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Short communication

Potential immunomodulatory and protective effects of the *Arthrospira*-based dietary supplement on shrimp intestinal immune defenses

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ABSTRACT

Herein, we evaluated the immunomodulatory and the antiviral protective properties of a cyanobacteria-enriched diet on the immune responses of the Pacific white shrimp *Litopenaeus vannamei* challenged with the White spot syndrome virus (WSSV). Shrimp were fed with an *Arthrospira platensis* supplemented feed during 20 days, and its effects were examined by evaluating well-known standardized shrimp immune parameters (total hemocyte counts, total protein concentration, phenoloxidase activity, and serum agglutination titer). Additionally, we assessed the expression of crucial genes involved in both hemolymph- and gut-based immunities related to the shrimp capacity to circumvent viral and microbial infections. Dietary supplementation improved shrimp survival rates after challenge with a median lethal dose of WSSV. From all immune parameters tested, only the serum agglutination titer was higher in treated animals. On the other hand, the expression of some representative marker genes from different immune response pathways was only modulated in the midgut and not in the circulating hemocytes, suggesting that this feed supplementation can be used as an attractive strategy to enhance immunity in shrimp gut. Altogether, our results evidence the immunomodulatory properties of *A. platensis* supplemented feed in shrimp humoral and intestinal defenses and highlight the potential use of cyanobacteria-based immunostimulants in shrimp farming for protection against infectious diseases.

1. Introduction

Arthrospira (*Spirulina*) *platensis* is a blue-green cyanobacterium with high protein content and antioxidants well known for its immunostimulant properties [1]. The administration of *A. platensis*-based supplements promotes several health beneficial physiological effects, such as immunomodulatory, antioxidant, anticancer, antimicrobial and antiviral activities [2]. Currently, these promising immunostimulants have shown applications not only in human health, but also in both veterinary medicine and aquaculture [3]. For instance, the administration of formulated diets containing extracts of *A. platensis* led to an improvement of the health status of different marine species, such as fish [3], pearl oysters [4] and shrimp [5].

The use of probiotics and immunostimulants in shrimp feeding has been widely used as prophylactic and therapeutic treatments against infectious diseases. Since its first appearance in the early 1990's, the White spot syndrome virus (WSSV) has been considered the major threat for penaeid shrimp farming worldwide [6]. To defend themselves against pathogens, shrimp rely on both cellular and humoral immune responses mediated by phagocytic immunocompetent cells named hemocytes. Shrimp hemocytes comprise a heterogeneous circulating cell population and are the main site of immune effectors production [7]. However, more attention has been recently paid to shrimp epithelial immune defenses, especially those occurring in the intestines [8–10]. Indeed, the shrimp gut is broadly considered as a route of entry for many pathogens and, like the hemocytes, all intestine portions are also important sources of immune molecules [9].

In the last years, the effectiveness of immunostimulants from different natural origins has been extensively studied in shrimp aquaculture concerning zootechnical and health performances. Surprisingly, the molecular mechanisms underlying the beneficial properties of immunostimulants as well as their modulatory effects on shrimp gut immunity are largely unknown. Here we have studied some unexplored effects of cyanobacteria-enriched feed on antiviral and intestinal

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immune defenses of the most important cultivated shrimp species, *Litopenaeus vannamei*. Our results showed for the first time that the oral administration of *A. platensis*-based diets promotes immunostimulation and modulates the expression of immune-related genes in the midgut that can be associated with shrimp antiviral protection against the WSSV.

2. Material and methods

2.1. Animals, experimental design and WSSV infection

Litopenaeus vannamei juveniles (12 ± 3 g) were obtained from the Laboratory of Marine Shrimp (Federal University of Santa Catarina, Brazil). Following acclimation (one week), shrimp (n = 144) were randomly divided into two groups (24 animals per group in triplicates) according to the diet used: SF (*Arthrospira platensis* supplemented feed) and CF (control feed). The cyanobacterial dry biomass was obtained from *A. platensis* cells cultivated in indoor tanks (50 L), containing the modified Jourdan medium at salinity 10, following by filtration (60 μm mesh) and dehydration at 40 °C for 24 h [11]. The supplemented feed (SF) was prepared by mixing a powered commercial feed (Guabi Vannamei, 35 EXT) with 0.6% of the dry biomass of *A. platensis* and 5% of carboxymethylcellulose (CMC) diluted in warm water (40 °C). The resulting mixture was pelleted and dehydrated overnight at 45 °C. This dry biomass concentration was defined based on a previous dose-response trial (0.1%, 0.3%, 0.6% and 1.2%) (data not shown). The CF was prepared following the same protocol, without the addition of the cyanobacterial biomass. Each experimental group was fed twice daily during 40 days at a rate of 3% of shrimp body weight. Uneaten food was removed by siphoning after 1 h, dried at 40 °C and weighed. To calculate the food consumption (1-, 10- and 20-day points), the mass difference between offered and uneaten food was divided by the weight in g of the live weight (6 animals per group in triplicates).

Following the 20-day feeding period, 36 animals of each experimental condition (12 shrimp per tank in triplicates) were sampled and processed for the evaluation of cellular and humoral immune parameters and gene expression analysis. Sample preparation for immune parameter measurements is described in section 2.2. For gene expression analysis, hemolymph was withdrawn into modified Alsever solution (MAS: 27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0) and hemocytes were collected and pooled (3 pools of 5 animals per condition) for total RNA extraction. Midguts (3 animals per condition) were collected just after the hemolymph withdrawn and immediately processed for total RNA extraction.

The remaining animals of each experimental group (36 animals per condition) were injected with 100 μL of a WSSV inoculum containing 3×10^2 viral particles (median lethal dose within 15 days, LD30/15). The WSSV inoculum was prepared as previously described [12]. After the viral challenge, shrimp were individually split within the same tanks (3 animals per tank) to avoid cannibalism. Each experimental group was fed with its respective diet (SF or CF) for more 20 days. Mortalities were monitored daily, and cumulative survival curves were created using Kaplan-Meier. Details of the experimental design are illustrated in Fig. 1A.

2.2. Assessment of shrimp immune parameters

The cellular and humoral shrimp defenses were compared between the experimental groups by evaluating the following well-known standardized immune parameters: total hemocyte counts (THC), total protein concentration (PC), phenoloxidase activity (POA) and serum agglutination titer (AGT) [13,14]. For THC, samples of hemolymph (3 pools of 5 animals per condition) were collected in MAS, fixed (4% formaldehyde) and counted using a Neubauer chamber. The other immune parameters were assessed in serum samples. For the serum preparation, hemolymph (3 pools of 5 animals per condition) was collected without MAS and left to coagulate for 24 h at 4 °C. The serum was collected by repeatedly centrifugation (2.000 × *g* for 10 min) of the resulting clot. The PC of the serum was quantified by the Pierce BCA protein assay kit (Thermo Fisher Scientific) using bovine serum albumin as a standard control. The serum was diluted 3.000 × and the absorbance measured in a microplate reader at 562 nm. For the determination of the POA, diluted serum samples were incubated for 5 min with an equal volume of 1 mg/mL trypsin (Sigma) and then with an equal volume of 3 mg/mL L-DOPA. After 5-min incubation, POA was measured spectrophotometrically by recording the formation of dopachrome from L-DOPA at 490 nm. POA was expressed as enzyme unit (U) and corresponded to an increase of 0.001 in the absorbance per min and per mg of protein at 20 °C. The basal POA was quantified by replacing trypsin with ultrapure water.

The agglutinating capacity of the hemolymph was determined by incubating 50 μL of serially diluted serum samples (diluted in TBS: 50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.4) with an equal volume of 2% suspension of dog erythrocytes (diluted in TBS) in U-shaped bottom 96-well microplates for 1.5 h at room temperature. In controls, shrimp serum was replaced by TBS. The agglutination titer (AGT) was expressed as the reciprocal of the highest serum dilution that shows a positive reaction. The AGT was converted into $\log_2$ values. All assays were performed in duplicates. For all immune parameters, differences were considered statistically significant at $P < 0.05$ using Mann-Whitney *t*-test.

2.3. Quantitative gene expression analysis

Total RNA was extracted from hemocyte and midgut samples using the TRIzol reagent (Thermo Fisher Scientific), treated with DNase I (Thermo Fisher Scientific) and precipitated with 0.3 M sodium acetate (pH 5.2) and isopropanol (1:1; v:v). RNA amount and quality were assessed by spectrophotometric analysis and the integrity of total RNA was analyzed by 0.8% agarose gel electrophoresis. First strand cDNA was synthesized from 1 μg of total RNA using RevertAid Reverse Transcriptase (Thermo Fisher Scientific) and oligo$(dT)_{12\text{-}18}$ primers.

Reverse Transcription quantitative real-time PCR (RT-qPCR) amplifications were performed in the StepOne Plus™ Real-time PCR System (Thermo Fisher Scientific) in a final volume of 15 μL containing 0.2 μM of each primer (Table S1), 7.5 μL of reaction mix (Maxima SYBR Green/ROX qPCR Master Mix 2 × ; Thermo Fisher Scientific) and 1 μL of cDNA. RT-qPCR assays were submitted to an initial denaturation step of 10 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. The *Lv*L40 (ubiquitin/ribosomal L40 fusion protein) and *Lv*RpS3A (S3A ribosomal protein) genes were used as references for RT-qPCR data normalization using the $2^{-\Delta\Delta Cq}$ method [15]. Differences in gene expression were considered statistically significant at $P < 0.05$ (cutoff of 1.5-fold change in expression level) using Student's t-test.

2.4. Total bacterial quantification

For bacterial quantification, genomic DNA (gDNA) was extracted from midguts using the DNAzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. gDNA amount and quality were assessed spectrophotometrically and the integrity of gDNA was analyzed by 0.8% agarose gel electrophoresis. The absolute quantification of total bacteria was performed by qPCR using the universal primers 926F and 1062R [16], that target a conserved region from the bacterial 16S rRNA gene. The absolute bacterial load in shrimp midguts was calculated using a standard curve derived from a 10-fold dilution series of a plasmid containing the DNA target sequence (10^7 to 10^3 plasmids/μL; $R^2 = 0.994$).

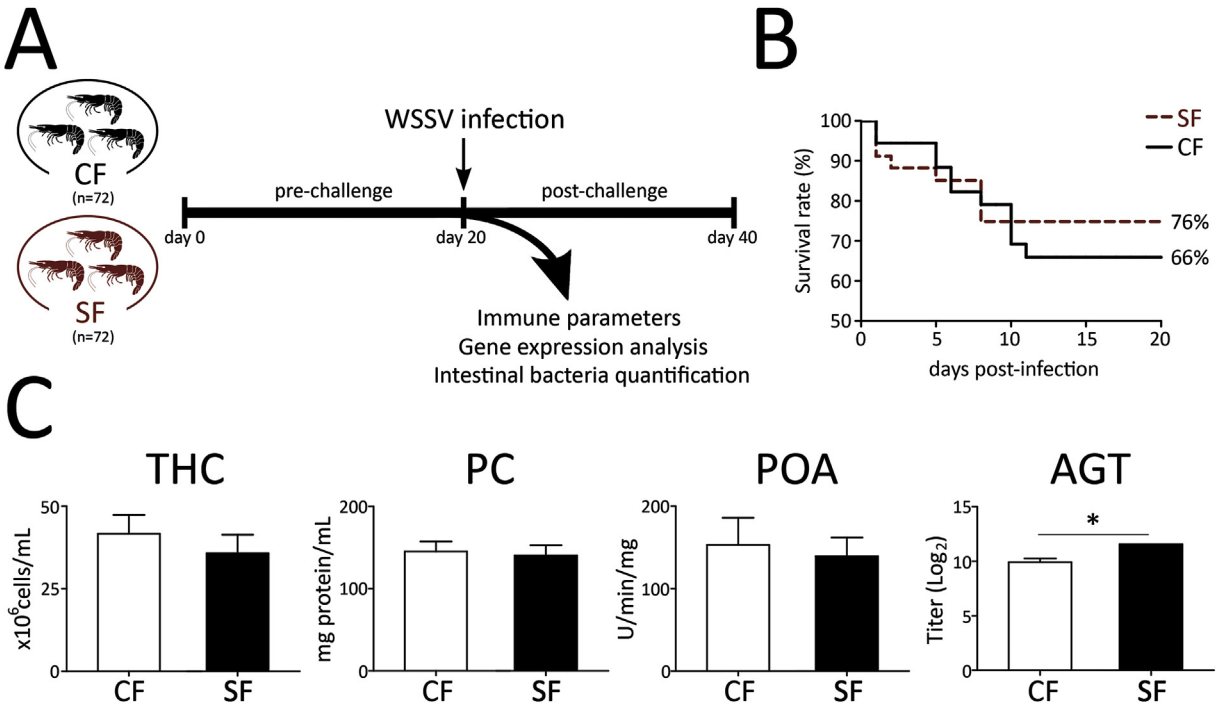

Fig. 1. (**A**) Details of the experimental design. Two experimental groups were set up, and shrimp were fed with a control (CF) or an *Arthrospira platensis* supplemented feed (SF) during 40 days. For immune parameters measurements and gene expression analysis, 36 animals of each group were sampled after 20 days of food supplementation. The remaining animals (36 animals per condition) were experimentally infected with WSSV and mortalities were monitored daily for 20 days. Each experimental group was fed with its respective diet (SF or CS) during all experimentation period (40 days). (**B**) Kaplan-Meier survival curves of shrimp after WSSV infection. (**C**) Comparison of four standardized shrimp immune parameters between the experimental groups: total hemocyte counts (THC), total protein concentration (PC), phenoloxidase activity (POA) and serum agglutination titer (AGT). Results are presented as the mean ± standard deviation, and asterisks indicate statistical differences (*) (Mann-Whitney *t*-test, $P < 0.05$).

3. Results and discussion

We have explored here the potential use of an *Arthrospira*-enriched diet as an immunostimulant to enhance shrimp immune defenses against viral infections. Indeed, the *Arthrospira*-based dietary supplement improved shrimp survival rates against the WSSV from 66% to 76% (Fig. 1B). This finding is particularly interesting given the potential antiviral protection conferred by this supplemented diet for at least 20 days post-WSSV-challenge. In addition to their antiviral effect, diets supplemented with extracts from *A. platensis* showed to be also useful in the protection against bacterial infections [5,17]. Effectively, compounds from cyanobacteria have been largely associated with antibacterial, antifungal, and antiviral defenses [18]. Notably, time of protection is one of the major bottlenecks for the use of immunostimulants in aquaculture and the identification of broad-spectrum and low-cost products is still the main goal to be achieved. Altogether, these results bring further support to the application of cyanobacteria-enriched diets for the prevention of different infectious diseases in shrimp aquaculture.

In order to better understand the promising protective antiviral effect associated with the administration of the immunostimulant feed, we evaluated four well-known standardized shrimp immune parameters after 20 days of feeding, a time point before the WSSV challenge. Dietary supplementation with *A. platensis* did not change the total hemocyte counts (THC) or the total protein concentration (PC) and the serum phenoloxidase activity (POA) (Fig. 1C). Both THC and POA are reliable indicators of shrimp health status [13], and this result is probably due to the time point of 20 days, which might have been too late to detect changes in those immune parameters. On the other hand, the serum agglutination titer (AGT) was higher in shrimp fed with supplemented diet than control animals (Fig. 1C).

The agglutinating capacity is commonly associated with a group of glycoproteins collectively termed as “agglutinins”, which can recognize and bind to specific carbohydrates. Most of the shrimp agglutinins, such as lectins [19] and βGBP (β-1,3-glucan binding protein) [20], are produced by the hepatopancreas and constitutively secreted to the hemolymph [9,19]. It is likely that the oral administration of *A. platensis* may have stimulated the hepatopancreas cells and, consequently, induced the synthesis and/or the release of soluble agglutinins. Many agglutinins, especially the lectins, are involved in different immunological functions, from microbial recognition and agglutination to antibacterial and antiviral effects [19]. Although we have not identified the lectin-type (from the seven groups found in shrimp) that has improved the agglutinating capacity of the hemolymph, it is widely known that C-type lectins are important microbial recognition proteins involved in shrimp antiviral defenses [21]. Indeed, while the expression of most C-type lectins is induced in response to viral infections [19], specific members can bind to the envelope proteins of the WSSV [22]. Our results bring new clues for the comprehension of the involvement of agglutinins/lectins in shrimp defenses against the WSSV. However, more functional studies are still required to understand their direct antiviral functions better.

The immunomodulatory effects of the *A. platensis* supplemented feed was further investigated at the molecular level by analyzing the expression of 12 key marker genes from different immune functional categories: (i) antimicrobial peptides (*Litvan* PEN3, *Litvan* ALF-A and *Litvan* ALF-C), (ii) microbial recognition (*Lv*Frep), (iii) immune signaling pathways (*Lv*Toll2, *Lv*IMD, *Lv*STAT, *Lv*Dorsal and *Lv*Relish), and (iv) proPO activating system (*Lv*proPO, *Lv*PPAE2 and *Lv*α2M-2) [9,12,23]. The expression levels of the selected genes were compared between the experimental conditions (CF × SF) in both circulating hemocytes and midgut by using fluorescence-based quantitative PCR analyses. In addition to hemocytes, which represent the primary site of immune-related genes expression, we have also analyzed

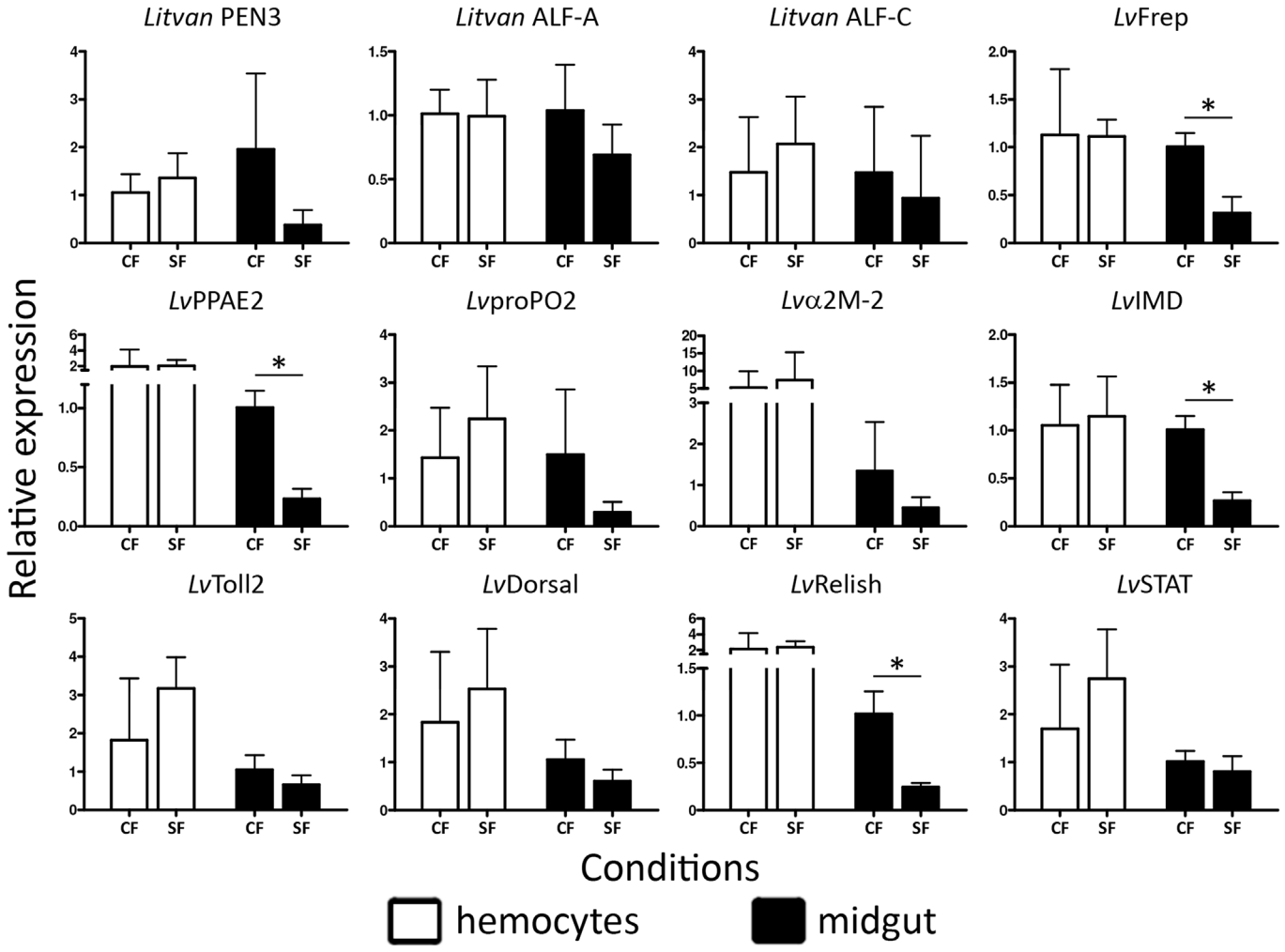

Fig. 2. Relative expression profile of 12 immune-related genes in circulating hemocytes (white bars) and midgut (black bars) of shrimp after 20 days of food supplementation. Results are presented as the mean ± standard deviation, and asterisks (*) indicate statistical differences (cutoff of 1.5-fold change in expression level; Student's t-test, $P < 0.05$). SF: *Arthrospira platensis* supplemented feed. CF: control feed.

transcriptional changes in the midgut, once this intestinal portion represents a potential route of entry for many pathogens into the shrimp hemocel.

While none of the 12 analyzed genes were modulated in circulating hemocytes, the expression of four genes was diminished in the midgut after 20 days of food supplementation (Fig. 2). The midgut transcript levels of *Lv*Frep (3.2 fold-change), *Lv*PPAE2 (4.3 fold-change), *Lv*IMD (3.8 fold-change) and *Lv*Relish (4.1 fold-change) were significantly lower in shrimp fed supplemented diet in comparison to controls (Fig. 2). In penaeid shrimp, the expression of both Frep (fibrinogen-related protein) and PPAE2 (proPO-activating enzyme 2) genes seems to be restricted to hemocytes [23,24]. Therefore, the reduction of their mRNA levels in the midgut of the SF group can be associated with a lower number of infiltrating hemocytes expressing both Frep and PPAE2. Since the THC was not altered between the experimental groups, it is plausive to argue that the proportion of Frep- and PPAE2-expressing hemocytes between tissues (hemocytes × midgut) has changed in response to the feed supplementation. Alternatively, one cannot rule out that the *A. platensis* supplemented feed led to a reduction of their expression levels in midgut-infiltrating hemocytes.

The same expression pattern was also observed for *Lv*IMD and *Lv*Relish (Fig. 2), two central genes involved in the IMD (immune deficiency) signaling pathway [7]. The IMD signaling has a crucial role in the intestinal defenses of arthropods by the regulation of immune-related genes involved in both antimicrobial and antiviral defenses, and also in the control of the gut microbiota [25,26]. Particularly in mosquitoes, the activation of the IMD pathway promotes a dysbiosis of the intestinal microbial populations that allows the replication of Sindbis virus [27]. In this framework, we have then investigated the effect of the feed supplementation on the abundance of shrimp intestinal microbiota. Absolute qPCR quantification assays showed that shrimp fed supplemented diet exhibited 6.45-fold ($P < 0.05$) more bacteria in their midguts than control animals (Fig. 3). In view of this result, one can hypothesize that this significant increase in bacterial abundance is directly associated with the administration of the *A. platensis* supplemented feed. However, the downregulation of the IMD pathway by the supplemented diet could have also an indirect effect on WSSV infection by modulating the shrimp intestinal bacterial populations. Actually, the gut bacteriome is a complex system that is highly sensitive to external influences (biotic and abiotic selection pressures), including WSSV infection [28].

Pieces of evidence suggest that *A. platensis* may be useful to improve both animal and human health status by changing the abundance and composition of gut microbiota [29]. For instance, the administration of *A. platensis*-based supplements showed to regulate the gut microbiota structure of juvenile great sturgeon (*Huso huso*) by increasing the abundance of specific beneficial bacteria [30] In shrimp, dietary supplementation with microalgae (*Porphyra haitanensis*) resulted in a significant change in the bacterial populations residing within the shrimp gut [31]. Actually, *P. haitanensis* supplemented feed led to a decrease in

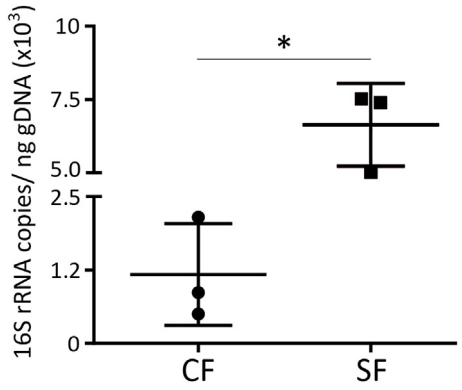

Fig. 3. Absolute quantification of total bacteria in the midgut of shrimp fed supplemented diet in comparison to control animals. Results are presented as the number of 16S rRNA gene copies per ng of total DNA (gDNA). The absolute quantification was assessed by qPCR using a standard curve derived from a 10-fold dilution series of a plasmid containing the DNA target sequence. Differences are indicated by asterisks (*) (Student's t-test, $P < 0.05$). SF: *Arthrospira platensis* supplemented feed. CF: control feed.

the abundance of intestinal *Vibrio* species (which includes many opportunistic shrimp pathogens) and improved shrimp survival to the WSSV infection [31].

In conclusion, our results provide pieces of evidence for the immunomodulatory effects of cyanobacterial-based feed on shrimp intestinal immunity. Dietary supplementation with *A. platensis* increased the hemolymph agglutination capacity and modulated the expression of essential immune-related genes, and the total bacterial abundance in the midgut, leading to a better global health state and higher survival rates facing WSSV infection. To our knowledge, this is the first study exploring the effects of an *A. platensis* supplemented feed on shrimp gut immunity and intestinal microbiota. A whole understanding of the effects of *A. platensis* supplementation on shrimp immune defenses will provide valuable information on the effectiveness and usefulness of immunostimulants in shrimp farming.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.02.062.

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