's test). Survival rate

expressed in percentage was arcsin square root transformed before statistical analyses. Significant differences in survival rate and in gene expression ratios during the time course of infection (0-24 h post injection) in fish fed with Control diet were detected by one-way ANOVA, and by T-test at each sampling time between the Control and VA3 dietary groups. When differences were significant by the ANOVA test, the Tukey multiple-comparison test was used to detect differences among experimental groups. Differences were considered to be significant when $P < 0.05$. The correlation between fish mortality and total VA levels in enriched *Artemia* metanauplius was evaluated by means of the Pearson Product Moment Correlation test. All statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc.).

3. Results and discussion

Although *Phdd* is a moderately virulent pathogen, it is one of the most common pathogens associated with newly cultured fish species [4, 27]. In the present study, we found a LD_{50} of 6 CFU per fish in Senegalese sole early juveniles (around 10-20 mg dry weight; Supplementary Figure S1) and therefore, this bacterial load was applied subsequently to decipher whether dietary VA level improved Senegalese sole early juvenile's immunocompetence.

3.1 Dietary VA supplementation improve the resistance of Senegalese sole early juveniles to an induced bacterial infection

The intensive culture of fish species has favored the occurrence of several outbreaks with *Photobacterium damselae* subsp. *damselae* resulting in moderate mortality [30]. Despite increasing knowledge about *Phdp* pathogenesis, relatively little is yet known about how *Phdd* induces fish mortality (reviewed in Labella et al. [4]). Further, the effectiveness of chemotherapy treatments remains limited [28, 29] when confronted with isolates of *Phdp* and *Phdd* possessing multidrug resistance genes which can be transferred horizontally. Therefore, immunoprophylaxis seems to be the best way to prevent pasteurellosis. Autovaccines against *Phdp* have been used in some farms [1], but their efficacy is dependent on the fish species,

fish size, vaccine formulation and immunostimulants used [30]. In addition, recent studies on probiotics to control Photobacteriosis have given encouraging results [31, 32]. Under current experimental conditions, Senegalese sole early juveniles fed with a VA-supplemented diet (203,000 total VA IU Kg^{-1}) showed a higher resistance to an induced bacterial infection with *Phdd* (Fig. 1; ANOVA, $P < 0.05$), with approximately a 50% reduction in endpoint mortality in comparison to the Control group (37,000 total VA IU Kg^{-1}; 36.6 ± 11.8 and 89.3 ± 13.7 % of cumulative mortality, respectively); and a statistically significant negative correlation between endpoint mortality rates and total VA dietary content (R= -0.971; $P = 0.02$; Pearson Product Moment Correlation test). This confirmed that VA has a role in Senegalese sole immunocompetence. Similar results were found regarding the enhancement of the immnune system by retinoids in freshwater [18-20] and marine fish species [21-23]. In order to confirm that fish mortality was caused by the intramuscular injection of *Phdd*, moribund challenged fish and healthy control ones injected with SSW were sampled. Confirmation of presence/absence of *Phdd* was done by PCR (Supplementary Figure S2).

3.2 VA might ameliorate the response to Phdd infection through different immune pathways

Phdd induced pathogenicity and mortality are known to be caused by excreted cellular protein components such as proteases, hemolysins, and siderophore-mediated iron sequestering proteins (reviewed in Labella et al. [4]). However, how the fish immnune system responds to this pathogen is still unknown. Thus, the time course response to *Phdd* infection in sole juveniles fed the Control and the highest VA supplemented (VA3) diets was evaluated by analyzing the expression of genes from the immune system.

All evaluated genes showed no differences in their expression levels between fish fed the Control and VA3 diets before the bacterial challenges (Figs. 2 and 3; T-test, $P > 0.05$), indicating that VA might prime the system to give an enhanced response upon stimulation by pathogen infection [33]. It is known that VA metabolites improve the immunocompetence in vertebrates [33]. Fish fed with synthetic or natural β-carotene and astaxanthin supplemented

diets had a higher serum complement activity, lysozyme activity, phagocytic activity and better non-specific cytotoxicity in peripheral blood lymphocytes, increasing the bio-defense mechanisms of the organism [18-20]. Furthermore, fish fed diets supplemented with astaxanthin showed higher resistance against infectious hematopoietic necrosis virus (IHNV) [20], while fish fed with high levels of retinol acetate, respiratory burst activity, leukocyte cytotoxic activity and total serum immunoglobulin M were enhanced [21-23].

Although additional analyses are required, the present work showed some potential underlying pathways by which VA might ameliorate fish immunocompetence. At 24 h post infection (hpi) a higher gene expression (ANOVA, $P < 0.05$) of the three main molecular players of the complement system was observed (Figure 2): *complement component 1 inhibitor* (*C1inh*; from the classical and lectin pathway), *complement component 3* (*C3*; from the alternative pathway) and *complement component 9* (*C9*; from the cytolytic pathway). Those findings were in partial agreement with those reported in Senegalese sole [34] and other fish species [35] after an infection with *Phdp,* where it was proposed that activation of complement provided protection against *Phdp* infection [35]. The common response among fish species (activation of complement) against multiple pathogens suggests that the supplementation of diet with VA can be used as an immunoprophylactic measure in different fish species and against more than one pathogen.

A higher expression of *C1inh* was observed in fish fed with supplemented VA diets at 8 and 12 hpi compared with that of infected soles fed with the Control diet (Fig. 2; T-test, $P < 0.05$). Interestingly, the classical and lectin pathway mainly relies on the activation of the C1 complex, which is under control of the C1-inhibitor that forms a proteolytically inactive stoichiometric complex with the C1r or C1s proteases. Although activation of the C1 complex plays crucial roles for successful host defense, excessive activation of complement can potentially cause serious damage to the host, leading to anaphylaxis and cell damage [36]. Similarly, a higher gene expression of *C3* in Senegalese sole fed VA3 diet was also reported, although it was only significant at 12 hpi (Fig. 2; T-test, $P < 0.05$). Complement C3 is the central protein of all three

activation pathways and essential in alerting the host of the presence of potential pathogens, contributing significantly to the orchestration and development of an acquired immune response [36, 37]. The highest gene expression of *C3* in Senegalese sole was found in the group fed with Control diet at 24 hpi, which is in accordance with results reported in different fish species after 24 h infection with *Phdp* [38]. In contrast, *C9* up-regulation in infected fish started at 4 hpi, but was only seen to be significantly different at 24 hpi in VA3 group. The ninth component of the complement is a single-chain glycoprotein that is involved in the formation of the membrane attack complex (MAC) on the surface of target cells [38].

The current study also provides new insights on other potential underlying pathways by which dietary VA content can improve fish immunocompetence. In this sense, expression of *lysozyme C* (*LysC*), *lectin galactoside-binding soluble 1 (galectin 1)-like 3* (*Lgals1*), *peroxiredoxin-1* (*Prdx1*, also known as *natural killer cell-enhancing factor*), *hepcidin* (*Hamp*), *transferrin* (*Transf*), and *metalloreductase six-transmembrane epithelial antigen of prostate 4* (*Steap4*; also known as *tumor necrosis factor, alpha-induced protein 9*) were also evaluated. Lysozymes exhibit antimicrobial properties by hydrolysis of N-acetylmuramic acid and N-acetylglucosamine, which are constituents of the peptidoglycan layer of bacterial cell walls [39]. Our results showed that from the initiation of infection onwards, *LysC* was significantly down-regulated in both experimental groups (Control and VA3; Fig. 3; ANOVA, $P < 0.05$) and no significant differences at any post injection times were observed between them regarding the effect of dietary VA level (T-test, $P > 0.05$). This is in contrast with previous reports regarding *LysC* in Senegalese sole after infection with *Phdp* [34, 40].

Similarly to gene expression results found for *LysC*, *Prdx1* did not differ between Senegalese soles fed Control and VA3 diets after the *Phdd* infection (Fig. 3; T-test, $P > 0.05$), being significantly down-regulated at all sampling points after infection (ANOVA, $P < 0.05$). Prdx enzymes efficiently reduce the increase in the intracellular concentration of H_2O_2, suggesting an important function in signaling cascades by removing H_2O_2 [41] and in the immunoregulation of the activity of natural killer cells (NK) [42]. Since NK cells are involved in

the recognition and destruction of host cells infected with pathogens, two hypotheses may be proposed. The first one is in relation to the lower infection suffered by VA3 fed fish due to the limited availability of iron for bacterial growth (see comments below), whereas the second hypothesis is that it could represent one mechanism of evasion of the immune response by *Phdd* due to the action of some proteases [43]. In contrast to *Prdx1* gene expression, we reported an increased gene expression of *Lgals1* in Senegalese sole early juveniles challenged with *Phdd* after 4 hpi (Fig. 3; ANOVA, $P < 0.05$), regardless of the dietary VA content. Lectins are proteins that bind to specific carbohydrate moieties on cell surfaces, assisting in the rapid clearance of pathogens by enhancing opsonization and phagocytosis [44]. Thus, present results were in agreement with their proposed function, the removal of pathogens after the onset of infection as previously found in infected fish [45].

Finally, we analyzed the expression of genes involved in the regulation of iron homeostasis since one of the main virulence factors of *Phdd* is its high-affinity siderophore-mediated iron-sequestering system [4]. The ability to acquire iron by pathogenic bacteria is essential for the growth and ability to cause infections within the host. Hepcidin (*Hamp*) is a small cysteine-rich protein with antimicrobial activity and an important role in iron homeostasis. Increased gene expression of *Hamp* in *Phdd*-challenged Senegalese sole early juveniles from 8 hpi onwards was found in this study (Fig. 3; ANOVA, $P < 0.05$). Similar results were observed in Senegalese sole exposed to LPS [46]. This gene expression pattern was observed regardless of the dietary VA content, with the exception of the 8 hpi sampling point, when fish fed with VA supplemented diets had a significantly lower gene expression of *Hamp* than those fed the Control diet (T-test, $P < 0.05$). However and in contrast to *Hamp* gene expression, *transferrin* (*Transf*) gene expression was significantly up-regulated only at 24 hpi in Senegalese sole fed with Control diet (ANOVA, $P < 0.05$), whereas fish fed the VA3 diet already exhibited a higher gene expression value compared to the Control fish at 4 hpi. This higher level of expression was maintained until 24 hpi (Fig. 3; T-test, $P < 0.05$). This increase in gene expression was in accordance with the presence of a VA response element in the promoter of *transferrin* [47].

Since tranferrin is a protein with a high affinity for iron, lowering the availability of iron in vertebrate tissues, VA might inhibit bacterial growth indirectly in this way [48], and thereby increase the survival of fish fed with the VA3 diet. In addition, the gene expression of *Steap4* (also known as *tumor necrosis factor, alpha-induced protein 9*) was also up-regulated in fish from the Control group infected with *Phdd* from 12 hpi onwards (Fig. 3; ANOVA, $P < 0.05$); which was in agreement with its up-regulation in sole exposed to LPS [46]. In contrast, there was a significant reduction of *Steap4* expression at each sampling point in infected fish from the VA3 group (T-test, $P < 0.05$). Although *Steap4* is capable of facilitating the cellular uptake of iron [49] it is induced by TNF-α as a protective anti-inflammatory factor [50] and it has been reported to be down-regulated by all-trans RA (the main active VA metabolite), thereby redirecting the immune balance towards immunoglobulin production and a humoral immune response [51]. This was in accordance with present results regarding a reduced expression of *Steap4* in soles fed with the VA3 diet from 4 hpi onwards (T-test, $P < 0.05$), concomitantly with the reported higher RA levels in VA3 diet fed soles than in those fed Control diet [15].

4. Conclusions

Sole fed higher doses of dietary VA were more resistant against a bacterial challenge with *Phdd*. The lower mortality rate in fish fed higher VA dietary content (203,000 total VA IU Kg^{-1}) was probably due to the stimulation by RA of the immune response system, and particularly by (i) avoiding cell damage and anaphylaxis by the excessive activation of the C1 complex through the control of C1 inhibitor, and (ii) reducing iron availability for growth and concomitant pathogenity of *Phdd* mainly through *transferrin* and *Steap4* regulation. Furthermore, this work highlights the need for integrative research on fish nutritional requirements, since the same dietary VA content that hampered skeletal development [15] stimulated fish immunocompetence (present study). Such opposed effects in both systems might be due to an osteoimmunology cross-talk that remains to be uncovered in fish species. Finally, feeding

345 metamorphosed Senegalese sole with 203,000 total VA IU Kg^{-1} might be an effective,
346 inexpensive and environmental-friendly method to improve Senegalese sole
347 immunocompetence while avoiding the inducement of skeletal deformities.
348
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356 **References**

357 [1] Morais S, Aragão C, Cabrita E, Conceição LEC, Constenla M, Costas B, Dias J, Duncan N,
358 Engrola S, Estévez A, Gisbert E, Mañanos E, Valente LMP, Yufera M, Dinis MT. New
359 developments and biological insights into the farming of *Solea senegalensis* reinforcing its
360 aquaculture potential. Rev Aquaculture 2014; 6:1-37.
361 [2] Martins P, Cleary DFR, Pires ACC, Rodrigues AM, Quintino V, Calado R, Gomes NCM.
362 Molecular analysis of bacterial communities and detection of potential pathogens in a
363 recirculating aquaculture system for *Scophthalmus maximus* and *Solea senegalensis*. Plos
364 One 2013; 8:1.
365 [3] Romalde JL. *Photobacterium damselae* subsp. *piscicida* an integrated view of a bacterial
366 fish pathogen. *Int Microbiol* 2002; 5:3-9.
367 [4] Labella A, Berbel C, Manchado M, Castro D, Borrego JJ. *Photobacterium damselae* subsp.
368 *damselae*, an emerging pathogen affecting new cultured marine fish species in Southern
369 Spain. Recent Advances in Fish Farms (Faruk A, ed.) 2011 pp. 135-142. InTech, Rijeka,
370 Croatia.

[5] Kiron V. Fish immune system and its nutritional modulation for preventive health care. Animal Feed Science and Technology 2012; 173:111-33.

[6] Oliva-Teles A. Nutrition and health of aquaculture fish. J Fish Dis 2012; 35:83-108.

[7] Ross SA, Caffery PJ, Draguer UC, De Luca LM. Retinoids in embryonal development. Physiol Rev 2000; 80:1021-54.

[8] Manicassamy S, Pulendran B. Retinoic acid-dependent regulation of immune responses by dendritic cells and macrophages. Semin Immunol 2009; 21:22-7.

[9] Fernández I, Gisbert E. The effect of vitamin A on flatfish development and skeletogenesis: a review. Aquaculture 2011; 315:34-48.

[10] Haga Y, Du S, Satoh S, Kotani T, Fushimi H, Takeuchi Y. Analysis of the mechanism of skeletal deformity in fish larvae using a vitamin A-induced bone deformity model. Aquaculture 2011; 315:26-33.

[11] Mazurais D, Darias M, Fernández I, Cahu C, Gisbert E, Zambonino-Infante JL. Gene expression pattern during European sea bass larvae development: Impact of dietary vitamins. Pp 91-111. *In*: Saroglia M and Zhanjiang L (Eds) 2012. Functional Genomics in Aquaculture. John Wiley & Sons, Oxford, UK.

[12] Fernández I. Hypervitaminosis A effects on *Sparus aurata* and *Solea senegalensis*: characterization of larval performance and the underlying gene expression of abnormal skeletogenesis. PhD Doctoral Thesis at Universidad de Barcelona 2011, Barcelona , Spain.

[13] Lie KK, Moren M. Retinoic acid induces two osteocalcin isoforms and inhibits markers of osteoclast activity in Atlantic cod (*Gadus morhua*) ex vivo cultured craniofacial tissues. *Comp Biochem Phys* A 2012; 161:174-84.

[14] Fernández I, Tiago DM, Laizé V, Cancela LM, Gisbert E. Retinoic acid differentially affects *in vitro* proliferation, differentiation and mineralization of two fish bone-derived cell lines: different gene expression of nuclear receptors and ECM proteins. J Steroid Biochem Mol Biol 2014; 140:34-43.

[15] Fernández I, Pimentel MS, Ortiz-Delgado JB, Hontoria F, Sarasquete C, Estévez A, Zambonino-Infante JL, Gisbert E. Effect of dietary vitamin A on Senegalese sole (*Solea senegalensis*) skeletogenesis and larval quality. Aquaculture 2009; 295:250-65.

[16] Fernández I, Gisbert E. Senegalese sole bone tissue originated from chondral ossification is more sensitive than dermal bone to high vitamin A content in enriched Artemia. J *Appl* Ichthyol 2010; 26:344-49.

[17] Marques CL, Fernández I, Rosa J, Viegas MN, Cancela ML, Laizé V. Spatiotemporal expression and retinoic acid regulation of bone morphogenetic proteins 2, 4 and 16 in Senegalese sole. J Appl Ichthyol 2014; 30:713-20.

[18] Amar EC, Kiron V, Satoh S, Watanabe T. Influence of various dietary synthetic carotenoids on bio-defence mechanisms in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Fish Shellfish Immunol 2001; 32:162-73.

[19] Amar EC, Kiron V, Satoh S, Watanabe T. Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss* Walbaum) associated with dietary intake of carotenoids from natural products. Fish Shellfish Immunol 2004; 16:527-37.

[20] Amar EC,Viswanath K, Tetsuya A, Satoh S, Watanabe T. Resistance of rainbow trout *Oncorhynchus mykiss* to infectious hematopoietic necrosis virus (IHNV) experimental infection following ingestion of natural and synthetic carotenoids. Aquaculture 2012; 330:148-55.

[21] Cuesta A, Ortuño J, Rodriguez A, Esteban MA, Meseguer J. Changes in some innate defence parameters of seabream (*Sparus aurata* L.) induced by retinol acetate. Fish Shellfish Immunol 2002; 13:279-91.

[22] Cuesta A, Esteban MA, J. Meseguer J. Tumoricidal activity of gilthead seabream (*Sparus aurata* L.) natural cytotoxic cells: the role played in vitro and in vivo by retinol acetate. Fish Shellfish Immunol 2003; 14:133-44.

[23] Cuesta A, Meseguer J, Esteban MA. Total serum immunoglobulin M levels are affected by immunomodulators in seabream (*Sparus aurata* L.). *Vet Immunol Immunop* 2004; 101:203-10.

[24] Derveaux S, Jo Vandesompele J, Hellemans J. How to do successful gene expression analysis using real-time PCR. Methods 2010; 50:227-30.

[25] Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: Bestkeeper-Excell-based tool using pairwise correlations. *Biotechnol Lett* 2004; 26:509-15.

[26] Infante C, Matsuoka MP, Asensio E, Cañavate JP, Reith M, Manchado M. Selection of housekeeping genes for gene expression studies in larvae from flatfish using real-time PCR. BMC Mol Biol 2008; 9:28.

[27] Labella A, Manchado M, Alonso MC, Castro D, Romalde JL, Borrego JJ. Molecular intraspecific characterization of *Photobacterium damselae* ssp *damselae* strains affecting cultured marine fish. J Appl Microbiol 2010; 108: 2122-32.

[28] Kim MJ, Hirono I, Kurokawa K, Maki T, Hawke J, Kondo H, Santos MD, Aoki T. Complete DNA sequence and analysis of the transferable multiple-drug resistance plasmids (R Plasmids) from *Photobacterium damselae* subsp. *piscicida* isolates collected in Japan and the United States. Antimicrob Agents Chemother 2008; 52:606-11.

[29] Nonaka L, Maruyama F, Miyamoto M, Miyakoshi M, Kurokawa K, Masuda M. Novel conjugative transferable multiple drug resistance plasmid pAQU1 from *Photobacterium damselae* subsp. *damselae* isolated from marine aquaculture environment. Microbes Environ 2012; 27:263-72.

[30] Arijo S, Rico R, Chabrillon M, Diaz-Rosales P, Martínez-Manzanares E, Balelona MC, Magariños B, Toranzo AE, Moriñigo MA. Effectiveness of a divalent vaccine for sole, *Solea senegalensis* (Kaup), against *Vibrio harveyi* and *Photobacterium damselae* subsp. *piscicida*. J *Fish Dis* 2005; 28:33-8.

448 [31] Garcia de la Banda I, Lobo C, Chabrillo M, Leon-Rubio JM, Arijo S, Pazos G, Lucas LM,
449 Moriñigo MA. Influence of dietary administration of a probiotic strain *Shewanella*
450 *putrefaciens* on Senegalese sole (*Solea senegalensis*, Kaup 1858) growth, body
451 composition and resistance to *Photobacterium damselae* subsp *piscicida*. Aquac Res 2012;
452 43:662-9.
453 [32] Batista S, Tapia-Paniagua ST, Moriñigo MA, Nunez-Diaz JA, Goncalves JFM, Barros R,
454 Cristovao B, Valente LMP, Ozorio ROA. Expression of immune response genes in sole
455 (*Solea senegalensis*, Kaup 1858) induced by dietary probiotic supplementation following
456 exposure to *Photobacterium damselae* subsp. *piscicida*. Fish Shellfish Immunol 2013;
457 34:1638-39.
458 [33] Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and
459 D take centre stage. Nat Rev 2008; 8:685-98.
460 [34] Costas B, Régo PCNP, Simões I, Marques JF, Castro-Cunha M, Afonso A. Cellular and
461 humoral immune responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) following
462 challenge with two *Photobacterium damselae* subsp. *piscicida* strains from different
463 geographical origins. J *Fish Dis* 2013; 36:543-53.
464 [35] Acosta F, Ellis AE, Vivas J, Padilla D, Acosta B, Deniz S, Bravo J, Real F. Complement
465 consumption by *Photobacterium damselae subsp. piscicida* in seabream, red porgy and
466 seabass normal and immune serum. Effect of the capsule on the bactericidal effect. Fish
467 Shellfish Immunol 2006; 20:709-17.
468 [36] Nakao M, Tsujikura M, Ichiki S, Vo TK, Somamoto T. The complement system in teleost
469 fish: progress of post-homolog-hunting researches. Dev Comp Immunol 2011; 35:1296-308.
470 [37] Boshra H, Li J, Sunyer JO. Recent advances on the complement system of teleost fish.
471 Fish Shellfish Immunol 2006; 20:239-62.
472 [38] Mauri I, Romero A, Acerete L, Mackenzie S, Roher N, Callol A, Cano I, Alvarez MC, Tort L.
473 Changes in complement responses in Gilthead seabream (*Sparus aurata*) and European

seabass (*Dicentrarchus labrax*) under crowding stress, plus viral and bacterial challenges. Fish Shellfish Immunol 2011; 30:182-88.

[39] Jollès P, Jollès J. What's new in lysozyme research? Always a model system, today as yesterday. Mol *Cell Biochem* 1984; 63:165-89.

[40] Fernández-Trujillo MA, Porta J, Manchado M, Borrego JJ, Alvarez MC, Bejar J. c-Lysozyme from Senegalese sole (*Solea senegalensis*): cDNA cloning and expression pattern. Fish Shellfish Immunol 2008; 25:697-700.

[41] Chang TS, Jeong W, Choi SY, Yu SQ, Kang SW, Rhee SG. Regulation of perosiredoxin I activity by Cdc2-mediated phosphorylation. J Biol Chem 2002; 277:25370-376.

[42] Shau HG, Buttlerfield LH, Chiu R, Kim A. Cloning and sequence-analysis of candidate human natural killer enhancing factor genes. Immunogenetics 1994; 40:129-34.

[43] Vivas J, Razquin BE, Lopez-Fierro P, Naharro G, Villena A. Correlation between production of acyl homoserine lactones and proteases in an *Aeromonas hydrophila aro*A live vaccine. Vet Microbiol 2004; 101:167-76.

[44] Dutta S, Bhattacharya B, Chatterjee B, Mazumder S. Characterization of a galactose binding serum lectin from the Indian catfish, *Clarias batrachus*: Possible involvement of fish lectins in differential recognition of pathogens. Comp Biochem Physiol C 2005; 141:76-84.

[45] Pellizzari C, Krasnov A, Afanasyev S, Vitulo N, Franch R, Pegolo S, Patarnello T, Bargelloni L. High mortality of juvenile gilthead sea bream (*Sparus aurata*) from photobacteriosis is associated with alternative macrophage activation and anti-inflammatory response: Results of gene expression profiling of early responses in the head kidney. Fish Shellfish Immunol 2013; 34:1269-1278.

[46] Osuna-Jiménez I, Williams TD, Prieto-Alamo MJ, Abril N, Chipman JK, Pueyo C. Immune- and stress-related transcriptomic responses of *Solea senegalensis* stimulated with lipopolysaccharide and copper sulphate using heterologous cDNA microarrays. Fish Shellfish Immunol 2009; 26:699-706.

[47] *Hsu SL, Lin YF, Chou CK. Transcriptional regulation of transferrin and albumin genes by retinoic acid in human hepatoma cell line Hep3B. Biochem J 1992; 283:611-5.*

[48] Ellis AE. Immunity to bacteria in fish. Fish Shellfish Immunol 1999; 9:291-08.

[49] Ohgami RS, Campagna DR, McDonald A, Fleming MD. The Steap proteins are metalloreductases. Blood 2006; 108: 1388-94.

[50] Moldes M, Lasnier F, Gauthereau X, Klein C, Pairault J, Feve B, Chambaut-Guerin AM. Tumor necrosis facor-alpha-induced adipose-related protein (TIARP), a cell-surface protein that is highly induced by tumor necrosis factor-alpha and adipose conversion. J Biol Chem 206; 33938-33946.

[51] Wang X, Allen C, Ballow M. Retinoic acid enhances the production of IL-10 while reducing the synthesis of IL-12 and TNF-alpha from LPS-stimulated monocytes/macrophages. J Clin Immunol 2007; 27:193-00.

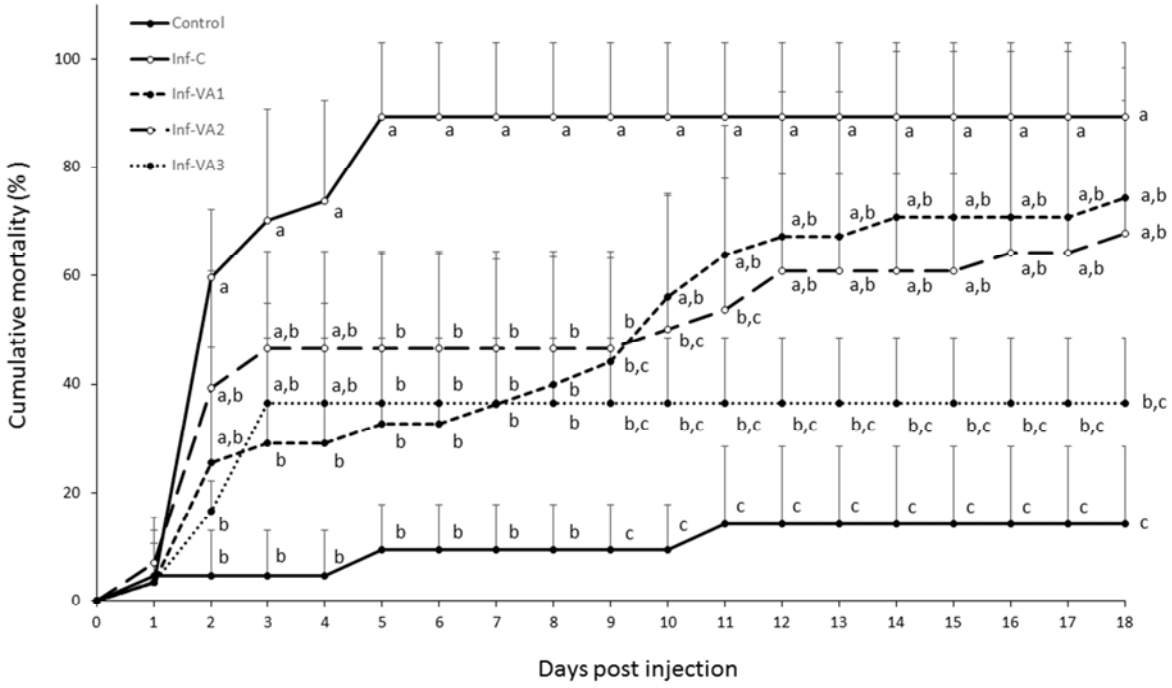
Figure captions

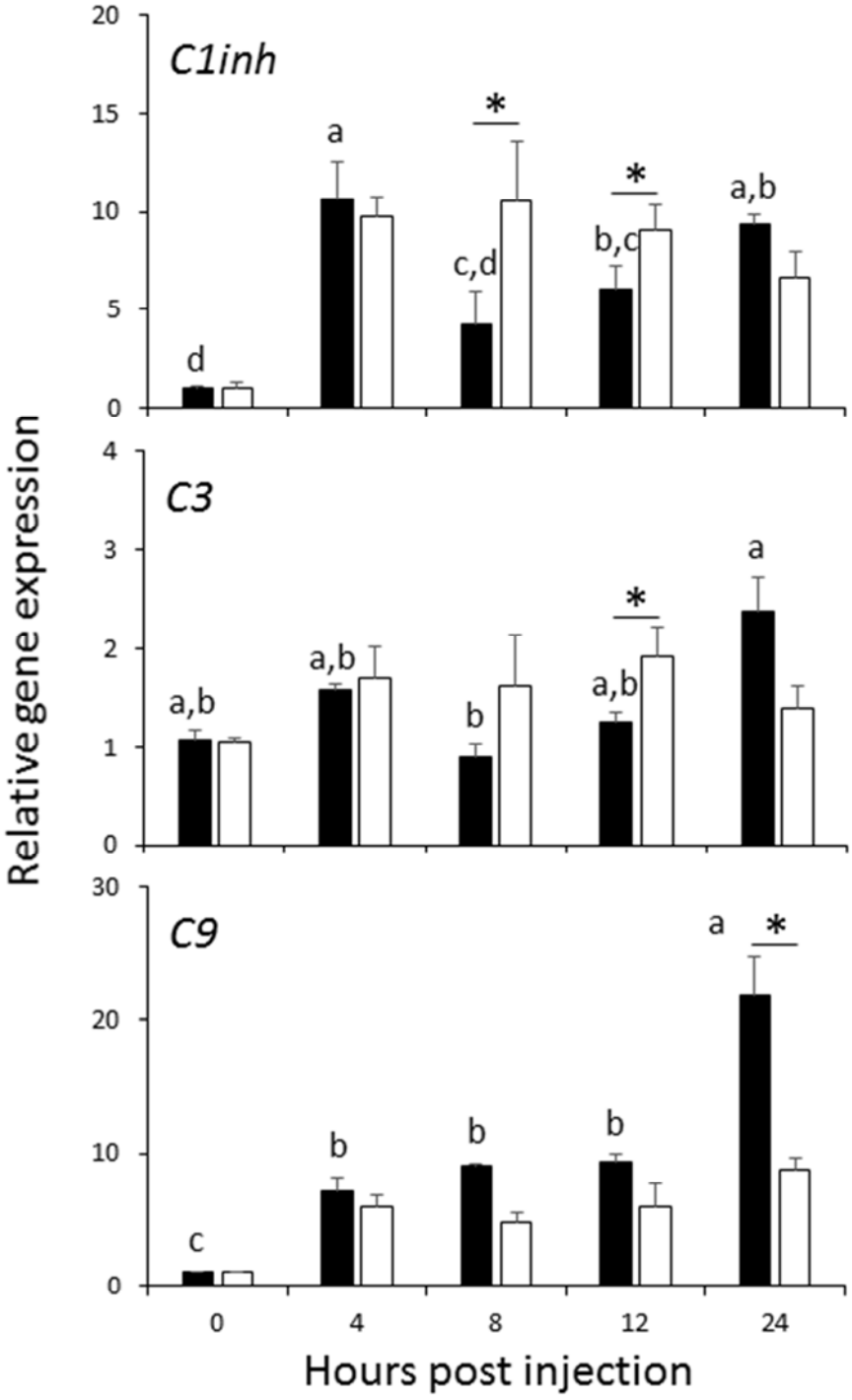
Figure 1. Fish mortality (%) during 18 days post injection of *Photobacterium damselae* subsp*. damselae* strain 33539 in Senegalese sole (*Solea senegalensis*) early juveniles fed with *Artemia* metanauplii containing increased levels of vitamin A (VA). Fish were fed with *Artemia* enriched with 37,000, 44,666, 82,666 and 203,000 total VA IU Kg^{-1} and infected by intramuscular injection of 10 µL of *P. damselae* subsp*. damselae* suspension ($6*10^2$ CFU mL^{-1} in SSW; Inf-C, Inf-VA1, Inf-VA2 and Inf-VA3, respectively); or fed with *Artemia* enriched with 37,000 total VA IU Kg^{-1} and injected intramuscularly with 10 µL of SSW (Control). Different letters at each day post injection denote significant differences in mortality among the different dietary regimes (ANOVA; $P < 0.05$). Each experimental group was done in quadruplicate (n = 7).

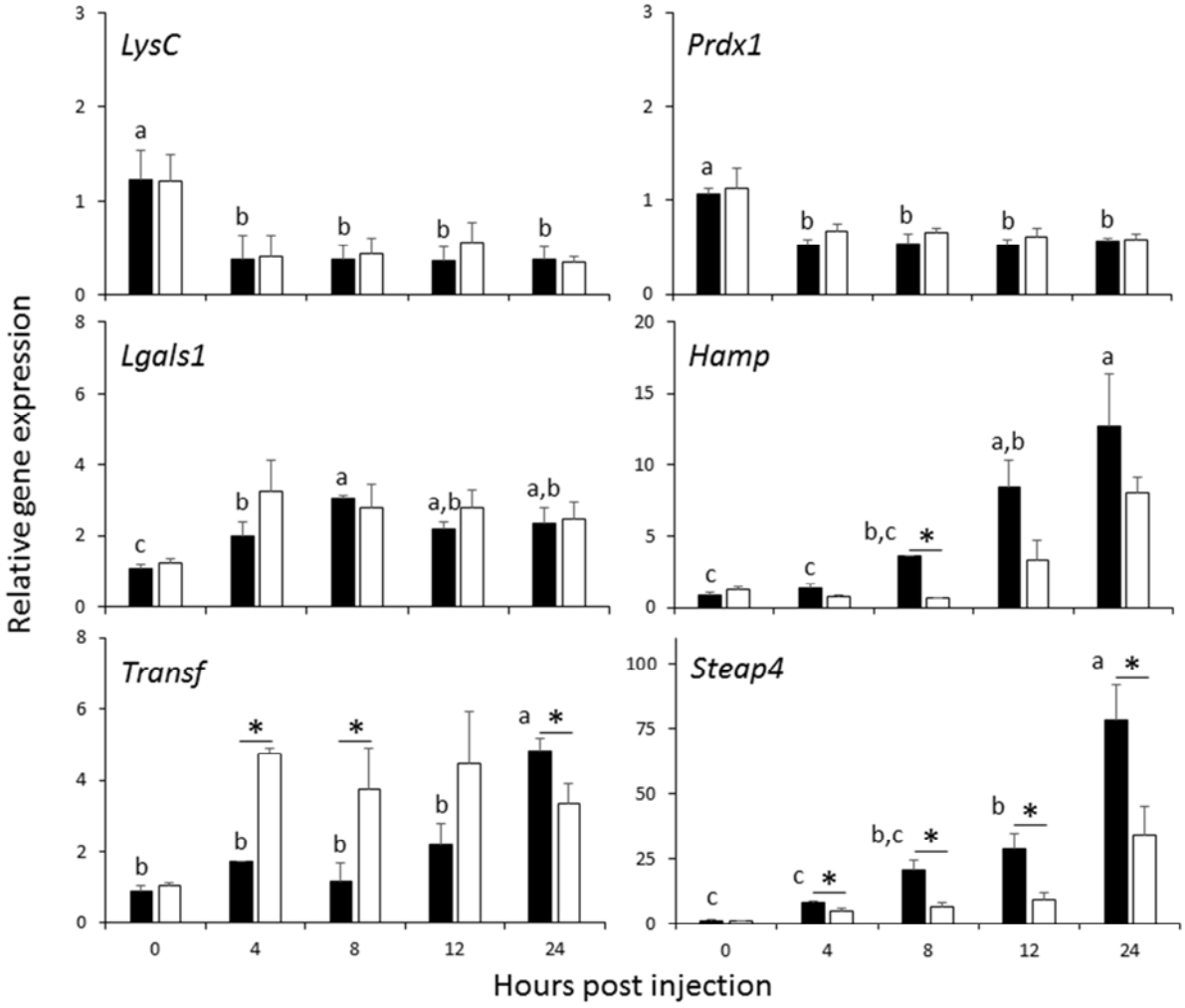
Figure 2. Time course of relative gene expression of some molecular players of the complement pathway in Senegalese sole (*Solea senegalensis*) early juveniles after injection with *Photobacterium damselae* subsp. *damselae* strain 33539. Relative gene expression was from fish fed with *Artemia* enriched with 37,000 (black bars; Control) or 203,000 total VA IU Kg^{-1} (white bars; VA3) before (0 hours post injection; hpi) and 4, 8, 12 and 24 hpi intramuscularly of 10 µL of suspension of *P. damselae* subsp. *damselae* strain 33539 ($6*10^2$ CFU mL^{-1} SSW). Different letters denote significant differences between sampling points of Control group (ANOVA, $P < 0.05$). Asterisk denotes significant differences between Control and VA3 groups at each sampling point (T-test, $P < 0.05$). Bars represent the relative gene expression mean values of three individuals from each experimental group (biological replicates; n = 3). Relative gene expression of each biological replicate was run in triplicate (technical replicates; n = 3).

554 Control fish at 0 hpi was the reference sample for relative gene expression analysis and set to
555 1. The housekeeping gene used was *ubiquitin* (*Ubq*). *C1inh*, C1 esterase inhibitor; *C3,*
556 Complement component C3; *C9,* Complement component C9*.*
557
558 **Figure 3.** Time course of relative gene expression of some other molecular players involved in
559 the immune system of Senegalese sole (*Solea senegalensis*) early juveniles after injection with
560 *Photobacterium damselae* subsp*. damselae* strain 33539. Relative gene expression was from
561 fish fed with *Artemia* enriched with 37,000 (black bars; Control) or 203,000 total VA IU Kg^{-1}
562 (white bars; VA3) before (0 hours post injection; hpi) and 4, 8, 12 and 24 hpi intramuscularly of
563 10 µL of suspension of *P. damselae* subsp*. damselae* strain 33539 ($6*10^2$ CFU mL^{-1} SSW).
564 Different letters denote significant differences between sampling points of Control group
565 (ANOVA, $P < 0.05$). Asterisk denotes significant differences between Control and VA3 groups
566 at each sampling point (T-test, $P < 0.05$). Bars represent the relative gene expression mean
567 values of three individuals from each experimental group (biological replicates; $n = 3$). Relative
568 gene expression of each biological replicate was run in triplicate (technical replicates; n=3).
569 Control fish at 0 hpi was the reference sample for relative gene expression analysis and set to
570 1. The housekeeping gene used was *ubiquitin* (*Ubq*). *Hamp*, Hepcidin; *Steap4,*
571 Metalloreductase six-transmembrane epithelial antigen of prostate 4; *Prdx1*, Peroxiredoxin-1;
572 *LysC*, Lysozyme C; *Transf,* Transferrin; *Lgals1*, Lectin, galactoside-binding, soluble, 1 (galectin
573 1)-like 3.
574
575 **Supplementary Figure S1.** Cumulative fish mortality (%) induced with intramuscular injection
576 (10 µL) of different suspensions of *P. damselae* subsp. *damselae* strain 33539 ranging from 0
577 (Control) to $6*10^5$ CFU mL^{-1} SSW (in quatriplicates) in Senegalese sole (*Solea senegalensis*)
578 early juveniles ($n = 10$) for lethal dose 50 (LD_{50}) determination.
579

Supplementary Figure S2. Agarose gel showing PCR products amplified with 16S-like ssrDNA degenerate primers from pure culture of *Photobacterium damselae* subsp*. damselae* strain 33539 (B), SSW injected (C), and 4 moribund infected Senegalese sole (*Solea senegalensis*) early juveniles (1, 2, 3 and 4). L, 1 Kb Plus DNA Ladder Invitrogen®. Note the corresponding amplicon of 1,424 bp from the 16S gene of *Phdd* from the pure culture, the absence of an amplicon from euthanized fish injected with SSW, and the same band in 4 different moribund infected juveniles. Amplicons from pure bacterial culture and infected fish were sequenced, analyzed using BLAST in GenBank at NCBI and identified as *P. damselae* subsp. *damselae* strain 33539.




C1inh
C3
C9
Relative gene expression
Hours post injection
0
4
8
12
24
d
a
c,d
b,c
a,b
a,b
a,b
b
a,b
a
c
b
b
b
a
*
*
*
*


LysC
Prdx1
Lgals1
Hamp
Transf
Steap4
Relative gene expression
Hours post injection
0
4
8
12
24

Highlights

- Dietary vitamin A (VA) enhances immune system in *Solea senegalensis* juveniles
- Sole fed 203,000 VA IU Kg^{-1} had higher resistance to an induced bacterial infection
- Complement system and iron homeostasis genes were differentially expressed
- Dietary VA levels impact on the fish osteoimmunological response

Supplementary Table S1. Gene name, accession numbers (GenBank or Unigene), primers and expected amplicon size used to perform the relative gene expression quantification in Senegalese sole (Solea senegalensis) early juveniles and confirming DNA *Photobacterium damselae* subsp*. damselae* presence in injected fish.

Gene name	Accession numbers*	Component	5' to 3' nucleotide sequences	Expected amplicon size (bp)
C1inh	unigene64193	Forward	ACCTGTGTGGCCTCTCCCCTG	107
	unigene526786	Reverse	ACTGGTAGCAGCAGCAGCCT	
C3	FJ345403	Forward	GGCTTACTAACTGGCTTCACTGTCA	110
		Reverse	CCTCTCTCTGACAGAACTGTGTCCA	
C9	unigene430267	Forward	ATCAGGAGGGTAACTGGACATGCTG	100
	unigene2945	Reverse	GCCCCACTCAGTCCGTCTGTGT	
Lgals1	unigene27503	Forward	TGACTCAGACAACATCGCAATGCAC	101
	unigene94321	Reverse	CACCCCAGCATCCCCCGGAC	
Hamp	FJ263548	Forward	ACACTCGTGCTCGCCTTTGTTTGCTTC	173
	AB455099	Reverse	TGACTCCAGCGTCTGTGTCTGACATGATTC	
LysC	DQ293993	Forward	AGCAGCTTAGGGAGAGAGACCGTC	254
		Reverse	TGTTGATCTGGAAGATGCC	
Prdx1	unigene65476_split_1	Forward	ACGGAGTGCTGAAGGAGGACGA	114
	unigene281027	Reverse	CCACAGAGCGACCCACAGGT	
Steap4	FJ263550	Forward	CTGGGACTGCTGGCACTTGGATTTG	160
		Reverse	CAGTTCTCCACGCTGACGTACTGTCGAAC	
Transf	FJ345407	Forward	TGCAGGGCTGGAACATTCCCATA	109
		Reverse	GCCCCGAAAAATGCACGCACG	
Ubq	AB291588	Forward	AGCTGGCCCAGAAATATAACTGCGACA	93
		Reverse	ACTTCTTCTTGCGGCAGTTGACAGCAC	
16S-like ssrDNA degenerate		Forward	AGAGTTTGATCMTGGCTCAG	1424
		Reverse	AAGGAGGTGATCCANCCRCA	

*GeneBank and/or Solea Data Base (http://www.juntadeandalucia.es/agriculturaypesca/ifapa/aquagenet/soleaDB) (solea_v4.1)