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Multienzymatic capacity of cultivable intestinal bacteria from captive *Litopenaeus vannamei* (Boone, 1931) shrimp reared in green water

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Abstract

Equilibrium between the gastrointestinal bacterial population and the environment is a critical factor for the health of captive aquatic animals. The bacterial enzymes are fundamental for proper nutrition and pathogen resistance in shrimp. Therefore, enzymatic profiles reveal essential characteristics for the selection of probiotic strains that can improve animal development. In this work, we analysed shrimp from a green water system where infectious myonecrosis virus (IMNV) was present. We isolated transient and intestine resident bacterial populations, characterising eight functional groups through different culture media. To identify each isolated bacteria, we used sequences from regions V6–V8 of the 16S rRNA. To determine viral load of shrimp samples, we used real-time PCR. The number of colony forming units (CFU) was similar between IMNV-infected and IMNV-uninfected shrimps. The growth of transient bacteria was higher than the growth of resident. In general, lipolytic bacteria presented higher frequency and genus diversity than the other functional groups. All groups showed higher frequency among transitory bacteria, except the amylolytic functional group, which was more frequent among the resident. We found two major orders of cultivable bacteria, Vibrionales and Bacillales. The genus *Vibrio* was predominant among the Vibrionales, while *Staphylococcus* and *Bacillus* were the most frequent among the Bacillales. Recorded Vibrionales and Bacillales included pathogenic and beneficial species of high importance for aquaculture. The results presented here will serve as a basis for improving the nutritional and health conditions of *Litopenaeus vannamei* in green water farming systems.

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Keywords *Litopenaeus vannamei* · Gut bacteria · Green water · IMNV · Enzymatic group · Probiotics

Abbreviations

IMNV	Infectious myonecrosis virus
WSSV	White spot syndrome virus
CFU	Colony forming unit
CHB	Cultivable heterotrophic bacteria

Introduction

In the last 10 years, Pacific whiteleg shrimp, *Litopenaeus vannamei*, has become one of the most globally important aquaculture species (FAO 2018). In typical aquaculture conditions, animals are subject to a number of stressing factors, such as high densities, deteriorating environmental conditions and exposure to pathogens (Xiong et al. 2017). Disease manifestation in farmed shrimp is normally associated to viruses and bacteria and their interactions. The relationship between virus and bacteria has a synergistic effect that has been associated with many cases of farmed shrimp mortality (Selvin and Lipton 2003; Phuoc et al. 2009).

Among the gut microorganisms from aquatic animals, intestinal bacteria are the most studied in connection with physiology, nutrition, and health. Many intestinal bacteria have the capacity to produce digestive enzymes. Moreover, the presence of these microbes, with their respective exogenous enzymes, was proved to stimulate the production of endogenous enzymes in the digestive tract in shrimp (Zhou et al. 2009; Luis-Villaseñor et al. 2015).

In the digestive environment, factors such as food composition may affect enzymatic activity. Shrimp from family Penaeidae are in general omnivorous, due to the production of a wide range of enzymes that allow them to use many energy sources in their diet (Tzuc et al. 2014). Many of these digestive enzymes are exogenous, having originated from intestinal bacteria. In addition, some intestinal bacteria seem to provide increased resistance against opportunistic pathogens (Tzuc et al. 2014; Hostins et al. 2017) and help maintain the immune system (Sha et al. 2016), making them capable of acting as probiotics (Adel et al. 2016).

Previous reports showed that the balance between the surrounding aquatic environment and the gut is critical for proper animal development (Xiong et al. 2017). Some bacteria may be indicative of shrimp health status, while digestive enzymes produced by gut bacteria may play an important nutritional role, affecting the rates of growth and survival on certain rearing systems (Adel et al. 2016).

In recent years, it was infectious myonecrosis (IMN) that appeared in Brazil in 2001, causing considerable losses worldwide. Shortly after its inception, IMN became a notifiable disease, in accordance with the recommendations of the World Organization for Animal Health (OIE) (Senapin et al. 2007; Lightner 2011). To this day, IMN causes periodical losses in Brazilian shrimp farming. In the face of a recent IMN outbreak, the objective of this study was to characterise intestinal bacteria from *L. vannamei* shrimp farmed in green water systems, according to enzymatic functional groups. After identifying genera of interest, we have genetically characterized them through 16S sequencing. We believe that this approach will provide new insight into *L. vannamei* microbiota capacity of producing exogenous enzymes under these specific conditions.

Materials and methods

Rearing system

The shrimp used here were reared at LANOA (Aquatic Organisms Nutrition Laboratory, Federal University of Ceará, Eusébio) on an outdoor green water (autotrophic) rearing system (described in Nunes et al. 2011; Façanha et al. 2016). For the experiment, we used 900 shrimps from a commercial specific pathogen resistant (SPR) strain. The animals had an average initial weight of 3.0 g and were stocked at 34 m^{-2}, in five 3.0-m^3 tanks, until they reached the final weight of 7.0 g. The system reached autotrophic conditions through natural fertilisation from faeces and feed remains, which allowed the formation of microalgae and heterotrophic microorganisms' biomass, characteristic of green water systems. Continuous aeration kept oxygen levels always close to saturation. During the whole experiment, pH was stable at 8.3 ± 0.2, temperature was 27 ± 1.5 °C, and salinity was 30.6 ± 2 g L^{-1}. The animals received a common commercial feed containing 35% crude protein, ad libitum. At the end of the rearing cycle, we transported selected animals from the rearing facility to the microbiology laboratory in 20-L plastic drums, at 27 °C ± 0.5 °C, under constant aeration, on a 40-min trip.

Microbiological sampling

All microbiological sampling, culturing and characterisation were conducted at LAMAP (Environmental and Seafood Microbiology Laboratory, Federal University of Ceará, Fortaleza). We selected and analysed 58 animals that presented healthy aspect and behaviour, in two separate sets. In the first set, we pooled samples from 45 shrimps in three groups of 15. Pooling samples reduces variation between individuals, which is useful when characterising functional groups in relation to the environment. In the second set, we sampled 13 animals individually and tested them for the presence of infectious myonecrosis virus (IMNV) and white spot syndrome virus (WSSV). The individual samples were prepared to assess the influence of IMMV on the functional groups, since the pooled samples tested positive for IMNV.

To obtain midgut samples, we numbed the animals in an ice bath and removed the intestine aseptically. To collect the transient microbiota (sensu Harris 1993), we carefully removed gut content with a sterile spatula and transferred it to sterile 10 g L^{-1} saline solution. To obtain the resident microbiota, we cleansed the remaining tissue by washing it successively with sterile saline solution, 70% ethanol, and sterile saline solution. After that, we homogenised the tissue mechanically in sterile 10 g L^{-1} saline solution. We diluted all samples serially to 10^{-5}, using 10 g L^{-1} sterile saline solution as diluent. We also collected, diluted, and analysed water samples in triplicate.

For IMNV molecular diagnosis of the individually sampled animals, before dissection, we collected 150 μL haemolymph in SIC-EDTA anti-clotting solution (450 mM NaCl, 10 mM KCl, 10 mM HEPES, 10 mM EDTA, pH 7.2). We also collected pleopods from the fifth abdominal somite for molecular diagnosis of WSSV.

Microbiological culture

We inoculated aliquots of each diluted sample in eight different types of culture media, specific to two genera and six functional categories (Table 1). We performed all analyses in duplicates, using 10 mg L^{-1} seawater as a diluent, except for TCBS (thiosulfate-citrate-bile salt-sucrose)

Table 1 Culture conditions and techniques used to grow *Litopenaeus vannamei* intestine bacteria from specific genera and enzyme functional groups

Target microorganism	Culture medium	Aliquot and seeding technique	Culture conditions
Bacillus spp.[1]	Plate count agar (PCA)	200 μL, spread-plate	72 h, 30 °C
Cultivable heterotrophic bacteria (CHB)[2]	Plate count agar (PCA)	1 mL, pour-plate	24–48 h, 30 °C
Vibrio spp.[3]	TCBS (thiosulphate citrate bile salt sucrose agar)	200 μL, spread-plate	48 h, 30 °C
Amylolytic[4]	Agar + starch	1 mL, pour-plate	2–5 days, 30 °C
Lactic acid[5]	Agar MRS (Agar Man, Rogosae Sharpe)	1 mL, pour-plate	48 h, 35 °C
Proteolytic[6]	Milk casein agar	200 μL, spread-plate	72 h, 30 °C
Lipolytic[7]	Bactopeptone, $CaCl_2 \cdot H_2O$, agar, Tween 80	200 μL, spread-plate	72 h, 30 °C
Cellulolytic[8]	Agar, mineral medium, carboxymethylcellulose	200 μL, spread-plate	72 h, 30 °C

[1] Modified from Defoirdt et al. (2011)

[2] APHA (2000)

[3] Donovan and Netten (1995)

[4] West and Colwell (1984)

[5] Ramírez et al. (2013)

[6] Sizemore and Stevenson (1970)

[7] Sierra (1957)

[8] Luczkovich and Stellwag (1993)

agar medium. We used both pour-plate (1 mL) and spread-plate (200 μL) inoculation. After specific temperature/time incubation for each microorganism group, we performed standard plate counts (Maturin and Peeler 2001) and recorded the results in terms of colony forming units (CFU) per unit of mass (CFU g^{-1} of intestine tissue) or volume (CFU mL^{-1} of water). Mann-Whitney *U* tests from the R Stats package v 3.5.1 were used to assess the differences between resident and transient counts and between IMNV positive and IMNV negative shrimp.

Microbial DNA extraction

To obtain bacterial biomass for DNA extractions, we conducted overnight incubations on seawater Luria-Bertani (20 g L^{-1}) broth at 35 °C and 120 rpm. After incubation, we centrifuged each sample at 13,000×*g* for 2 min and dissolved the resulting pellet in 500 μL EDTA (50 mM) and lysozyme (2 mg mL^{-1}), keeping the solution in a water bath at 37 °C for 60 min, to ensure complete digestion of the bacterial cell wall. After these initial steps, we used Wizard® SV Genomic DNA Purification System kits (Promega Co.) for extractions and a NanoDrop® 2000 (Thermo Fisher Scientific) for DNA quantification.

RNA/DNA extraction and cDNA synthesis

For total RNA extraction, we isolated haemocytes from the hemolymph by centrifugation at 1200×*g* for 10 min at 4 °C and used TRIzol™ reagent (Invitrogen) according manufacturer's

instructions. We treated total RNA with DNAase I, incubating the samples at 37 °C for 15 min, followed by inactivation at 65 °C for 10 min. We assessed the nucleic acid concentration of all samples with Qubit™ Quantification Assay kits (Invitrogen). We synthesised cDNA using High-Capacity cDNA Reverse Transcription kits (Applied Biosystems™) in 20 μL reactions containing 2 μL RT 10× buffer, 0.8 μL dNTP mix (25×), 2 μL RT random primers (10×), 1 μL MultiScribe™ Reverse Transcriptase (50 U) and 1 μg total RNA. We then incubated the reactions in a thermocycler successively at 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min. We extracted total DNA from pleopods preserved in 95% ethanol using a standard proteinase K/PCI protocol (Sambrook et al. 1989). We stored all cDNA and DNA samples at − 20 °C until analysis by real-time PCR (qPCR).

Molecular identification

To amplify 16S rRNA V6–V8 hypervariable regions (~ 430 bp), we used primers U968 (AAC GCG AAG AAC CTT AC) and L1401 (CGG TGT GTA CAA GAC CC) (Nübel et al. 1996). All 12.5 μL PCRs contained 1x proprietary buffer, 0.8 mM $MgCl_2$, 0.016 mM dNTPs, 0.08 pmol each primer, 0.5 U Taq DNA polymerase (Invitrogen) and 50–100 ng de DNA. Thermal cycling conditions included initial denaturing at 94 °C/2 min, followed by 30 cycles of 94 °C/60s, 52 °C/60 s and 72 °C/60 s, followed by a final extension step at 72 °C/8 min. We sequenced the resulting PCR products directly, using BigDye® Terminator v3.1 Cycle Sequencing kits (Applied Biosystems) and an ABI 3500 Genetic Analyser (Thermo Fisher Co.).

To assemble and analyse sequences, we used Geneious 10.3 (Kearse et al. 2012). To search for similarities, we used Ribosomal Database Project (RDP) tools (Cole et al. 2014) and NCBI's BLAST (Morgulis et al. 2008). RDP searches used *Sequence Match* and *Classifier* to select matches with similarities and S-ab scores close to 1 and 80% confidence threshold (Wang et al. 2007). We assessed BLAST results in terms of similarity (≥ 90%) and *E* values (~ 0.0).

qPCR

We performed fluorogenic probe qPCR using TaqMan™ to estimate IMNV and WSSV copy number in each sample. We used primers and probes described by Andrade et al. (2007) and Poulos and Lightner (2006). We performed qPCRs in 10 μL volumes containing 5 μL Platinum Quantitative PCR SuperMix-UDG (Invitrogen®), 0.3 μM each primer, 0.15 μM Taqman® probe, 0.2 μL 50× buffer, 3.05 μL ultrapure water (Invitrogen®) and either 1 μL (10–25 ng) cDNA, for IMNV assays, or 1 μL (100 ng) DNA, for WSSV assays. We used a 7500 Real-Time PCR System (Applied Biosystems) with the following cycling conditions for all samples: incubation at 50 °C for 2 min, followed by Platinum® Taq DNA polymerase activation at 95 °C for 2 min, followed by 40 cycles of 95 °C/15 s and 60 °C/30 s, and then a final extension of 10 min at 60 °C. We performed each assay in duplicate, and the sample was considered positive only when both reactions where positive. We used SDS 1.3.1 (Applied Biosystems®) for data analysis and copy number determination.

Results

In the pooled sample analysis, the total number of cultivable bacteria from the resident microbiota was 40 times lower than that of transient microbiota and 20 times higher than that

of the water. The CFU values for water were in general lower than those observed for transient and resident microbiota, except in the case of Amylolytic bacteria (Fig. 1). When comparing transient and resident bacteria, we observed that, again, amylolytic bacteria were the only discordant group, presenting considerably lower numbers in the transient microbiota (Fig. 1). The most numerous groups in the resident microbiota were lipolytic, amylolytic and CHB functional groups. However, the three most abundant groups among transient cultivable bacteria were lipolytic, CHB and lactic acid (Fig. 1). The differences observed among *Bacillus*, amylolytic and acid lactic bacteria were significant ($p = 0.05$) for one-sided tests. In the individualised analysis, five animals were positive for IMNV, showing between 6.76×10^2 and 1.14×10^5 copies IMNV μg^{-1} RNA. WSSV was not detectable in any of the samples. The differences in bacterial counts among infected and non-infected animals were not significant ($p > 0.05$) for all bacterial groups analysed (Fig. 2).

Unique sequences produced from a subset of randomly selected isolates are available at GenBank under the accession numbers MG722642-MG722675. Detailed results of the homology searches are available as supplementary material (Table S1). *Vibrio* was present in all enzymatic groups, while lipolytic bacteria were the most diverse in a number of genera (Fig. 3). Besides the selective medium of growth, *Bacillus* also showed proteolytic activity (Fig. 3). *Vibrio* was the dominant genus among Vibrionales, while *Staphylococcus* and *Bacillus* were the most frequent among Bacillales (Fig. 4). Most of the cellulolytic and amylolytic bacteria were species of *Vibrio*.

Vibrio sequences showed high homology to a number of different species, namely *V. corallilyticus*, *V. proteolyticus*, *V. azureus*, *V. mytili*, *V. natriegens*, *V. nigripulchritudo*, *V. parahaemolyticus*, *V. probioticus*, *V. rumoiensis*, *V. variabilis*, *V. vulnificus*, *V. hispanicus* and *V. harveyi*. Among *Staphylococcus*, *S. cohnii*, *S. warneri* and *S. hominis* were identified, and among *Bacillus*, *B. subtilis*, *B. thuringiensis* and *B. cereus* (Table S1).

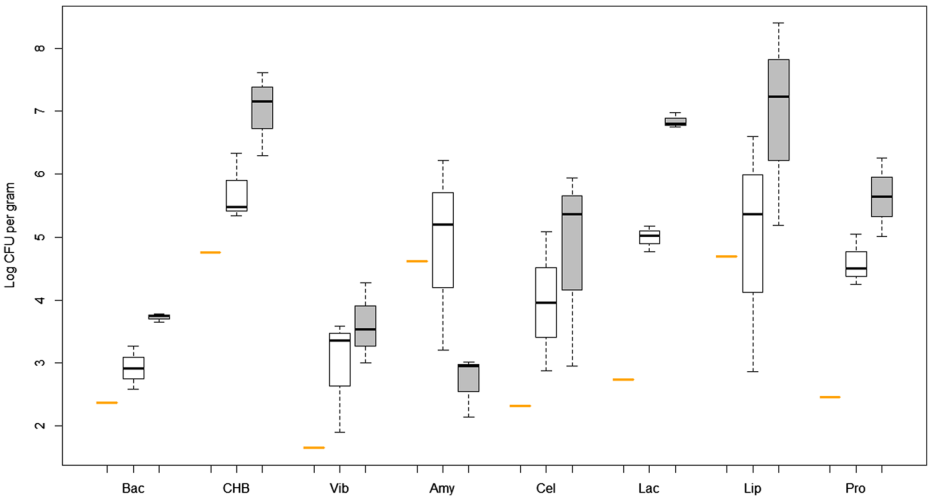

Fig. 1 Bacterial counts per functional group, from pooled sample analysis of midgut transient and resident microbiota, from *Litopenaeus vannamei* reared in a green water system. CFU number of colony forming units. Orange bar refers to water; white boxes, transient; grey boxes, resident. Functional groups: Bac *Bacillus*, CHB cultivable heterotrophic bacteria, Vib *Vibrio*, Amy amylolytic, Cel cellulolytic, Lac lactic acid, Pro proteolytic

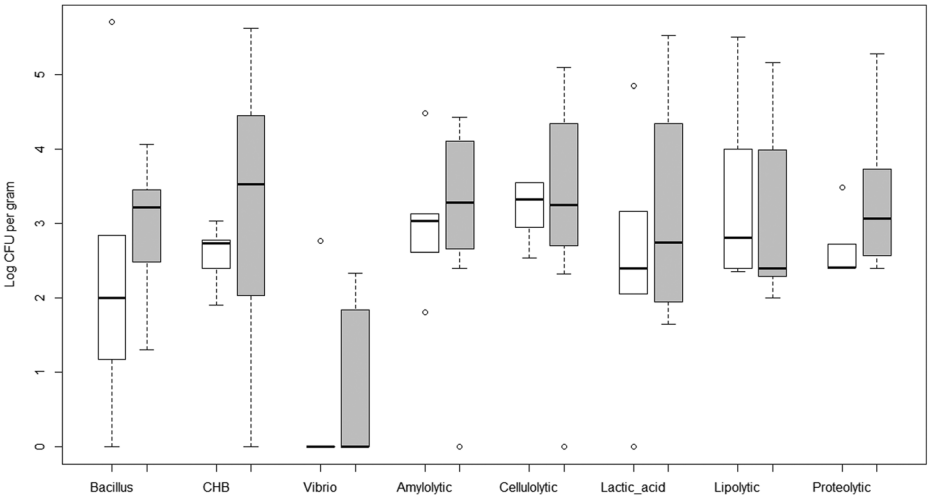

Fig. 2 Resident bacteria counts per functional group, for *Litopenaeus vannamei* intestines individually analysed against IMNV presence. CFU number of colony forming units. White boxes represent IMNV-free shrimp; grey boxes, IMNV-positive; white circles, outliers

Discussion

In the present work, we describe the cultivable transient and resident bacteria found in the intestine of *L. vannamei* reared in green water in tropical South America, examining the effects of natural infection by IMNV over growth of intestinal cultivable bacteria, under eight enzymatic functional groups. Bacterial species or strains capable of producing visible colonies on solid media are considered cultivable (Austin 2017). Cultivability is a characteristic of great interest in probiotics prospection and development (Tzuc et al. 2014; Adel et al. 2016).

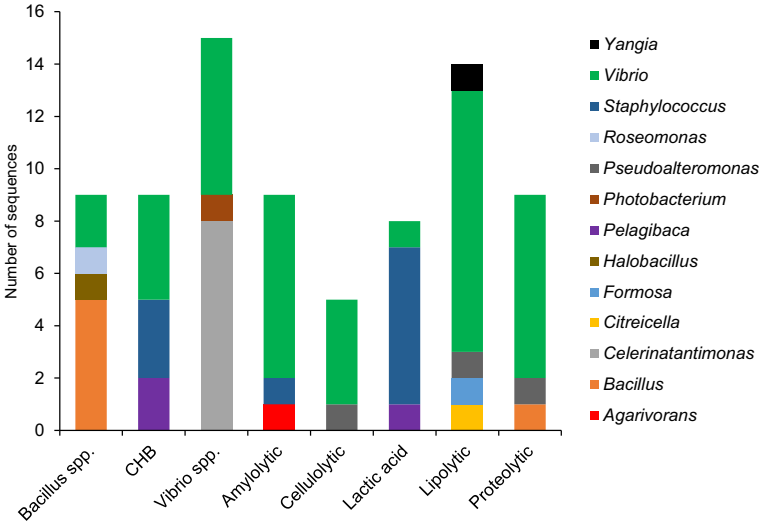

Fig. 3 Composition of the studied bacterial groups based on a random sample of isolates from the resident microbiota

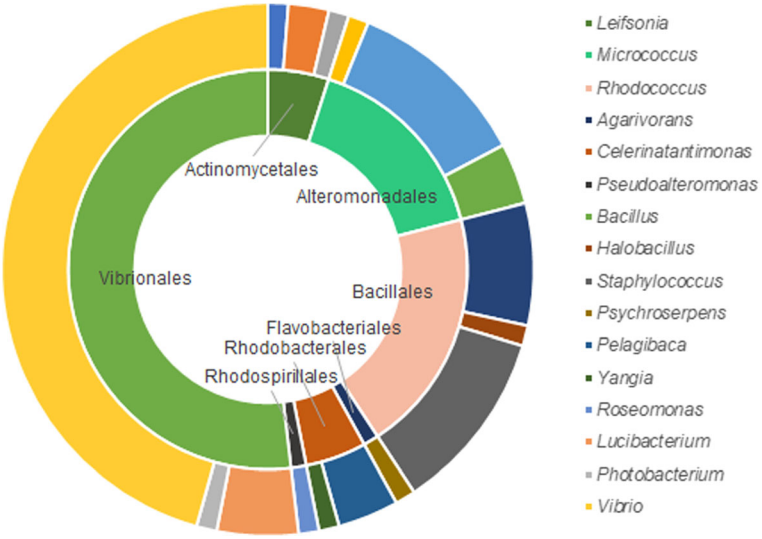

Fig. 4 Identification doughnut chart, based on BLAST and RDP (please refer to Table S1 for further details)

In aquatic organisms, there is an important distinction between the resident, possibly symbiotic gut bacteria and the transient, commensal ones (Harris 1993). The intestinal tract of farmed shrimp represents a selective environment that adapts the bacterial community to intestinal conditions (Zhang et al. 2014). Resident and transient microorganisms are both sensitive to external environment interactions, such as dietary administration, pathogenic infections, and farming environment (Cardona et al. 2016; Qiao et al. 2017; Xiong et al. 2017). Green water conditions, such as those used the present study, are reached by the free development of photosynthesising microalgae under sunlight (Moriarty 1997). The phytoplankton community in this environment mostly consists of species belonging to the Dinophyceae and Cyanobacteria classes, while trophic net established may provide significant nutritional supplement in shrimp farming (Alonso-Rodríguez and Paéz-Osuna 2003; Cremen et al. 2007). In the present study we did not assess the planktonic community of the system used; therefore, the effects of different diversities and abundances on shrimp gut microbiota remain to be addressed.

Intestinal bacteria present ample enzymatic potential and bring many benefits to the intestinal physiology of the host, in terms of digestion and nutrition (Tzuc et al. 2014; Sha et al. 2016). In this study, the presence of bacteria with multienzymatic capacity in the intestinal tract indicates the ability to digest and absorb various dietary components in *L. vannamei*. Shrimp have a relatively short and straight digestory system, which therefore requires rapid digestion (Beseres et al. 2006). The total amount of transient bacteria we observed here was many times higher than that of resident bacteria. The higher counts observed for the enzymatic groups in the transient bacterial community are likely to improve feed degradation before reaching the intestinal epithelium. On the other hand, the relationship between the water and the resident bacteria was particularly clear for the group of Amylolytic bacteria.

Previous studies reported a strong interaction between the intestine microbiota and the aquatic environment. Aquaculture water in general presents higher diversity than the gut, and the constant water flux through the digestive system affects the resident microbiota, as well as

the production of digestive enzymes (Gatesoupe 1999; Johnson et al. 2008; Tzuc et al. 2014; Cardona et al. 2016).

Among the enzymatic groups studied, the superiority of lipase-producing bacteria in the intestine is indicative of greater lipid assimilation for *L. vannamei* reared in green water. This characteristic is linked to both the dietary factors as well as the environment where the host lives (Stecher and Hardt 2011). A higher nutritional bioavailability of lipids may be of interest when preparing diets for *L. vannamei* because fatty acids, besides being an important source of energy, are fundamental components of cell membranes (Glencross 2009).

In the present study, Amylolytic bacteria were the only group more frequent in the resident microbiota. Previous reports affirm that the influence of different sources of carbohydrates in the microbial population is still unclear (Qiao et al. 2017). However, the specific selection of carbohydrate sources is very important for *L. vannamei* growth, because this organism uses the extra energy source to compensate for the loss of energy caused by stress (Li et al. 2017). Diseases cause significant physiological stress, and we have confirmed the presence of IMNV. Nevertheless, we observed no difference between bacteria counts from infected and non-infected individual shrimp, for any of the groups, including amylolytic bacteria.

The results showed that the presence of IMNV did not seem to influence the intestinal bacterial community. However, we should note that the animals studied were asymptomatic. In *L. vannamei*, some immunological effects are produced only at the stage of advanced infection (Costa et al. 2009). Evaluations of immunological parameters of *L. vannamei* during the progression of IMNV infection seem to indicate that if the Prophenoloxidase system is not triggered, the bacterial community is not likely to be directly affected (Costa et al. 2009).

We reported here the presence of *Vibrio* in all functional groups, corroborating the well-known multienzymatic capacity of this genus. We also found *Bacillus cereus* among the proteolytic bacteria. Bacteria with the ability to produce various extracellular enzymes are of great probiotic potential (Tzuc et al. 2014). The genus *Vibrio* includes many pathogenic strains; however, the role of *Vibrio* in the regular metabolism is not well established. *Vibrio* and *Bacillus* are frequent and abundant in shrimp gut (Huang et al. 2016; Rungrassamee et al. 2016). Previous studies have reported on the ability of *Vibrio* spp. to hydrolyse a wide variety of polysaccharides, such as cellulose and starch in fish and shrimp (Raghul and Bhat 2011; Tzuc et al. 2014). *Bacillus* has shown proteolytic activity in fish intestines, especially *Bacillus cereus*, identified in this study (Mondal et al. 2010).

Some species identified here, such as *Vibrio vulnificus*, *V. parahaemolyticus*, *V. harveyi* and *Photobacterium damselae*, have been described as relevant pathogens in aquaculture (Wang et al. 2014; Kumaran and Citarasu 2016). However, some *Vibrio* species, such as *V. proteolyticus*, *V. natriegens* and *V. mytilli*, offer benefits to farmed shrimp and fish. These benefits include the degradation of proteins and assimilation of organic carbon in biofloc systems (De Schrijver and Ollevier 2000; Luis-Villaseñor et al. 2015). We also found many *Bacillus* commonly used as probiotic agents, such as *Bacillus subtilis*, *B. thuringiensis*, *B. cereus* and *B. aquimaris*. These *Bacillus* species present inhibitory activity against pathogens and seem to improve digestion, immune response and survival in *L. vannamei* (Ngo et al. 2016; Vargas-Albores et al. 2017).

Comprehending the dynamics of functional bacterial groups in farmed shrimp gut can provide a better understanding of the influence of environment and farming systems over transitory and resident bacterial populations. This approach will eventually allow us to control microbiota through diet, prebiotics and probiotics in farmed shrimp. In conclusion, the present work characterised bacteria from eight functional groups under typical tropical rearing

conditions, identifying genera and species of potential probiotic use. The results described here will serve as a basis for improving aquaculture strategies of *L. vannamei* reared in green water.

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Compliance with ethical standards

All shrimp were collected and maintained in accordance with ethical standards in animal research.

Conflict of interest The authors declare that they have no conflict of interest.

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