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Short communication

Environmental rearing conditions are key determinants of changes in immune gene expression patterns in shrimp midgut

Mariana Rangel Pilotto[a], Nicolas Argenta[a], Jamille Martins Forte[b], Bárbara Hostins[c], Francisca Gleire R. Menezes[d], Rodrigo Maggioni[b], Oscarina Viana de Sousa[e], Wilson Wasielesky Jr.[c], Rafael Diego Rosa[a], Luciane Maria Perazzolo[a,*]

[a] Laboratory of Immunology Applied to Aquaculture, Department of Cell Biology, Embryology and Genetics, Federal University of Santa Catarina, CEP 88040-900, Florianópolis, SC, Brazil
[b] Center of Studies and Diagnosis of Aquatic Organism Diseases, Marine Sciences Institute, Federal University of Ceará, CEP 60165-081, Fortaleza, CE, Brazil
[c] Aquaculture and Marine Biotechnology Group, Institute of Oceanography, Federal University of Rio Grande, CEP 96201-900, Rio Grande, RS, Brazil
[d] Fisheries Engineering Department, Federal University of Ceará, CEP 60455-760, Fortaleza, CE, Brazil
[e] Laboratory of Fish and Environmental Microbiology, Marine Sciences Institute, Federal University of Ceará, CEP 60165-081, Fortaleza, CE, Brazil

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ABSTRACT

The super-intensive BioFloc Technology (BFT) system has been highlighted as a promising eco-friendly alternative to the traditional shrimp rearing systems. To gain insight into the impact of environmental rearing conditions on shrimp intestinal immunity, we assessed the expression profile of key immunological genes in the midgut of *Litopenaeus vannamei* shrimp reared in two contrasting culture systems: the indoor super-intensive BFT and the outdoor intensive Green-Water System (GWS). From the 30 analyzed genes, the expression levels of 25 genes were higher in the midgut of shrimp reared in BFT than in GWS. The main functional categories represented in BFT-shrimp were the prophenoloxidase-activating system, immune signaling, antimicrobial peptides, and RNA interference pathway. Comparatively, only the RNAi pathway gene Dicer-1 (*Lv*Dcr1) was more expressed in animals from the GWS group. However, despite the differences in gene expression, the total midgut bacterial abundance was similar between the experimental groups. Altogether, our results suggest that the microbial-rich environment offered by the BFT system can be acting as an immunostimulant by altering the immune expression profile of the midgut. The gene expression level found in GWS animals could be related to the chronic presence of the IMNV in the Brazilian Northeast. Knowing the effects of environmental stress factors on the intestinal immune defenses can provide an in-depth understanding of the relationship between cultivated shrimp and the major pathogens affecting the shrimp industry.

1. Introduction

Two threatening viral diseases, the white spot syndrome (WSS) and the infectious myonecrosis (IMN), have caused notable economic losses in both Northeast (WSS and IMN) and Southern (WSS) regions of Brazil, where the shrimp farming is most concentrated (Dantas et al., 2018; Marques et al., 2015). Shrimp farming is usually performed in soil ponds that require regular water exchange due to the immense amount of organic material generated in the system (Ahmad et al., 2017; Rego et al., 2018). Typically, the pond waste is released in the environment without previous treatment, and this unfriendly environmental practice has caused the rapid dissemination of pathogens in farms and coastal areas (Moss et al., 2012).

The green-water system (GWS) is a photoautotrophic shrimp culture usually conducted in outdoor tanks naturally fertilized by food wastes and shrimp feces generated into the system. Consequently, microalgae and other organisms freely grow in this environment, decreasing the transparency and defining the greenish of the water. This outdoor rearing system is continuously subjected to fluctuations in water parameters (oxygen, pH, temperature, salinity) (Nunes et al., 2011), as commonly observed for other traditional shrimp farming systems. In contrast, the super-intensive BioFloc Technology (BFT) system has appeared as a promising eco-friendly alternative to the traditional shrimp cultures (Ahmad et al., 2017). This closed system is based on the principle of waste nutrients recycling, which is responsible for maintaining good water quality (De Schryver et al., 2008). The stability of

* Corresponding author.
E-mail address: l.m.perazzolo@ufsc.br (L.M. Perazzolo).

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this system depends on the dynamic interaction among bacterial communities and other organisms such as microalgae, fungi, protozoans, nematode, and rotifers (Emerenciano et al., 2017). Also, animals are stocked at high densities and the system operates with little or no water exchange (Bossier and Ekasari, 2017; Emerenciano et al., 2017). Besides, the microbial bioflocs present in this culture can also represent an additional food source for the animals, and their consumption can increase the shrimp growth performance and robustness (Burford et al., 2004; Maicá et al., 2017).

Our current knowledge on the shrimp immunity is practically restricted to the hemolymph defenses mediated by the immunocompetent cells (hemocytes) and soluble plasma immune effectors/inducers. However, the shrimp midgut is an important source for the expression of immune genes and a potential region for pathogen ingress, considering its lack of a protective chitinous cuticle layer (McGaw and Curtis, 2013; Wang et al., 2012). In line with this notion, it is proposed that some key immune responses may occur in the midgut in order to limit the spreading of pathogens. On the other hand, the cellular and molecular mechanisms of the shrimp immunity associated with the intestinal epithelium remain to be elucidated.

Current studies have suggested that BFT improves shrimp immunity leading to higher survival rates under both bacterial and viral infections (Crab et al., 2010; Ekasari et al., 2014), probably due to an immunostimulatory effect associated to this microbial-rich environment (Ahmad et al., 2017; Crab et al., 2012). Thus, it is of prime importance to understand the impacts of the BFT rearing system on shrimp immune defenses and its influence on shrimp-microbe interactions. In order to fill this research gap, we have explored the expression profile of key immune gene markers in the midgut of shrimp reared in two contrasting culture systems: the indoor super-intensive BFT and the outdoor intensive GWS. Our results showed for the first time that the BFT system can be acting as an immunostimulant by altering the immune gene expression profile of the shrimp midgut.

2. Material and methods

2.1. Shrimp and rearing conditions

Litopenaeus vannamei juveniles were reared in two contrasting culture systems: BioFloc Technology system (BFT) and Green-Water System (GWS). The BFT system was performed in indoor tanks inoculated with a mature biofloc at the Marine Aquaculture Station (EMA-FURG, Rio Grande, Brazil). Shrimp were stocked at an initial density of 300 animals/m^2 in tanks of 35 ton. The amount of solids present in BFT tanks was maintained in 369.6 mg/L and the evaporated water was daily replaced by the same volume of dechlorinated freshwater. The GWS was performed in outdoor tanks exposed to sunlight and weather changes at the Laboratory of Aquatic Animal Nutrition (Federal University of Ceará, Eusébio, Brazil). Shrimp were stocked at an initial density of 34 animals/m^2 in tanks of 3 ton, with a daily water exchange of 50%. Finally, animals reared in Clear seaWater System (CWS) at the Laboratory of Marine Shrimp (UFSC, Florianópolis, Brazil) served as controls. For that, shrimp were stocked at an initial stocking density of 20 post larvae/m^3 in indoor tanks continuously aerated and kept under controlled temperature (29 ± 1 °C) and salinity (34–35), with a daily water exchange of 80%, as detailed in (Pilotto et al., 2018).

Water quality parameters were maintained within the ideal for each culture, and systems were provided with constant aeration supplied from mechanical blowers. Shrimp were fed *ad libitum* with a commercial shrimp diet (containing at least 35% of crude protein), 3 times a day. After four months of rearing and when shrimp reached the juvenile stage (7–10 g), midgut samples were individually collected from randomly selected animals and combined in pools (10–30 animals per culture system in triplicates) for subsequent extraction of total RNA and genomic DNA (gDNA).

2.2. Molecular diagnosis of WSSV and IMNV

Midguts were harvested by dissection and washed in ice-cold Tris-saline solution (10 mM Tris, 330 mM NaCl, pH 7.4) for removal of the intestinal content. Both total RNA and gDNA samples were extracted using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. After extraction, RNA samples were treated with DNase I (Thermo Fisher Scientific) at 37 °C for 15 min while gDNA samples were treated with 50 μg/mL RNase A (Fermentas) at 37 °C for 30 min. Quantification and quality of nucleic acids were assessed by spectrophotometry and 0.8% agarose gel electrophoresis, respectively. Total RNA samples of 1 μg were used as templates for complementary DNA (cDNA) synthesis using oligo $(dT)_{12\text{-}18}$ primer and the RevertAid Reverse Transcriptase (Thermo Fisher Scientific). Molecular diagnosis of WSSV was carried out by nested-PCR using 50 ng of gDNA as previously described by (Lo et al., 1996). The molecular detection of IMNV was performed by RT-PCR using 1 μL of synthesized cDNA as described by (Senapin et al., 2007).

2.3. Fluorescence-based reverse transcription real-time quantitative PCR (RT-qPCR)

RT-qPCR amplifications were performed in the StepOnePlus 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. Average values of three reference genes (*Lv*EF1α, *Lv*Actin, and *Lv*RpS3A) were used for RT-qPCR data normalization as previously described (Farias et al., 2019). The relative expression levels of the target genes (BFT × GWS) were calibrated with the midgut expression profile of shrimp reared in CWS (control group). Differences in gene expression were considered statistically significant at $P < 0.05$ (cutoff of 2-fold change in expression level) using unpaired Student's t-test. The heat map was generated using the ClustVis software (https://biit.cs.ut.ee/clustvis/).

2.4. Bacteria quantification by real-time quantitative PCR (qPCR)

The absolute quantification of total bacteria was performed by real-time quantitative PCR (qPCR) using universal primers (926F: 5′-AACTCAAAKGAATTGACGG-3'; 1062R: 5′-CTCACRRCACGAGCTGAC-3′) targeting a conserved region from the bacterial 16S ribosomal RNA gene (De Gregoris et al., 2011). The qPCR amplifications were performed in triplicate using the GoTaq qPCR Master Mix (Promega) according to the manufacturer's reaction parameters. Bacterial counts in shrimp midguts were 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; $R^2 = 0.999$). Difference in total bacteria counts was considered statistically significant at $P < 0.05$ using unpaired Student's t-test.

3. Results and discussion

In order to explore the impacts of environment rearing conditions on shrimp intestinal immunity, we have evaluated and compared the expression profile of 30 key immune markers encoding genes in the midgut of shrimp reared in two contrasting culture systems practiced by the aquaculture sector: the indoor super-intensive BioFloc Technology system (BFT) and the outdoor intensive Green-Water System (GWS). For that, we covered the main functional categories involved in shrimp immune defenses: (i) microbial recognition, (ii) cytokines and immune signaling pathways, (iii) antimicrobial peptides (AMPs), (iv) prophenoloxidase (proPO)-activating system, (v) RNA interference (RNAi)

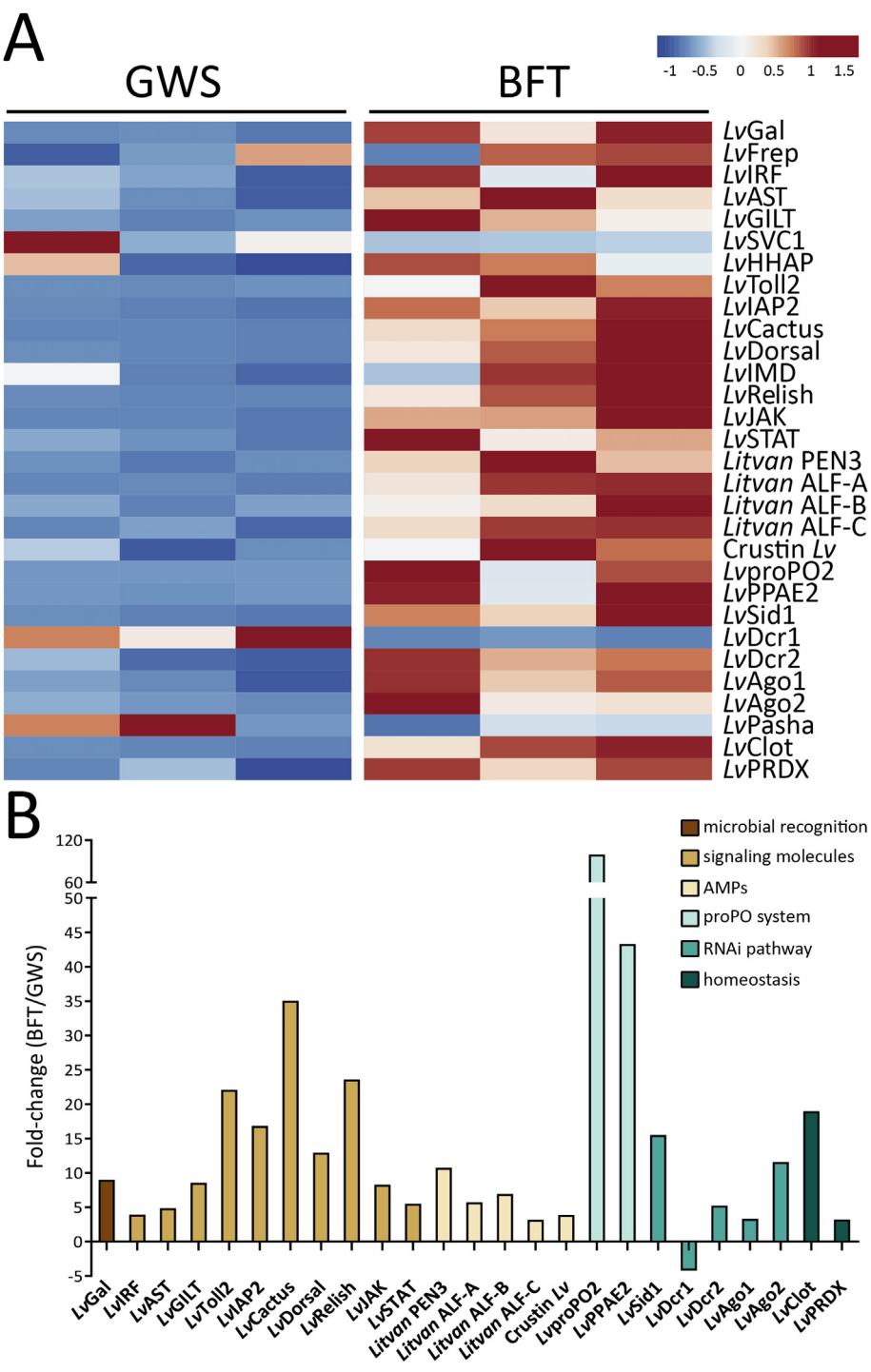

Fig. 1. (A) Heat map showing the expression profile of 30 immune-related genes (Table S1) in the midgut of *Litopenaeus vannamei* shrimp reared in two contrasting culture systems: GWS (green-water system) and BFT (Biofloc technology). The relative expression levels of the target genes were calibrated with the midgut expression profile of shrimp reared in CWS (control group). Each cell in the matrix corresponds to the expression level of a given gene in an experimental condition and the intensity of the color (from blue to red) indicates the magnitude of expression, based on the color scale at the right top of the heat map. (B) Differences in the transcript levels of the genes whose expression showed significant changes (> 2-fold change; unpaired Student's t-test, $P < 0.05$) between the BFT and GWS groups. Values are expressed in fold-change (BFT/GWS). *P* value and fold-change for each evaluated gene are shown in Table S2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

pathway and (vi) homeostasis (clotting and redox system). The expression levels of the selected genes were compared between the experimental rearing conditions (BFT × GWS) in the midgut because it is a major shrimp immune organ (Pilotto et al., 2019; Silveira et al., 2018) in close contact with the external environment and therefore with potentially infectious microorganisms.

Remarkably, the expression levels of most genes, from all functional categories, were higher in the midgut of shrimp reared in BFT than in animals reared in GWS (Fig. 1A). Indeed, only five genes (*Lv*Frep, *Lv*HHAP, *Lv*SVC1, *Lv*IMD, and *Lv*Pasha) showed no changes in their expression levels between the rearing conditions. Particularly, it is worth highlighting that most of those genes displayed a variation in expression levels among the biological replicates within the same experimental condition (Fig. 1A). Such variation in gene expression has been already described for shrimp (*L. vannamei*) hemocytes (Goncalves et al., 2014). Regarding the differentially expressed genes, 24 showed to be more expressed in the midgut of BFT-reared shrimp while only the *Lv*Dcr1 gene showed to be more expressed in animals cultured in the GWS (Fig. 1B).

The microbial richness and diversity of the BFT environment continuously provides MAMPs (microbial-associated molecular patterns) that can act as potential immunostimulants (Bossier and Ekasari, 2017). Since bioflocs also serve as a food source for shrimp, the constant ingestion of MAMPs may induce the expression of immune-related genes in the digestive system. To appraise this hypothesis, we analyzed the expression levels of two main genes involved in the recognition of

MAMPs and, consequently, in the trigger of different immune responses: the galectin *Lv*Gal (Hou et al., 2015) and the fibrinogen-related protein *Lv*Frep (Coelho et al., 2016). While *Lv*Frep mRNA levels were not changed, the *Lv*Gal gene showed higher expression levels in the midgut of shrimp reared in BFT (8.9 fold-change) than in GWS (Fig. 1B). Interestingly, a recombinant galectin from *L. vannamei* (r*Lv*Gal) was able to recognize and agglutinate *Vibrio anguillarum* (Hou et al., 2015). Actually, galectins display a remarkable functional diversity in shrimp hemolymph-based immunity (Wang and Wang, 2013), and it is possible that they are also intimately involved in microbial sensing in the gut.

After MAMP recognition by pattern recognition proteins, a variety of cellular and humoral mechanisms can be activated, such as the antimicrobial defenses mediated by gene-encoded AMPs and the proPO-activating system. These antimicrobial defenses are generally regulated by secreted cytokines and the immune signaling pathways Toll, IMD and JAK/STAT (Tassanakajon et al., 2018). Most genes from this functional category were more expressed in the midgut of shrimp reared in BFT than in animals cultured in GWS: *Lv*IRF (3.91 fold-change), *Lv*AST (4.86 fold-change), *Lv*GILT (8.58 fold-change), *Lv*Toll2 (22.1 fold-change), *Lv*IAP2 (16.84 fold-change), *Lv*Cactus (35 fold-change), *Lv*Dorsal (12.9 fold-change), *Lv*Relish (23.5 fold-change), *Lv*JAK (8.2 fold-change) and *Lv*STAT (5.5 fold-change) (Fig. 1B). A same result was also observed for all analyzed AMPs: *Litvan* PEN3 (10.7 fold-change), *Litvan* ALF-A (5.7 fold-change), *Litvan* ALF-B (6.9 fold-change), *Litvan* ALF-C (3.1 fold-change) and the Type IIa Crustin *Lv* (3.8 fold-change) (Fig. 1B). In a previous work, we demonstrated that the expression of both *Litvan* ALF-A and *Litvan* ALF-C was induced in *L. vannamei* midgut in response to a *Vibrio* infection (Silveira et al., 2018), suggesting an important role for gene-encoded AMPs in shrimp intestinal defenses (Farias et al., 2019).

As observed for AMPs, the transcript abundance of genes from the proPO-activating system was also greater in shrimp from the BFT group. In arthropods, the melanization process is dependent of the phenoloxidase (PO) enzyme whose activation is mediated by proPO-activating enzymes (PPAEs) (Cerenius et al., 2010). Interestingly, *Lv*proPO2 (99.5 fold-change) and *Lv*PPAE2 (43.3 fold-change) were the genes with the highest differential expression levels between the two environmental rearing conditions (Fig. 1B). Indeed, previous studies demonstrated that genes from the proPO cascade are generally over-expressed in the digestive system of shrimp reared in BFT (Kim et al., 2014; Panigrahi et al., 2018). However, since hemocytes are the main site of production of shrimp antimicrobial effectors (AMPs and proPO system), the highest expression levels of those genes are likely a consequence of a significant number of infiltrating hemocytes in the midgut of BFT-reared shrimp. The importance of infiltrating hemocytes in the crosstalk between shrimp hemolymph-based and gut-based immunities was previously confirmed (Silveira et al., 2018).

The high expression levels of genes related to antimicrobial defenses, including also the clotting protein *Lv*Clot (18.96 fold-change) and the peroxiredoxin *Lv*PRDX (3.23 fold-change), in the midgut of shrimp reared in BFT could be associated with the rich consortia of microorganisms present in this environment. Considering this issue, we quantified and compared the total bacterial counts present in the midgut of shrimp reared in both culture systems. Absolute qPCR quantification assays showed that the midgut bacterial abundance was quite similar between the experimental groups ($P = 0.37$) (Fig. 2). Indeed, the BFT environment is not only composed by bacteria, but also by diverse populations of fungi, protozoans, nematodes and rotifers (Ahmad et al., 2017; Emerenciano et al., 2017). In contrast, green-water systems harbor predominantly phytoplankton, such as diatoms (Nunes et al., 2011). In view of this result, one can hypothesize that the immunostimulatory status promoted by the BFT environment could be associated to MAMPs provided by other microorganisms than bacteria. Thus, it is of great interest to determine the different microbial consortia (such as archaea, fungi and virus) that inhabit the midgut of shrimp reared in BFT, as previously performed for the bacterial communities (Pilotto et al., 2018).

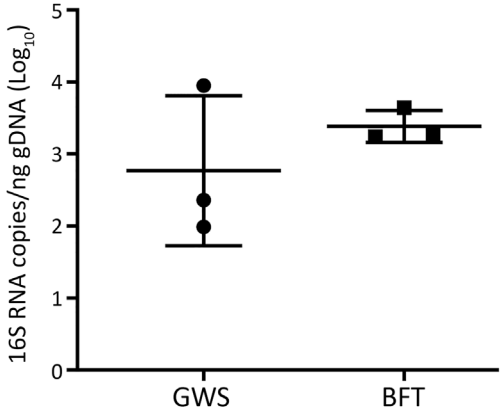

Fig. 2. Absolute quantification of total bacteria in the midgut of shrimp reared in GWS (green-water system) and BFT (Biofloc technology). The absolute quantification was assessed by qPCR using a standard curve derived from a 10-fold dilution series of a plasmid containing the DNA target sequence. Values are expressed in log10-scale copies of the bacterial 16S ribosomal RNA gene per ng of gDNA.

Finally, we investigated the impact of the environmental rearing conditions on the shrimp ability to trigger antiviral defenses by evaluating the expression of six genes associated to the RNAi pathway. As observed for antimicrobial-related genes, the expression of the RNAi pathway genes *Lv*Sid1 (15.5 fold-change), *Lv*Dcr2 (5.23 fold-change), *Lv*Ago1 (3.3 fold-change) and *Lv*Ago2 (11.5 fold-change) was higher in BFT-reared shrimp when compared to the GWS group (Fig. 1B). Despite this potential antiviral defense status, shrimp reared in BFT were diagnosed as negative for both WSSV and IMNV (Fig. S1). It is widely known that some RNAi-associated molecules display unconventional functions, although the RNAi machinery has been proved to be crucial for shrimp antiviral defenses (Wang et al., 2014). For instance, while the Dicer-2 gene (*Lv*Dcr2) showed to be induced in shrimp midgut after *Vibrio* infections (Silveira et al., 2018), in fruit flies this gene was shown to play an unconventional function by modulating the Toll signaling, which is associated to insect antibacterial defenses (Wang et al., 2015). In line with this notion, we can expect a similar antibacterial defense occurring in shrimp midgut associated to the microbial richness found in the BFT environment.

Unlike the other analyzed genes, only the expression of the RNAi pathway gene Dicer-1 (*Lv*Dcr1) was higher (4.1 fold-change) in the midgut of shrimp reared in GWS than in BFT-reared animals (Fig. 1B). In crustaceans, Dicer-1 is involved not only in the RNAi pathway, but also in the maturation of microRNAs (miRNAs), an extensive category of small noncoding RNAs involved in the regulation of almost all life processes. The miRNAs play important roles in the endogenous regulation of genes at the post-transcriptional level and in crustacean antiviral responses (He et al., 2015). Besides, the Dicer-1 gene also showed to be an important component of shrimp miRNA biogenesis during WSSV infections, which is responsible for inhibiting virus replication (Huang and Zhang, 2012). It would be interesting to further investigate the mechanisms involved in the regulation of *Lv*Dcr1 and miRNAs during infections caused by RNA viruses, such as IMNV.

Interestingly, shrimp reared in GWS were diagnosed as positive for IMNV but negative for WSSV (Fig. S1), although none of them presented clinical signs of the IMN disease (Prasad et al., 2017). It has been previously demonstrated an upregulation of the *Lv*Dcr1 gene in the circulating hemocytes and gills of *L. vannamei* shrimp infected with the Taura syndrome virus (TSV) (Yao et al., 2010), which is an RNA virus like IMNV (Prasad et al., 2017). Thus, it is possible that the over-expression of the *Lv*Dcr1 gene in the midgut of GWS-reared shrimp was due to the presence of the IMNV, rather than the environmental

conditions. Indeed, the GWS used in this study was performed in the Northeast of Brazil, a shrimp production region officially declared as endemic for IMNV infection (Prasad et al., 2017). Previous studies have reported IMNV-positive diagnosis to asymptomatic and chronically infected shrimp in its natural condition of farming (Feijó et al., 2015, 2013; Vieira-Girão et al., 2012).

Indeed, shrimp can harbor multiples viral pathogens with no gross signs of disease and be ordinarily active (Flegel et al., 2004). Viral presence in shrimp does not necessary means disease (Flegel, 2007). Additionally, the occurrence of persistent viral infections is common in shrimp without signs of illness. After some years of severe mortality in shrimp farms caused by viral pathogens, the severity of the disease can decline, and despite the survivors remain infected, no signs of the disease are observed (Flegel et al., 2004). This finding is attributed to the development of tolerance to a viral agent, phenomenon common in Asian farms known as viral accommodation (Flegel, 2007). Once it has been frequent to find asymptomatic IMNV-positive shrimp in Brazilian Northeast farms (Feijó et al., 2015, 2013; Vieira-Girão et al., 2012), it is reasonable to suppose that a viral accommodation to the IMNV has occurred.

As far as we know, there are no reports on the expression of genes in shrimp midgut chronically infected with IMNV. There is only one study evaluating the expression of four immunological genes in the gills of IMNV-positive shrimp, but they did not compare their results to an IMNV-free group (Vieira-Girão et al., 2012). Our study is the first one to cover the expression of 30 genes from different immune functional categories, including antiviral defense, in the midgut of shrimp chronically infected with IMNV. However, further research needs to be conducted to gain insights into the effect of chronical-IMNV infection and its relationship with the shrimp rearing condition.

4. Conclusion

In conclusion, we provide here evidence of the impact of environmental rearing conditions on the global immune gene expression patterns in the midgut of the most important penaeid species cultured worldwide, *L. vannamei*. The microbial-rich environment offered by the BFT system can be acting as an immunostimulant by altering the shrimp gut immunity and therefore preventing systemic dissemination of pathogens. The GWS gene expression pattern could be related to the low microbial diversity found in this system, as well as the typical fluctuations in the water parameters inherent to this rearing condition. Also, the gene expression level found in GWS animals could be related to the chronic presence of the IMNV in rearing farms from the Brazilian Northeast. Knowing the effects of environmental stress factors on the intestinal immune defenses can provide an in-depth understanding of the relationship between cultivated shrimp species and the major pathogenic microorganisms affecting the aquaculture industry. Further investigation is needed to understand the relation between the chronic presence of IMNV in shrimp and illness progression, tolerance, and/or latent viral infection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2020.103618.

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