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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 damsela* subsP. *damsela*, 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.

Keywords

Vitamin ; Retinoic acid ; Immune system ; Gene expression ; Senegalese sole *Solea senegalensis* ; *Photobacterium damsela* subP. *damsela*

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 synthesise 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 this is 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 immunerelated 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 Coruna, 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™) 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™), 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 damsela* subsP. *damsela* (ATCC® 33539) (*Phdd*) was grown at 22° C in tryptic soy broth (TSB) or tryptic soy agar (TSA) supplemented with NaCl to a final concentration of 2% (w/v) and then stored at - 80° C 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° C 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° C 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 LD₅₀ for *Phdd* in Senegalese sole early juveniles (LD₅₀ - 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 LD₅₀ 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 ° C 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 anesthetic (Tricaine methanesulfonate, MS-222, Sigma). Fish sampled during the *Underlying pathways - challenge* were individually frozen in RNA later (Ambion®) and stored at -80° C 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° C until DNA extraction.

2.4. DNA extraction and confirmation of presence of *P. damsela* subsP. *damsela* 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° C for 8 min, followed by 40 cycles of a 1 min denaturation at 94° C, annealing at 58° C for 1 min, elongation at 72° C for 1 min and a final extension of 10 min at 72° C) with 16S-like ssrDNA degenerate primers (Supplementary Table S1) and subsequent sequencing of the resulting amplicon.

2.5. 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 (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 genespecific 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 μ L of a 1:10 dilution of cDNA template. Standard amplification parameters were: 95° C for 5 min followed by 45 amplification cycles (95° C for 45 s, 59° C for 1 min and 70° C for 1min). A final dissociation reaction (melting curve) was performed (95° C for 15 s, 60° C for 20 s, 95° C for 15 s and 60° C for 15 s) to evaluate the specificity of the amplicons. A calibrator sample was included in each qPCR plate [24].

2.6. 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 (*Ubp*), 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.

2.7. Statistical analysis

Results are given as mean \pm standard deviation. All data were checked for normality (KolmogoroveSmirnov 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 (0e24 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* metanauplii 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 damsela* subsP. *damsela* 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 $89.3 \pm 13.7\%$ of cumulative mortality, respectively). A statistically significant negative correlation between endpoint mortality rates and total VA dietary content was also found ($R = -0.971$; $P = 0.02$; 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 immune system by retinoids in freshwater [18e20] and marine fish species [21e23]. 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 immune 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 in 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 (Fig. 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 *C1inhibitor* 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; Ttest, $P < 0.05$). 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 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 downregulated 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, to 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. Hpcidin (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 transferrin 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 downregulated 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 highest 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 pathogenicity 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 crosstalk that remains to be uncovered in fish species. Finally, feeding metamorphosed Senegalese sole with 203,000 total VA IU Kg⁻¹ might be an effective, inexpensive and environmental-friendly method to improve Senegalese sole immunocompetence while avoiding the inducement of skeletal deformities.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at [http:// dx.doi.org/10.1016/j.fsi.2015.08.007](http://dx.doi.org/10.1016/j.fsi.2015.08.007).

Figure 1. Fish mortality (%) during 18 days post injection of *Photobacterium damsela* subsP. *damsela* 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 mL of *P. damsela* subsP. *damsela* suspension (6*10² 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).

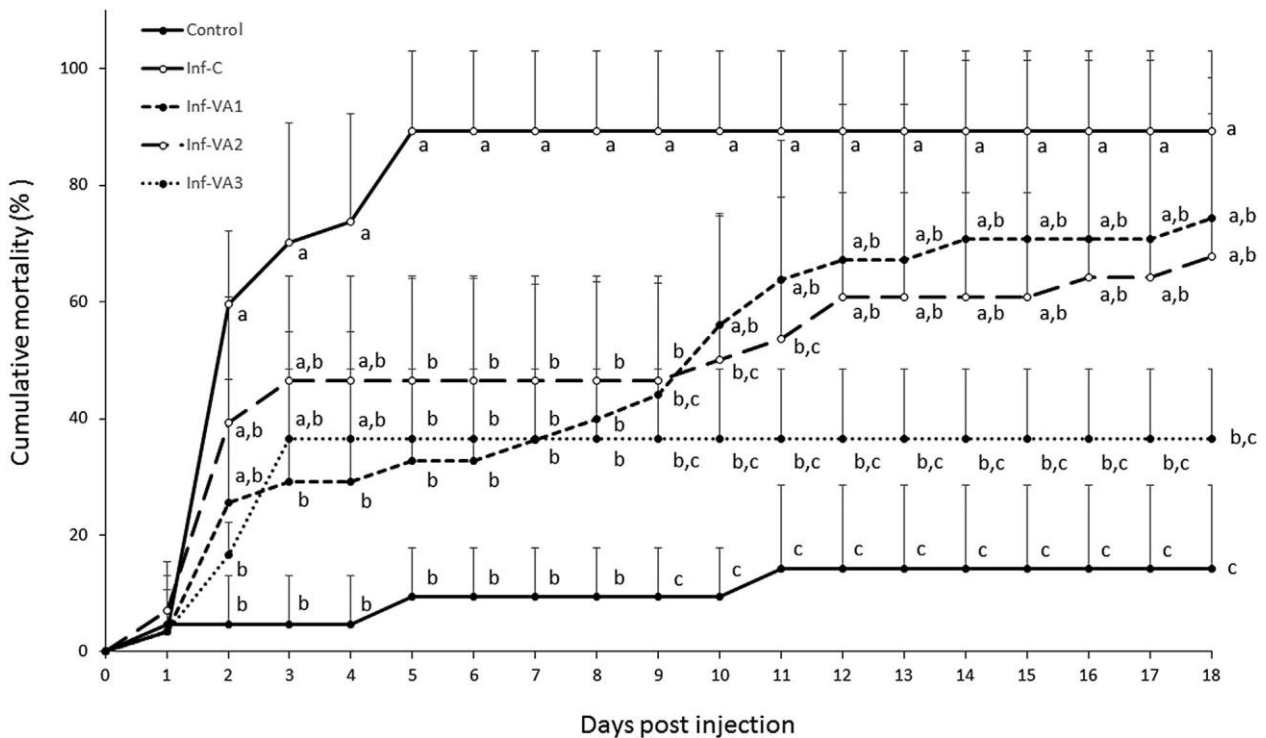


Figure 2. Time course of relative gene expression of some molecular players of the complement pathway in Senegalese sole (*Solea senegalensis*) early juveniles after injection with *Photobacterium damselae* subsP. *damselae* strain 33539. Relative gene expression was from fish fed with *Artemia* enriched with 37,000 (black bars; Control) or 203,000 total VA IU Kg⁻¹ (white bars; VA3) before (0 h post injection; hpi) and 4, 8, 12 and 24 hpi intramuscularly of 10 µL of suspension of *P. damselae* subsP. *damselae* (6*10² 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). Control fish at 0 hpi was the reference sample for relative gene expression analysis and set to 1. The housekeeping gene used was *ubiquitin (Ubg)*. *C1inh*, C1 esterase inhibitor; *C3*, Complement component C3 ; *C9*, Complement component C9.

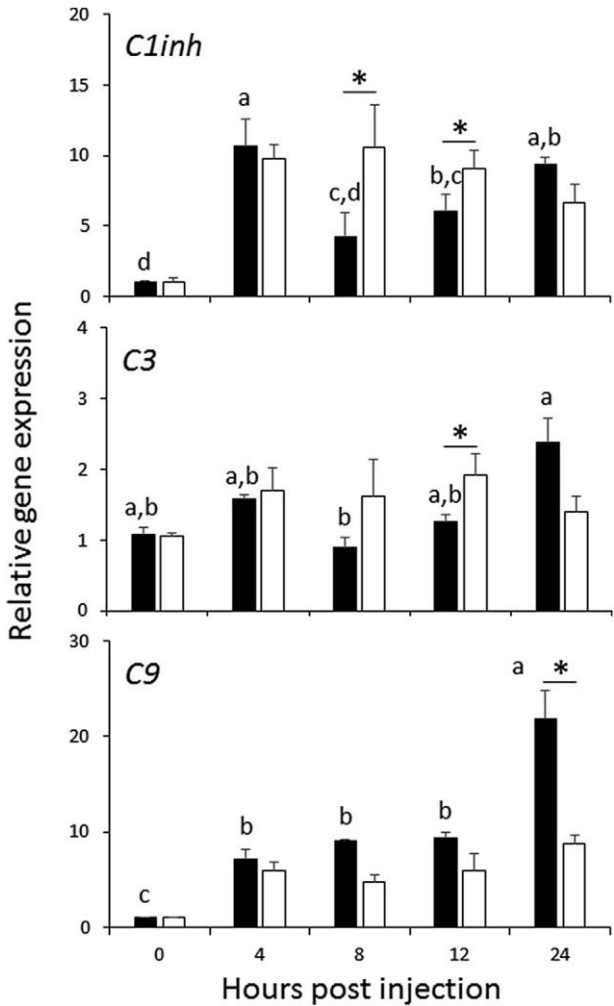
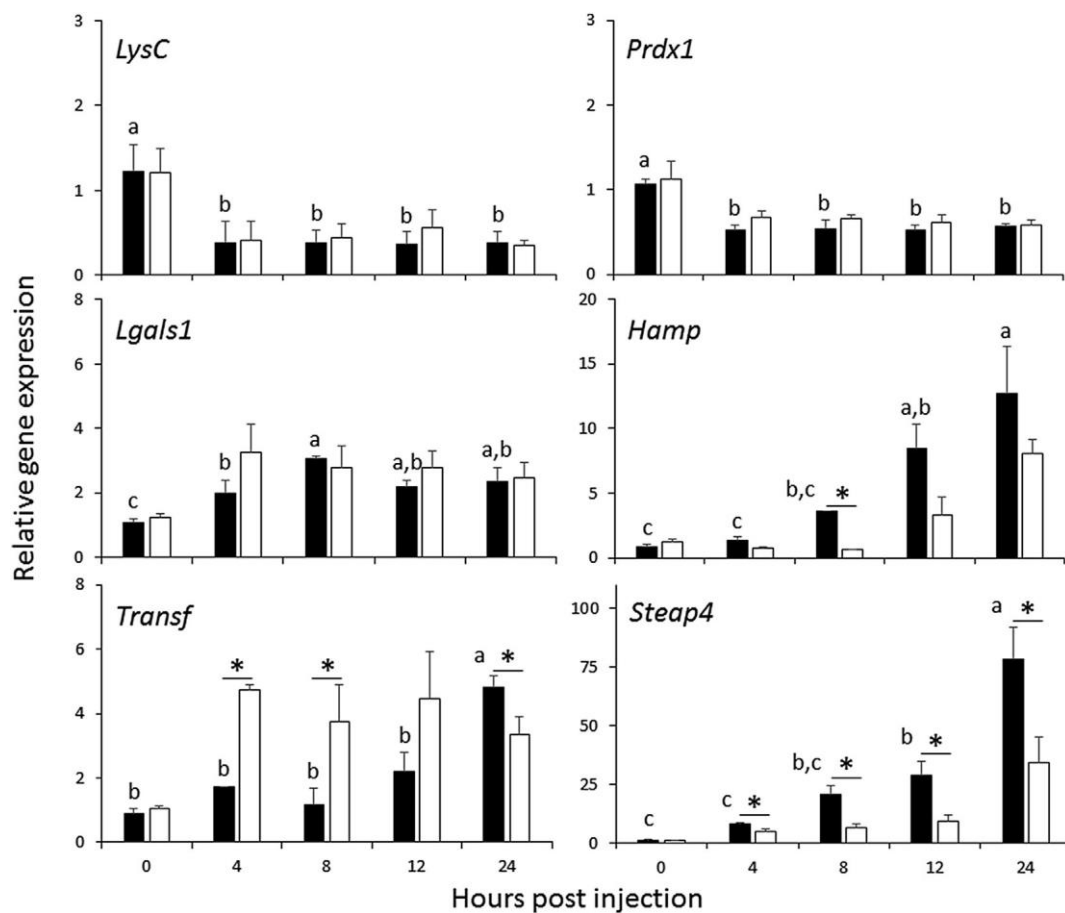


Figure 3. Time course of relative gene expression of some other molecular players involved in the immune system of Senegalese sole (*Solea senegalensis*) early juveniles after injection with *Photobacterium damsela* subsP. *damsela* strain 33539. Relative gene expression was from fish fed with Artemia enriched with 37,000 (black bars; Control) or 203,000 total VA IU Kg⁻¹ (white bars; VA3) before (0 h post injection; hpi) and 4, 8, 12 and 24 hpi intramuscularly of 10 µL of suspension of *P. damsela* subsP. *damsela* (6*10² 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). Control fish at 0 hpi was the reference sample for relative gene expression analysis and set to 1. The housekeeping gene used was *ubiquitin (Ubq)*. *Hamp*, Hecpudin; *Steap4*, Metalloreductase six-transmembrane epithelial antigen of prostate 4; *Prdx1*, Peroxiredoxin-1; *LysC*, Lysozyme C; *Transf*, Transferrin; *Lgals1*, Lectin, galactoside-binding, soluble, 1 (galectin 1)-like 3.



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