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Short communication

An immune-related gene expression atlas of the shrimp digestive system in response to two major pathogens brings insights into the involvement of hemocytes in gut immunity

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ABSTRACT

Much of our current knowledge on shrimp immune system is restricted to the defense reactions mediated by the hemocytes and little is known about gut immunity. Here, we have investigated the transcriptional profile of immune-related genes in different organs of the digestive system of the shrimp *Litopenaeus vannamei*. First, the tissue distribution of 52 well-known immune-related genes has been assessed by semiquantitative analysis in the gastrointestinal tract (foregut, midgut and hindgut) and in the hepatopancreas and circulating hemocytes of shrimp stimulated or not with heat-killed bacteria. Then, the expression levels of 18 genes from key immune functional categories were quantified by fluorescence-based quantitative PCR in the midgut of animals experimentally infected with the Gram-negative *Vibrio harveyi* or the White spot syndrome virus (WSSV). Whereas the expression of some genes was induced at 48 h after the bacterial infection, any of the analyzed genes showed to be modulated in response to the virus. Whole-mount immunofluorescence assays confirmed the presence of infiltrating hemocytes in the intestines, indicating that the expression of some immune-related genes in gut is probably due to the migratory behavior of these circulating cells. This evidence suggests the participation of hemocytes in the delivery of antimicrobial molecules into different portions of the digestive system. Taken all together, our results revealed that gut is an important immune organ in *L. vannamei* with intimate association with hemocytes.

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1. Introduction

Aquatic environments are rich in both organic and inorganic nutrients and harbor a dense and diverse microbial community, including prokaryotes, protozoans, fungi and a wide range of enveloped and nonenveloped viruses. These microorganisms are easily dispersed by water and can interact with all multicellular organisms in symbiotic or pathogenic relationships. Penaeid shrimp, like other invertebrates, defend themselves from invading microorganisms by the combination of effective antimicrobial and antiviral responses mediated mainly by the circulating immunocompetent cells, the hemocytes (Cerenius et al., 2010). The recognition of the invader by soluble and/or cell surface pattern recognition proteins (PRPs) leads to hemocyte mediated reactions, such as phagocytosis, nodule formation, encapsulation, tissue infiltration (Jiravanichpaisal et al., 2006) and, in some cases, to the release of nucleic acid extracellular traps (Robb et al., 2014). Besides, hemocytes are also involved in the production of humoral factors, cytotoxic free radicals (reactive oxygen and nitrogen species) and antimicrobial peptides (AMPs) (Tassanakajon et al., 2013). AMPs are major components of the innate immune response and four gene-encoded AMP families have been characterized in shrimp: penaeidins, crustins, anti-lipopolysaccharide factors (ALFs) and stylicins (Destoumieux-Garzón et al., 2016).

Genomic and transcriptomic technologies have significantly improved our knowledge on shrimp immunity and responses to infectious diseases affecting the farming industry worldwide. However, most of studies have been focused on both cellular and

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humoral immune responses occurring in the hemolymph (Tassanakajon et al., 2013). Whereas gut is an important route for pathogen ingress, colonization and transmission, surprisingly little attention has been paid to shrimp epithelial responses and intestinal immunity. Basically, the shrimp gut is a tube (beginning at the esophagus and ending at the anus) divided into three main portions, the foregut, midgut, and hindgut. Whilst both foregut and hindgut are lined with a chitinous cuticle, the midgut is composed by a glandular (columnar) epithelium destitute of cuticle and lined with a peritrophic membrane (McGaw and Curtis, 2013; Wang et al., 2012). Other important component of the shrimp digestive system is the hepatopancreas, a digestive and endocrine organ comparable to the liver in vertebrates. The hepatopancreas is a large gland occupying the dorsal region of the cephalothorax, which is also considered as an important site for the expression of immune-related genes in shrimp (Robalino et al., 2007; Zeng et al., 2013).

In this study, we have performed a gene expression analysis in the most important penaeid species cultured worldwide, *Litopenaeus vannamei*, with the goal of characterizing the gut molecular responses triggered by two unrelated pathogens, the Gram-negative *Vibrio harveyi* and the White spot syndrome virus (WSSV). Firstly, the expression and distribution of well-known immune-related genes were investigated in three portions of the gut (foregut, midgut and hindgut) and in hepatopancreas in comparison to hemocytes (the main expression site of shrimp immune-related genes). Further, we showed that genes involved in both antimicrobial and antiviral defenses were induced in the midgut in response to a *Vibrio* infection but not against the WSSV. The presence of infiltrating hemocytes in the intestines, as revealed by whole mount immunofluorescence staining, suggests that these cells participate in shrimp gut immunity, likely through the delivery of antimicrobial molecules into different portions of the digestive system.

2. Materials and methods

2.1. Animals, microbial challenges and tissue collection

Litopenaeus vannamei juveniles (10 ± 2 g) were obtained from the Laboratory of Marine Shrimp (Federal University of Santa Catarina, Brazil). Following acclimation (one week), shrimp (n = 5) were stimulated by the injection of 5 × 10^7 CFU/animal of heat-killed (70 °C for 20 min) *Vibrio harveyi* ATCC 14126 under 100 μL sterile seawater (SSW). Naïve (unchallenged) animals (n = 5) were used as control. At 48 h post-stimulation, hemocytes were obtained as previously described (Goncalves et al., 2014) and the digestive organs (foregut, midgut, hindgut and hepatopancreas) were harvested by dissection. Tissue samples were washed in a Tris-saline solution (10 mM Tris, 330 mM NaCl, pH 7.4), homogenized in TRIzol reagent (Thermo Fisher Scientific) and processed for RNA extraction and semiquantitative RT-PCR analysis.

The experimental infections were performed with two unrelated shrimp pathogens: the Gram-negative bacterium *V. harveyi* ATCC 14126 and the WSSV. For the bacterial infection, shrimp were injected with 6 × 10^7 CFU/animal (under 100 μL SSW) of live *V. harveyi* ATCC 14126 (median lethal dose within 2 days, LD50/2) or with 100 μL SSW (injury control). For the viral infection, shrimp were injected with 100 μL of a WSSV inoculum containing 3 × 10^2 viral particles (median lethal dose within 15 days, LD50/15). The WSSV inoculum was prepared as previously described (Goncalves et al., 2014). Control animals for the viral infection (injury control) were injected with 100 μL of a tissue homogenate prepared from WSSV-free shrimp. At 48 h post-injection, midguts were harvested by dissection, washed in Tris-saline solution, pooled (3 pools of 5 animals per condition) and immediately processed for gene expression analysis. Unchallenged animals (shrimp at time 0 h) were used as control for all experimental conditions.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis for tissue distribution of gene expression

Total RNA was extracted by using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA samples were then treated with DNase I (Thermo Fisher Scientific) for 15 min at 37 °C to eliminate contaminating genomic DNA. After DNase I inactivation (10 min at 65 °C), samples were precipitated with 0.3 M sodium acetate. RNA amount and quality were assessed by spectrophotometric analysis and the integrity of total RNA was analyzed by 0.8% agarose gel electrophoresis. Following heat denaturation (70 °C for 5 min), reverse transcription was performed using 2 μg of purified total RNA with 50 ng/μL oligo(dT)$_{12\text{-}18}$ in a 20-μL reaction volume containing the RevertAid Reverse Transcriptase (Thermo Fisher Scientific), according to the manufacturer's instructions. PCR reactions were conducted in a 15-μL reaction volume using 1 μL of synthesized cDNA (diluted 1:10) as template. Details of all primers used are listed in the Table S1. The PCR amplification protocol consisted of an initial denaturation at 95 °C for 10 min followed by 30 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s and a final extension step of 72 °C for 10 min. The PCR products were analyzed by electrophoresis in 1.5% agarose and stained by ethidium bromide. The expression of the β-actin gene (*Lv*Actin) was used to normalize the RT-PCR data for comparison. The heatmap was generated using MultiExperiment Viewer (MeV) analysis software.

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 0,3 μM of each primer, 7,5 μL of reaction mix (Maxima SYBR Green/Rox qPCR Master Mix 2 × ; Thermo Fisher Scientific) and 1 μL of cDNA (diluted 1:20). Primer sequences are listed in Table S1. 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. Melt curve analysis (60–95 °C at a temperature transition rate of 0.05 °C/s) for each primer pair was performed to ensure primer specificity with continuous fluorescence acquisition. The eukaryotic translation elongation factor 1-alpha (*Lv*EF1α), the β-actin (*Lv*Actin) and the ubiquitin/ribosomal L40 fusion protein (*Lv*L40) were used as reference genes for RT-qPCR data normalization using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). Differences in gene expression were considered statistically significant at $P < 0.05$ (cutoff of 1.5-fold change in expression level) using one-way ANOVA and Tukey's multiple comparison test.

2.4. Whole mount immunofluorescence staining

Midguts from naïve (unchallenged) shrimp (n = 5) were harvested by dissection, washed in ice-cold Tris-saline solution and immediately fixed in 4% paraformaldehyde. The intestinal content was gently scraped with a sterile object glass and the midguts were longitudinally opened with a scalpel. Midguts were blocked in PBS-T solution (1 × PBS, 1% BSA, 0.1% Triton X-100) for 3 h at room temperature followed by 16 h incubation with rabbit anti-penaeidin polyclonal antibodies (10 μg/mL) (Destoumieux et al., 2000) at 4 °C with gentle rocking. Midguts were subsequently washed 3 times with PBS-T solution for 20 min at room

temperature and incubated with FITC-conjugated anti-rabbit secondary antibodies diluted at 1:500 and DAPI (Thermo Fisher Scientific) for 4 h at room temperature. Negative controls consisted in replacing anti-penaeidin antibodies with PBS-T solution. The sections were mounted and sealed. The experiments were repeated three times, and representative images were taken by confocal microscope (Leica DMI6000 B Microscope). Routine hematoxylin and eosin (H&E) staining was performed for histological examination with light microscopy.

3. Results and discussion

3.1. The expression of genes from different immune functional categories is differentially distributed in shrimp gut

To go further insight into the shrimp gut immunity, we have assessed the expression of 52 well-known immune-related genes in the three portions of the gastrointestinal tract (foregut, midgut and hindgut) and in the hepatopancreas, a digestive gland analogous to vertebrate liver. Since hemocytes are the main site of expression of immune-related genes in penaeid shrimp (Tassanakajon et al., 2013), we also considered circulating hemocytes from which most of these genes have been initially characterized. We covered immune key functional categories with genes involved in both antimicrobial and antiviral defenses: (i) antimicrobial peptides or AMPs, (ii) immune signaling pathways, (iii) proPO activating system, (iv) microbial recognition, (v) RNAi pathway, (vi) cytokine-like and (vii) redox system (Table S1).

The first striking information given by the gene expression screening is that gut (foregut, midgut and hindgut) and circulating hemocytes displayed closed expression patterns (Fig. 1A). Most of the gene-encoded AMPs (penaeidins and ALFs) and the components of the RNAi and the immune signaling pathways (Toll, IMD and JAK/STAT) showed to be constitutively transcribed in those tissues. However, the highest expression level of those genes was clearly observed in hemocytes (Fig. 1A). Genes involved in antimicrobial defenses were also found to be constitutively expressed in the gut of the black tiger shrimp *Penaeus monodon* (Soonthornchai et al., 2010). Like observed for *L. vannamei*, the expression of AMPs in the digestive system of *P. monodon* was mainly detected in the anterior gut than in the hepatopancreas (Soonthornchai et al., 2010).

The other striking observation was that hepatopancreas displayed a distinct expression pattern with few expressed genes common with the three gut portions and hemocytes (Fig. 1; Table S2). Genes exclusively expressed in hepatopancreas were in particular the invertebrate (i-type) lysozyme, likely involved in digestive functions, and C-type lectins (*Lv*Lectin-1, *Lv*CTL3 and *Lv*LT) known to be involved in carbohydrate binding and microbial recognition (Wang and Wang, 2013). Only few genes, namely the interferon-like antiviral cytokine *Lv*SVC1 (a shrimp Vago homolog), the glutathione S-transferase *Lv*GST and the C-type lectin *Lv*AV were expressed in both gut and hepatopancreas but not in hemocytes. No expression of genes involved in signaling pathways was observed in hepatopancreas that differs from insects where fat body expresses those genes including AMPs (Imler and Bulet, 2005).

We further evaluated the transcriptional profile of the 52 immune-related genes in shrimp stimulated by the injection of heat-killed *V. harveyi*. The global gene expression pattern observed for each portion of the gut and for the hepatopancreas and circulating hemocytes of unchallenged shrimp changed in response to the microbial stimulation (Fig. 1B; Table S2). Interestingly, while the expression of some genes was only detected after the challenge, the expression of others could be seen in unchallenged animals only.

Fig. 1. (**A**) Heat map showing the expression profile of 52 immune-related genes in hemocytes (HE), foregut (FG), midgut (MG), hindgut (HG) and hepatopancreas (HP) of naïve (N) or heat-killed *Vibrio*-stimulated (S) shrimp. The figure (not to scale) shown at the top of the heat map indicates the anatomic location of the four digestive organs analyzed in this study. Gene expression analysis was performed by semiquantitative RT-PCR using the β-actin gene (*Lv*Actin) as an endogenous expression control. Each cell in the matrix corresponds to the expression level of a given gene in an experimental condition (n = 5 animals/condition) and the intensity of the color (from white to red) indicates the magnitude of expression, based on the color scale at the right top of the heat map. The full gene annotation is provided in Supplemental Table S1. (**B**) Venn diagrams showing the number of overlapping and unique genes whose expression was detected in the five analyzed tissues of naïve (unchallenged) and *Vibrio*-stimulated shrimp. The list of the overlapping and the unique genes indicated in the Venn diagrams is provided in Supplemental Table S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This can be seen, for instance, in the foregut, where the expression of α2-macroglobulins (*Lv*α2M-1 and *Lv*α2M-2) and of the RNAi pathway genes *Lv*Sid1, *Lv*Dcr2 and *Lv*Ago2 was only detected in *Vibrio*-stimulated shrimp and the expression of n*Lv*ALF1 was only detected in unchallenged animals. Likewise, in both midgut and hindgut, the expression of genes from distinct functional categories (AMPs, proPO activating system, microbial recognition, cytokine-like …) was also only detected upon the bacterial stimulation (Fig. 1). Following challenge, the expression levels of different immune-related genes showed to be induced in the gut of other shrimp species, confirming the importance of the digestive system in shrimp local immune responses (Watthanasurorot et al., 2012; Yang et al., 2016).

Finally, *Lv*NOS encoding a nitric oxide synthase was seen to be expressed exclusively in the intestines (Fig. 1A). Many studies have highlighted the importance of the redox system in different host-pathogen interactions (Ferrari et al., 2011). In *Anopheles gambiae*, the heme peroxidase/NADPH oxidase 5 (HPX2/NOX5) system showed to be involved in nitric oxide (NO) toxicity and, consequently, in the mosquito successful response to *Plasmodium* infections (Oliveira et al., 2012). Up to date, little is known about the role of NO in shrimp epithelial defenses, but recent works have evidenced that the production and scavenging of reactive oxygen species (ROS) by dual oxidases and catalases, respectively, can play a critical role in shrimp intestinal immunity and in the regulation of the gut microbiota (Yang et al., 2016, 2015).

3.2. Infiltrating hemocytes contribute to the expression of immune-related genes in shrimp midgut

In order to investigate the presence of hemocytes in shrimp gut tissues, midguts from naïve (unchallenged) animals were analyzed by whole mount immunofluorescence staining using polyclonal antibodies raised against penaeidins, AMPs exclusively found in hemocytes (Muñoz et al., 2002). Confocal images clearly confirmed the presence of penaeidin-expressing hemocytes in the midgut epithelium (Fig. 2). No signals were observed in other cell types found in the midgut, suggesting that the expression of some immune-related genes in the midgut, and probably in all gastrointestinal tract and in the hepatopancreas, is due to hemocyte infiltration. Furthermore, the non-detection of transcripts for other hemocyte-expressed genes such as the glutathione peroxidase *Lv*GPx (Fig. 1A) suggests that hemocytes display distinct migratory behavior in shrimp tissues. Our results suggest that infiltrating hemocytes homed to digestive tissues play a central role in shrimp gut immunity in a similar way as observed for both myeloid- and lymphoid-derived immune cells in human intestines (Coombes and Powrie, 2008; Hirota et al., 2013).

Taking into account the presence of hemocytes in the midgut, one can assume that the expression of genes from some immune-related categories (AMPs, immune cell signaling …) is only detectable because of these infiltrating cells and not because of shrimp gut tissues. Indeed, penaeidins (Muñoz et al., 2002), ALF members from Group B (Somboonwiwat et al., 2008) and the proPO enzyme (Söderhäll et al., 2003) are exclusively produced by granular hemocytes. However, we cannot rule out that shrimp gut tissues (such as gastric and intestinal epithelia) are involved in the synthesis of immune-related molecules whose expression is also found in hemocytes. For instance, in *Crassostrea gigas* oysters, the bactericidal/permeability-increasing protein (*Cg*-BPI) is constitutively expressed in different surface epithelia of unchallenged animals and induced in circulating hemocytes in response to bacterial challenge (Gonzalez et al., 2007). Besides, the expression of the ALF*Pm*7 gene, a novel member of Group C ALFs recently identified in *P. monodon*, was mainly detected in stomach and lymphoid organs than in hemocytes (Soonthornchai et al., 2016). In agreement with these findings, we have showed in a previous study that the expression profile of some immune-related genes (including Group C ALFs) during shrimp ontogenesis could be the result of different sites of expression such as the digestive system (Quispe et al., 2016).

We cannot discard the hypothesis of the existence of specific tissue-resident hemocyte populations in shrimp gut distinct from those found in the hemolymph. The presence of tissue-specific subsets of a particular immune cell type was previously reported in mammals (Davies and Taylor, 2015). However, only few works have been devoted to the localization of immune-related gene expression in shrimp tissues by using physical mapping techniques such as *in situ* hybridization and immunohistochemistry (Muñoz et al., 2002; Somboonwiwat et al., 2008). The application of these techniques will be helpful to confirm the existence of specific tissue-resident hemocytes in crustaceans, but also to verify if the shrimp midgut is also divided in distinct functional domains, in terms of host gene expression profile and microbial colonization, as observed for the midgut of flies (Royet and Charroux, 2013).

3.3. Genes involved in antimicrobial and antiviral defenses are induced in shrimp midgut in response to Vibrio infection

From the first data obtained in the screening of gene expression, we have selected 14 genes involved in shrimp antimicrobial (antimicrobial peptides: *Litvan* PEN1/2, *Litvan* PEN3, *Litvan* PEN4, *Litvan* ALF-A, *Litvan* ALF-B, *Litvan* ALF-C, *Litvan* ALF-D and c-type lysozyme) and antiviral defenses (RNAi pathway-related genes: *Lv*Dcr1, *Lv*Dcr2, *Lv*Ago1, *Lv*Ago2, *Lv*Sid1 and *Lv*TRBP1) and 4 genes that appeared to be modulated in gut in response to the heat-killed bacterial stimulation (*Lv*α2M-2, *Lv*AV, *Lv*SVC1 and *Lv*NOS). Their expression levels were quantified by fluorescence-based quantitative PCR (RT-qPCR) in the midgut at 48 h after infections with two important pathogens for shrimp aquaculture, the Gram-negative *V. harveyi* and the WSSV. We focused on the midgut because it lacks the cuticular lining found in the other gut portions (foregut and hindgut) and, as observed for most insect vectors, it could represent an important route of entry for many pathogens into the hemocel (Saraiva et al., 2016).

While the expression of three genes (*Litvan* ALF-A, *Litvan* ALF-C and *Lv*Dcr2) were up-regulated in response to the *Vibrio* infection, the WSSV did not affect the expression of the analyzed genes at 48 h post-infection (Fig. 3). Differences in gene expression patterns were observed between the *Vibrio*-infected group and the aseptic injury control, indicating that the modulation of those genes was triggered by bacterial molecular patterns and not by tissue damage. Curiously, the expression of most genes showed to be modulated in shrimp midgut in response to the microbial challenge, but not after the *Vibrio* infection (Fig. 1A; Fig. 3). In this context, it is likely that the gene expression regulation observed in the midgut of shrimp stimulated with heat-killed bacteria was due to the recognition of microbe-associated molecular patterns (MAMPs), while the expression pattern observed in response to the *Vibrio* infection was probably the result of host-pathogen interactions.

The antimicrobial peptides *Litvan* ALF-A (2.3 fold-change) and *Litvan* ALF-C (3.1 fold-change) showed to be induced in shrimp midgut after the *Vibrio* infection (Fig. 3). Interestingly, both ALF genes showed to be directly involved in shrimp survival to infectious diseases. In L. *vannamei*, RNAi-mediated knockdown of *Litvan* ALF-A increased the susceptibility of shrimp to *Vibrio penaeicida* and to the fungal pathogen *Fusarium oxysporum* (de la Vega et al., 2008). Besides, RNAi experiments also revealed an essential *in vivo* role of ALF*Pm*6 (Group C ALF) in the antimicrobial defense of *P. monodon* against *V. harveyi* (Ponprateep et al., 2012). Taken

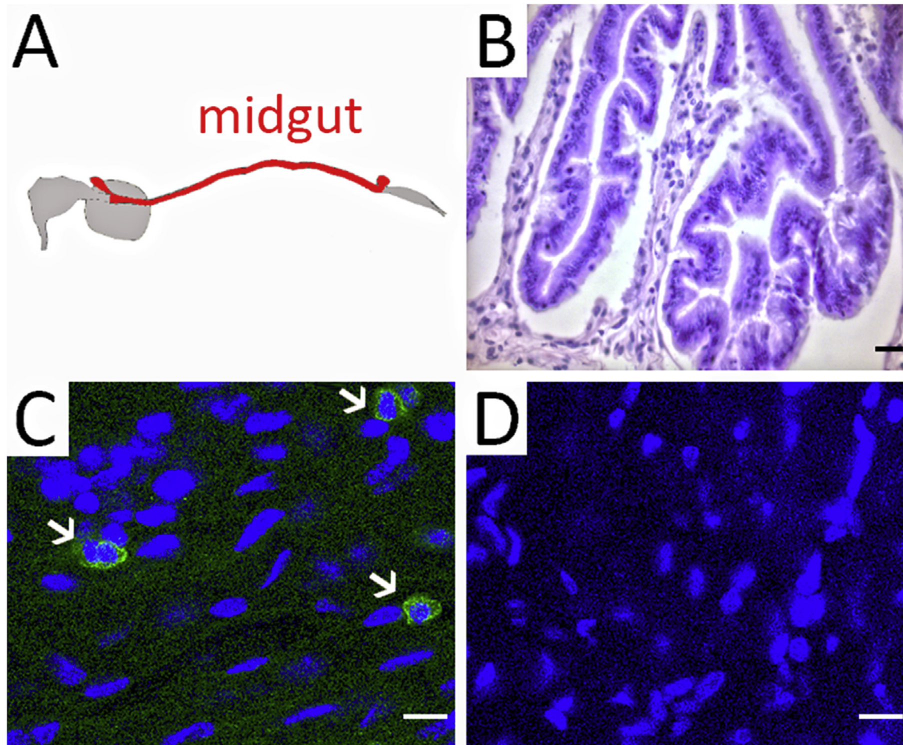

Fig. 2. (**A**) A not-to-scale representation of the digestive system of penaeid shrimp. The midgut is highlighted in red. (**B**) Histological sections of midgut stained with hematoxylin and eosin (H&E). (**C**) Scanning confocal microscopy images of the detection of infiltration hemocytes in the midgut of naïve (unchallenged) shrimp by whole mount immunofluorescence using anti-penaeidin polyclonal antibodies. The white arrows indicate the presence of penaeidin-expressing hemocytes in the midgut epithelium. The nuclei are stained in DAPI, blue. (**D**) Primary anti-penaeidin antibodies were omitted for control staining. Bars = 20 μm.

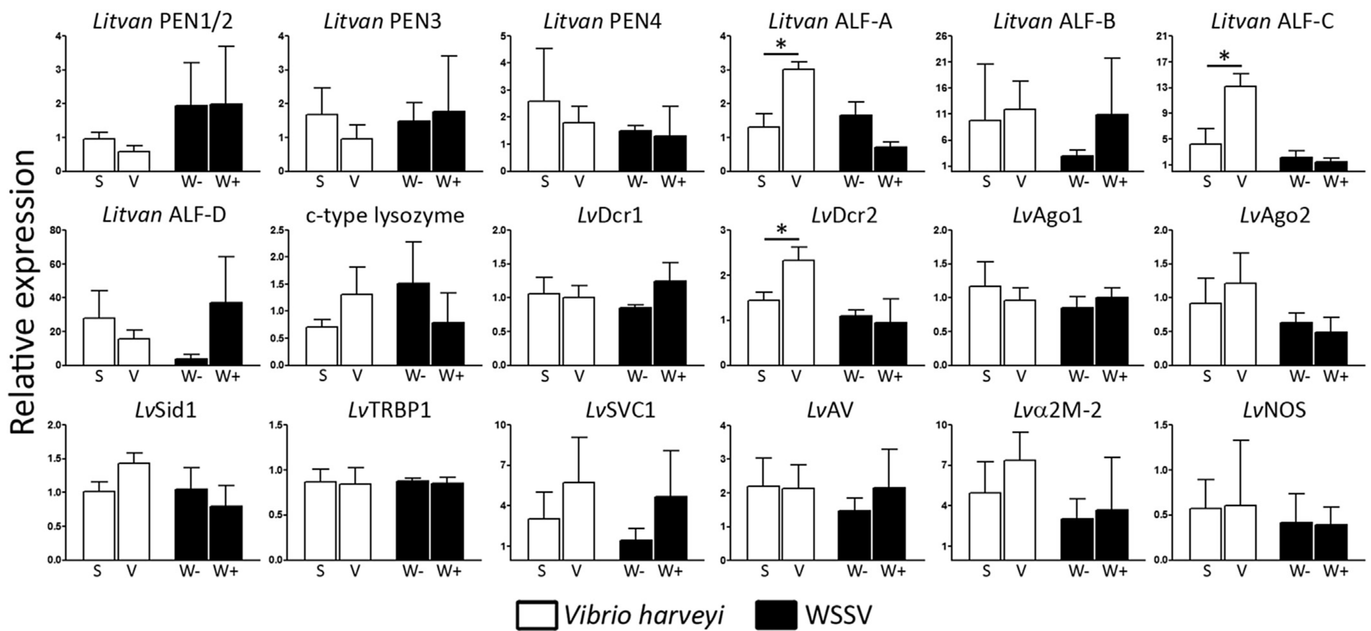

Fig. 3. Relative expression profile of 18 immune-related genes in shrimp midgut at 48 h after experimental infections with *Vibrio harveyi* ATCC 14126 (white bars) or WSSV (black bars). Results are presented as mean ± standard deviation of relative expressions (three biological replicates) and statistical differences are indicated by asterisks (*) (one-way ANOVA/Tukey, $P < 0.05$). V: *V. harveyi* ATCC 14126 (6×10^7 CFU/animal), S: sterile seawater injury control, W+: WSSV (3×10^2 viral particles/animal); W-: tissue homogenate inoculum prepared from WSSV-free shrimp.

together with our findings, ALFs appear to play a critical role in cellular and humoral antimicrobial defenses but also in shrimp gut immunity. Indeed, the knockdown of the ALF*Pm*3 gene (Group B ALF from *P. monodon*) leads to the proliferation of bacteria in the hepatopancreas and hemolymph, resulting in shrimp death (Ponprateep et al., 2012). Furthermore, it has been shown that

Group C ALFs, but not ALFs from Group A, participate in the maintenance of the hemolymph microbiota of the kuruma shrimp *Marsupenaeus japonicus* (Wang et al., 2014). In view of these results, it would be of particular importance to investigate the role of the ALF diversity (Rosa et al., 2013) in the selection and maintenance of the *L. vannamei* gut microbiota.

Like ALFs, *Lv*Dcr2 gene expression was also induced in shrimp midgut (1.6 fold-change) in response to the bacterial infection (Fig. 3). Actually, Dicer-2 plays a central role in exogenous small interfering RNA (siRNA) processing and, consequently, in shrimp antiviral defenses (Labreuche and Warr, 2013). Until now, the role of RNAi pathway genes in shrimp response to bacterial infections was not yet investigated and it is probably that these genes could also be involved in the posttranscriptional regulation of genes involved in antibacterial defenses as observed in insects (Wang et al., 2015).

All other immune-related genes analyzed in this study were not modulated at 48 h after infections and probably this result is partly due to the time course response of each gene in terms of gene expression regulation in the midgut. Likewise, it may be also due to the route of infection. We have performed our experimental infections by intramuscular injection in order to standardize a same inoculum load per animal, but natural infection routes, such as *per os* or immersion, could trigger specific patterns of gene expression. In *P. monodon*, the expression of AMPs and lectins showed to be modulated in the midgut following an immersion of shrimp in a live bacterial suspension (Soonthornchai et al., 2010). Besides, it has been shown that hemocytes massively migrate to shrimp tissues at the first 12–24 h after microbial infections (Muñoz et al., 2002) and it is likely that in our experiment, modulation of hemocyte-expressed genes in the midgut has occurred earlier (before the 48-h time period).

4. Conclusion

We provided here a gene expression atlas of the immune functions likely involved in penaeid shrimp gut immunity. Results from our analyses showed that gut is an important site for the expression of immune-related genes in the shrimp *L. vannamei* and that infiltrating hemocytes can play an essential role in gut immunity in particular through the delivery of antimicrobial molecules into different portions of the digestive system. Our results bring new insights into the role of infiltrating hemocytes in the crosstalk between hemolymph-based and gut-based immunities. Thus, the involvement of both antimicrobial- and antiviral-related genes in the control of gut microbiota and in the response to pathogens could be assessed by posttranscriptional gene silencing using the RNAi approach. The elucidation of the molecular mechanisms driving shrimp gut response to major pathogens is a prerequisite for understanding the role of the gut in preventing and controlling infectious diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.dci.2017.10.005.

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