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Efficacy and variations in bacterial density in the gut of *Litopenaeus vannamei* reared in a BFT system and in clear water supplemented with a commercial probiotic mixture

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ABSTRACT

The purpose of this study was to evaluate the colonization ability of a commercial probiotic mixture in the gut of *Litopenaeus vannamei* reared in BFT and a clear water culture and to verify the effects of the probiotic on the gut bacterial abundance of *Vibrio* sp. and *Bacillus* sp. when added to the biofloc or clear water culture system. The 45-day experiment contained four treatments: Biofloc with no Probiotic application (**BFT**); Biofloc with Probiotic application (**BFT + P**); Clear Water with no Probiotic application (**CW**); and Clear Water with Probiotic application (**CW + P**). The commercial probiotic mixture (Sanolife PRO-W® - INVE Aquaculture) was composed of *Bacillus subtilis*-complex and *Bacillus licheniformes* strains and was added to the water every 48 h. The bacterial abundance in the gut of shrimp was assessed using fluorescence in situ hybridization (FISH) in which rRNA-targeted oligonucleotide probes were used to identify *Bacillus* sp. and *Vibrio* sp. Total bacterial abundance was higher in the probiotic application treatments (BFT + P and CW + P). However, the percentage of *Bacillus* sp. of the total bacterial abundance was higher in clear water than in BFT when the probiotic mixture was added. The percentage of *Vibrio* sp. was higher in the treatments without probiotic addition. The use of Sanolife PRO-W® decreased the bacterial abundance of *Vibrio* sp. in both the BFT and clear water culture systems, while the abundance of *Bacillus* increased. The results obtained in this study can contribute to our understanding of the microbial ecology of biofloc, which is currently considered a key issue of this culture system.

1. Introduction

The use of probiotics has been widely explored in aquaculture practice. Therefore, its supplementation is a result of the current investigation to find antibiotic-free and environmental friendly alternatives in order to promote the healthy development of aquaculture (Sha et al., 2016).

By definition, probiotics are live microorganisms that provide health benefits to the host when administered at appropriate levels (Moriarty, 1998). These microorganisms can colonize the intestinal tract and multiply, promoting the efficient modulation of various biological systems in the aquatic host (Gatesoupe, 1999; Mohapatra et al., 2013). They can be administered via the water or as feed additives (Kumar et al., 2016; Moriarty, 1998; Skjermo and Vadstein, 1999) with either single or a combination of probiotics in a mixture (Villaseñor et al., 2013).

Different bacterial species are used as probiotics in penaeid cultures, especially bacteria of the genus *Bacillus* (Ninawe and Selvin, 2009; Sapcharoen and Rengpipat, 2013). The ability to form spores, providing protection against acid and bile salts and other environmental conditions, make *Bacilli* efficient probiotics. In addition, species of this genus have a high capacity to produce compounds of biotechnological importance such as antibiotics, enzymes, amino acids, and vitamins. They are also capable of degrading cholesterol, influencing the immune status of the host and have antiviral activity (Sorokulova, 2013).

Bacillus species have been used as a dietary supplement in humans and animals for years. Probiotic *Bacillus*has a higher growth and survival rate (Balcazár et al., 2007; Zhou et al., 2009; Nimrat et al., 2012; Krummenauer et al., 2014a; Zokaeifar et al., 2014), improves the activities of digestive enzymes (Wang, 2007; Zhou et al., 2009), enhances the immune response and confers disease resistance against pathogenic *Vibrio*species (Gullian et al., 2004; NavinChandran et al., 2014; Sha

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et al., 2016) in shrimp farming.

Vibrio spp. occur naturally in marine and estuarine environments. They can also be part of the intestinal bacterial community of L. *vannamei*, and some species are even probiotics (Verschuere et al., 2000; Lakshimi et al., 2013; Liu et al., 2015; Huang et al., 2016). However, most species are considered opportunistic bacteria and potential pathogens that cause vibriosis outbreaks and huge production losses (Saulnier et al., 2000).

The gut is considered as the primary pathogen transmission route (De Schryver and Vadstein, 2014). In this context, the probiotic bacteria compete with potential pathogenic bacteria for adhesion sites, nutrients and chemical compounds in the water and in the gut of the host (Gatesoupe, 1999; Mohapatra et al., 2013; Hamza et al., 2016). Hence, they can stick to the mucosal epithelium of the gastrointestinal tract and help to resist pathogens, by decreasing their numbers in the host (Villaseñor et al., 2011; Akhter et al., 2015; Lazado et al., 2015). It is important to monitor the microbiota during probiotic addition to determine the efficiency of gut colonization and the alterations that occur in the bacterial community due to the administration of these microorganisms (Merrifield et al., 2010; Verschuere et al., 2000).

Positive effects of *Bacillus* on L. *vannamei* have been discovered by observing the antagonistic activity, modulation of the microbiota, a decrease in the number, and an increase in the resistance against *Vibrio* in conventional - clear water (Li et al., 2009; Nimrat et al., 2011; Zokaeifar et al., 2014; Sha et al., 2016) and in Biofloc Technology culture systems (Aguillera-Rivera et al., 2014; Ferreira et al., 2015).

Biofloc Technology (BFT) is based on the establishment of a microbial community that is responsible for maintaining water quality and improving growth and survival rates (McIntosh et al., 2001; Wasielesky et al., 2006; Avnimelech, 2007). In addition, this microbial community can inhibit the proliferation of pathogens by competitive exclusion for food and space in the water or in the gut (Crab et al., 2010). Hu et al. (2016) verified that the combined use of *Bacillus* and molasses as a carbon source helped to increase the diversity of the microbial community. In addition, this treatment effectively inhibited pathogens and promoted the formation and development of a beneficial microbial community structure in biofloc-rich water.

The interaction between the microbial community present in biofloc-rich water as well as the associated effects of the addition of external probiotic bacteria as a commercial product is still unclear, as is the modulation of the microbial community in terms of the presence, bacterial abundance and composition under these conditions. Knowledge about such interactions is important for establishing strategies for disease control, water quality and microbial community management in biofloc culture systems.

The primary objective of this study was to compare the efficiency of colonization of a commercial probiotic mixture in the gut of L. *vannamei* reared in BFT and clear water culture and to verify the effects of this probiotic *Bacillus* mixture on the abundance of the putative pathogenic *Vibrio*in the presence of a Biofloc Technology Culture System.

2. Material and methods

2.1. Animal origin

Post-larval L. *vannamei* were obtained from the commercial hatchery Aquatec Ltda (Canguaretama, Rio Grande do Norte, Brazil) and were transferred to the hatchery sector of the Marine Station of Aquaculture, Institute of Oceanography, Federal University of Rio Grande. In the laboratory, shrimp were cultured until they reached an average weight of 0.6 ± 0.02 g before the experimental units were stocked.

2.2. Probiotic mixture

The commercial probiotic mixture (Sanolife –PRO-W® - INVE Aquaculture) used in this study was composed of *Bacillus subtilis*-complex and *Bacillus licheniformis* strains, at a bacterial concentration of 5×10^{10} CFU g^{-1}. The product was added to the culture water every 48 h, at a concentration of 0.5 ppm or 0.5 mg L^{-1} and activated according to the manufacturer's recommendations.

2.3. Experimental design

The trial was carried out in 12 rectangular polyethylene tanks with a bottom area of 0.20 m^2 and an effective volume of 50 L, allocated to an acclimatized room. Shrimp (0.64 ± 0.02 g) were transferred and stocked at 150 shrimp m^{-2} (30 shrimp per tank). The experimental setup contained four treatments with three replicates each: Biofloc with no Probiotics application (**BFT**); Biofloc with Probiotics application (**BFT + P**); Clear Water with no Probiotics application (**CW**); and Clear Water with Probiotics application (**CW + P**).

The biofloc experimental units were filled with 5 L of biofloc-rich water (10% reuse water) obtained from an L. *vannamei* grow-out study in nine 35-m^3 tanks, plus 45 L of filtered (sand filter) natural seawater treated with a chlorine solution (10 ppm measured immediately after chlorination) and dechlorinated using ascorbic acid powder (1 ppm). For the clear water treatments, the tanks were filled with 50 L filtered (sand filter) and pre-treated sea water. Dechlorinated municipal freshwater was used to compensate for evaporative losses and to maintain salinity throughout the experiment.

Sugar cane molasses (C:H:*N* = 30.05:2.78:0.79) was added as an organic carbon source when the total ammonia level reached 1.0 mg $L^{-1}$to foster the development of microbial aggregates in the biofloc treatments. Organic carbon supplementation was based on the addition of 6 g of carbon for each 1 g of total ammonium nitrogen (TA-N) in the water. This procedure followed the methods described by Avnimelech (2009), Ebeling et al. (2006) and Samocha et al. (2007).

A water exchange of 80% was performed every 48 h in the clear water treatments. Hence, natural, filtered and pre-treated sea water at the same temperature as in the experimental units was used for the renewal procedure.

The shrimp were fed a 40% crude protein (CP) commercial diet (Potimar 40 J, Guabi®, Brazil) twice a day (0800 and 1700 h) for 45 days. The feeding rate was adapted from table presented of in review by Jory et al. (2001) and was initially 10% of the total biomass.

2.4. Water quality and sampling

Dissolved oxygen (DO), temperature and pH were measured twice daily (0800 and 1700 h) using a YSI 556 MPS meter (YSI Inc., Yellow Springs, United States). Total ammonium nitrogen (UNESCO, 1983) and nitrite-nitrogen (NO_2-N) (Aminot and Chaussepied, 1983) were measured twice a week. Salinity (using a manual optical refractometer), nitrate-nitrogen (NO_3-N) (Aminot and Chaussepied, 1983), total suspended solids (TSS) (Strickland and Parsons, 1972) and alkalinity (APHA, 1998) were measured weekly.

Shrimp (5) from each tank (15 shrimp per treatment) were killed by thermal shock in an ice bath and aseptically necropsied to remove the intestinal tract at the end of the experimental period (45 days). The intestinal tract samples were fixed in a final concentration of 2% paraformaldehyde and individually weighed for further analysis.

2.5. Analysis of the presence and efficiency of the commercial probiotic mixture and putative pathogenic bacteria by fluorescence in situ hybridization (FISH)

The intestine samples were processed according to a protocol adapted from Epstein and Rossel (1995). A Tween solution (0.0001%) was added to each sample, which was then sonicated (Vibra Cell VCX 130 PB, Sonics & Materials ®) three times (range 110.7 μm per 60 s). After sonication, the samples were centrifuged three times at 500G for five minutes. The supernatant was removed, and the remaining

Table 1
rRNA-targeted oligonucleotide probes of different bacterial species used in this study. All probes were labeled with fluorochrome Cy3.

Probe	Specificity	Sequence 5–3′	%FA[a]	Reference
NON	Negative control	TAGTGACGCCGTCGA	30	Yokokawa and Nagata, 2005
Bacil1	*Bacillus*	GCCGCCTTTCAATTTCGAAC	35	Ichijo et al., 2010
Vib519a	*Vibrio*	ACCACCTGCATGCGCTT	40	Huggett et al., 2008

[a] Percentage of formamide (FA) in in situ hybridization buffer.

contents were washed twice with ultrapure water. Three supernatant fractions were placed in the same bottle and shaken vigorously. The material was then centrifuged as previously described. Aliquots of each sample were filtered on polycarbonate filters (Nuclepore® - 0.2 μm) and refrigerated until hybridization.

Bacillus genus components of the probiotic mixture added to the biofloc-rich water and putative pathogenic bacteria (*Vibrio sp.*) were identified with rRNA-targeted oligonucleotide probes (Table 1). Sample processing and Fluorescent in situ Hybridization was realized as described by Del'Duca et al., 2013. A negative control made with a probe with no specificity for bacteria was used to evaluate the hybridization efficiency. All probes were labeled with the Cy3 fluorochrome. In addition to each specific probe, DAPI was used to determine the total bacterial abundance. The counting of the bacterial cells was carried out in ten random fields with an Olympus BX60 epifluorescence microscope equipped with a 41,007 filter for the Cy3 marker, and a 31,000 filter for DAPI at a magnification of 1000 ×. The results found for the hybridized samples using a probe without specificity were discounted from the densities of the specific probes. The total density of microorganisms was calculated by counting the cells stained with DAPI.

2.6. Statistical analyses

One-way analysis of variance (ANOVA) was used to identify significant differences in bacterial abundance and water quality parameters among treatments. The ANOVA was followed by Tukey's posthoc comparison when significant differences were found. Statistical significance was taken as $P < 0.05$.

3. Results

3.1. Water quality

The water quality parameters are shown in Tables 2 and 3, and they were divided according to the biofloc (BFT) and clear water (CW) groups. Temperature, dissolved oxygen, pH, salinity and alkalinity did not differ significantly among treatments (Table 2).

The levels of nitrogen compounds, total ammonia nitrogen (TAN) and nitrite (NO_2 – N) were significantly higher in the CW and CW + P treatments. On the other hand, the nitrate (NO_3-N) concentrations were significantly higher in the BFT and BFT + P treatments. A total suspended solids measurement was performed only for the BFT and BFT + P treatments (Table 3) because no accumulation of significant amounts of particulate organic matter or suspended solids occurred in the clear water systems.

3.2. Bacterial density

The total bacterial abundance in the gut of L. *vannamei* cultured in the BFT + P treatment ($3.67 \pm 0.18{\cdot}10^8$ cells g^{-1}) was significantly higher, followed by the CW + P ($2.12 \pm 0.3{\cdot}10^{-8}$ cells g^{-1}) treatment. The treatments with no ProW® supplementation (BFT – $1.65 \pm 0.9{\cdot}10^8$ cells g^{-1}; CW – $1.53 \pm 0.04{\cdot}10^8$ cells g^{-1}) did not differ between each other and had the lowest bacterial abundance (Fig. 1).

Regarding the bacterial density of *Bacillus* as the component of the probiotic mixture, a higher number of cells was detected in the treatments containing probiotics (BFT + P – $0.63 \pm 0.06{\cdot}10^8$ cells g^{-1}; CW + P – $0.69 \pm 0.09{\cdot}10^8$ cells g^{-1}; BFT- $0.18 \pm 0.18{\cdot}10^8$ cells g^{-1}; CW- $0.07 \pm 0.07{\cdot}10^8$ cells g^{-1}) (Fig. 2A). On the other hand, the abundance of *Vibrio* was higher in both treatments without the probiotic (BFT- $0.23 \pm 0.04{\cdot}10^8$ cells g^{-1}; CW- $0.27 \pm 0.04{\cdot}10^8$ cells g^{-1}; BFT + P – $0.06 \pm 0.01{\cdot}10^8$ cells g^{-1}; CW + P – $0.07 \pm 0.02{\cdot}10^8$ cells g^{-1}) (Fig. 2B).

Regarding the percentage of *Bacillus* and *Vibrio* to the total bacterial abundance, the most significant contribution of *Bacillus* was in the CW + P treatment, followed by the BFT + P treatment. The BFT and CW treatments showed the lowest contribution of *Bacillus* sp. to the total bacterial abundance. On the other hand, *Vibrio* sp. percentage was significantly higher in the treatments with no probiotic application (BFT and CW) and lower in the treatments in which the probiotic mixture was administered (Table 4).

4. Discussion

In addition to promoting direct host benefits, a probiotic can control water quality and modify the aquatic community structure by interacting with other microorganisms (Verschuere et al., 2000; Newaj-Fyzul et al., 2014; Dawood and Koshio, 2016). The application of a probiotic mixture did not change the water quality parameters analyzed in this study. Water temperature, dissolved oxygen, pH, salinity and alkalinity were similar between treatments with and without probiotic in the clear water and BFTsystems. These values remained within the proper range for rearing L. *vannamei* (Van Wyk and Scarpa, 1999; Furtado et al., 2011). However, the mean values of total suspended solids (TSS) in the biofloc treatments were below the range recommended by Samocha et al. (2007) for L. *vannamei*.

No differences were observed in the concentration of nitrogen compounds (TAN, NO_2-N and NO_3-N) between treatments with and without probiotic. However, in the BFT treatments, lower concentrations of ammoniacal nitrogen and higher nitrate levels were observed, indicating that nitrification occurred, but it did not occur in the clear water treatments. However, the nitrogen concentrations observed in the clear water treatments were maintained in the optimal range for L. *vannamei* (Lin and Chen, 2001; Lin and Chen, 2003; Kuhn et al., 2010) via water exchanges performed every 48 h.

The nitrification observed in the BFT treatments is possibly performed by the microbial community present in the floc, as a diversity of

Table 2
Mean (± standard deviation) values of water quality parameters throughout the experiment for*L. vannamei* culture in biofloc (BFT) and in clear water (CW) with and without probiotic application. Different letters indicate statistical differences ($P < 0.05$).

Treatment	Temp. (°C)	D.O_2 (mg L^{-1})	pH	Salinity	Alkalinity (mg L^{-1})
BFT	27.77 ± 0.87	5.72 ± 0.31	7.83 ± 0.12	32.50 ± 1.95	181.25 ± 18.74
BFT + P	27.74 ± 0.92	5.72 ± 0.34	7.82 ± 0.11	32.47 ± 2.00	175.00 ± 19.71
CW	27.63 ± 0.85	5.86 ± 0.40	7.93 ± 0.07	29.22 ± 2.55	181.25 ± 15.30
CW + P	27.66 ± 0.91	5.88 ± 0.42	7.94 ± 0.07	29.08 ± 2.44	175 ± 13.69

Table 3
Mean (± standard deviation) values of nitrogen compounds (total ammonia nitrogen –TAN, nitrite – NO_2-N and nitrate –NO_3-N) and total suspended solids (TSS) of L. *vannamei* culture in biofloc (BFT) and clear water (CW) with and without probiotic application. Different letters indicate statistical differences ($P < 0.05$).

Treatment	TAN(mg L^{-1})	NO_2-N (mg L^{-1})	NO_3-N (mg L^{-1})	TSS (mg L^{-1})
BFT	0.04 ± 0.007 [a]	0.18 ± 0.09 [a]	50.36 ± 12.41 [b]	420.50 ± 89.44
BFT + P	0.04 ± 0.005 [a]	0.16 ± 0.09 [a]	53.06 ± 12.45 [b]	425.50 ± 102.41
CW	0.38 ± 0.16 [b]	0.43 ± 0.21 [b]	0.10 ± 0.22 [a]	–
CW + P	0.40 ± 0.13 [b]	0.42 ± 0.25 [b]	0.12 ± 0.23 [a]	–

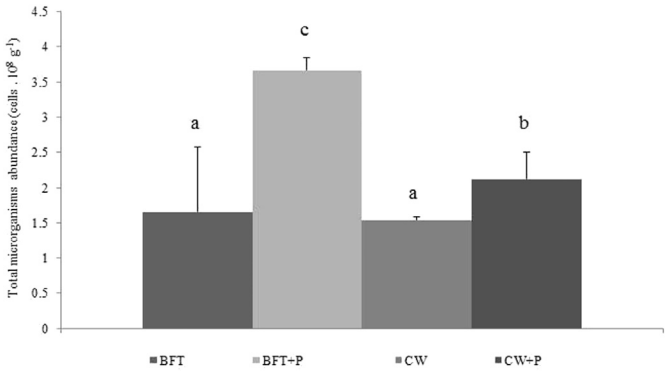


Fig. 1. Total abundance of microorganisms ± Standard Deviation (cells $\cdot 10^8$ g^{-1}) in the *L. vannamei* gut in the BFT, BFT + ProW, CW and CW + ProW treatments. Different letters indicate statistical differences ($P < 0.05$).

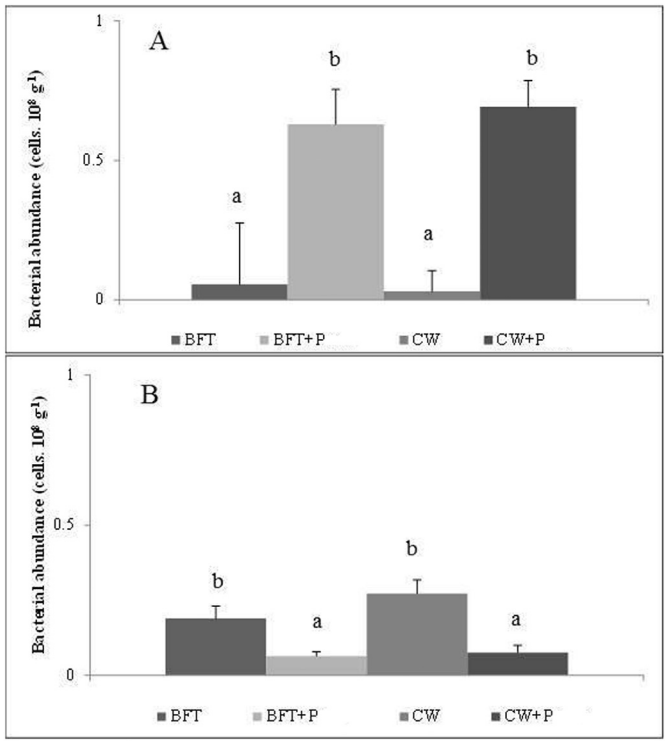


Fig. 2. Specific bacterial abundance ± Standard Deviation (cells $\cdot 10^8$ g^{-1}) of *Bacillus sp.* (A) and *Vibrio sp.* (B) in the *L. vannamei* gut in the BFT, BFT + ProW, CW and CW + ProW treatments. Different letters indicate statistical differences ($P < 0.05$).

photoautotrophic, chemoautrotrophic and heterotrophic microorganisms is found in this culture system (Ebeling et al., 2006). The occurrence of this process is evidenced in the present study by the accumulation of NO_3-N, which has similarly been reported by Krummenauer et al. (2014a, b) for the culture of L. *vannamei* in a Biofloc system. Besides, some heterotrophic microorganisms have been reported to nitrify and/or transform many types of nitrogen compounds (Yang et al., 2011). These mechanisms occur by converting the nitrogen in the microbial biomass in the presence of oxygen and an organic carbon source, which is the main principle of biofloc formation (Avinimelech, 1999; Ebeling et al., 2006).

Normally, the colonization of the mucosal membrane by *Bacillus* is not permanent. The density of these bacteria can be controlled by varying the dose of probiotic (Abriouel et al., 2011; Al Gburi et al., 2016; Sorokulova, 2013). When added to the water, the probiotic can be absorbed via hostosmoregulation processes and through the feed (Kesakordi-Watson et al., 2008). The establishment of the probiotic in the gastrointestinal tract of the host is directly related to the dosage and ingestion time. The higher the concentration of probiotic in the water, the shorter is the ingestion time for the shrimp (Riquelme et al., 2001). In the present study, the probiotic mixture was added to the water at a concentration of $12.5.10^5$ CFU L^{-1} every 48 h. The intestine of the shrimp was colonized by the probiotic *Bacillus* mixture added to both the BFT and CW systems at the end of the 45-day experiment.

Hence, the addition of the probiotic mixture to the water can facilitate the colonization of *Bacillus* in the gut by increasing its numbers (Nimrat et al., 2012) and can decrease the presence of *Vibrio* (Silva et al., 2013). De Schryver and Vadstein (2014) suggested that aquatic organisms have a closer association between the host intestinal microbiota and the environmental microbiota. Likewise, Del'Duca et al. (2015) verified that a strong similarity in the bacterial community exists among the gut, the water and the pond sediment in cultured tilapia. Thus, a probiotic can be used as a strategy for manipulating the microbial diversity and controlling pathogenic infections in the gut of the host when applied on a rational basis.

The use of an experimental or commercial probiotic, predominantly *Bacillus spp.*, promoted resistance against *Vibrio* infections in previous studies. These results were confirmed by challenge tests (Balcazár et al., 2007; Villaseñor et al., 2015), antagonistic activity in vitro (Liu et al., 2015) or presumptive *Vibrio* counts (Silva et al., 2013). These confirmations are in concordance with the present study in which similar results for the bacterial abundance of *Vibrio* and *Bacillus* in the gut of L. *vannamei* reared in BFT + P and CW + P were obtained.

As previously mentioned, the quantification of specific bacteria of the *Bacillus* and *Vibrio* genera in the gut of *L.vannamei* showed an antagonist tendency. Higher numbers of *Bacillus* (BFT + P and CW + P) were associated with a lower abundance of *Vibrio* and vice versa. One of the principal modes of action of a probiotic is competition for adhesion sites and colonization of the intestine, as well as competition for chemicals or available energy, avoiding the establishment of harmful pathogens (Gatesoupe, 1999; Mohapatra et al., 2013), and also exerting anti-adhesive activity against *Vibrio harveyi* (Hamza et al., 2016). Besides, another important property of probiotics is the production of substances that have antagonistic properties, such as organic acids and bacteriocins (Ringø et al., 2010; Ringø et al., 2012). These substances can alter the metabolism of microbiota (Sakata et al., 1999), and support the health of the host, providing protection against infections by stimulating the immune system (Lara-Flores and Aguirre-Guzman, 2009).

The presence and the number of probiotic organisms in this study induced a higher total bacterial abundance in the intestinal tract of L. *vannamei* in a BFT system than in clear water (CW), which would probably affect the bacterial diversity and the composition of the

Table 4
Percentage (Mean ± Standard Deviation) of *Bacillus* spp. and *Vibrio* spp. in the total bacterial abundance observed in BFT and clear water with and without probiotic application. Different letters indicate statistical differences ($P < 0.05$).

	BFT	BFT + P	CW	CW + P
Bacillus spp	3.58 ± 3.08[a]	17.26 ± 4.14[b]	1.90 ± 1.08[a]	32.85 ± 2.46[c]
Vibrio spp	17.77 ± 9.80[b]	1.75 ± 0.49[a]	17.74 ± 3.24[b]	3.54 ± 0.53[a]

microbiota, which can be affected by the contact with the surrounding environment and the feed intake (Tzuc et al., 2014). The addition of a commercial probiotic mixture containing *Bacillus* was able to modify the gut bacterial community in L. *vannamei*, enhancing the bacterial diversity and decreasing the number of *Vibrio* in clear water (Villaseñor et al.,2013; Li et al., 2009). The probiotic seemed to have greater ability to colonize the intestinal tract in this study, and it represented > 30% of the total bacteria in the shrimp gut. Such fact can possibly lead to positive effects to the host as previously mentioned, which results in disease resistance and hence better results of shrimp production.

The use of BFT as a culture system contributed to an increase in the bacterial abundance in the gut of L. *vannamei*, with or without the addition of the probiotic mixture. Similarly, Hu et al. (2016) assessed the effect of the combined use of a probiotic and molasses as a carbon source on the microbial community structure and verified that this combination promoted the formation and development of a beneficial microbial community structure in a high-density intensive aquaculture of L. *vannamei*. These results corroborate those of the present study in which a combination of molasses and a probiotic enhanced the total bacterial abundance in the BFT + P treatments.

Bacillus species such as *B. subtilis* and *B. amyloliquefaciens* can auto-aggregate and co-aggregate (Al Gburi et al., 2016). Auto-aggregation of probiotic strains may be required for their adhesion to host epithelial cells in the gut. As in a biofloc, co-aggregation may facilitate the integration of exogenous bacteria, allowing the development of a multi specific biofilm. Additionally, this ability may also allow probiotic organisms to create a barrier, which could effectively prevent colonization by pathogens (Kos et al., 2003; Al Gburi et al., 2016).

The use of a commercial probiotic mixture can contribute to homeostasis and prevent outbreaks of opportunistic pathogenic bacteria in a BFT system and clear water. A synergistic effect is possible between the probiotic and the heterotrophic bacterial community in the biofloc for L. *vannamei*, which could prevent the development of lesions in the hepatopancreas caused by pathogenic *Vibrio* bacteria in the water (Aguillera-Rivera et al., 2014).

Ferreira et al. (2015) investigated the probiotic properties of *Bacillus* sp. isolated from biofloc-rich water and supplemented to L. *vannamei*. They verified a lower number of *Vibrio* in the water in the experimental units treated with *Bacillus*, which showed antagonism against *Vibrio harveyi*. Isolated *Bacillus* sp. bacteria from the biofloc-rich water of an L. *vannamei* culture were assessed for antagonistic in vitro activity and the total number of *Vibrio* and *Bacillus* in the water by a plating technique. The authors observed in vitro inhibitory characteristics of *Bacillus* against the putative pathogen *Vibrio alginolyticus* and a reduction of the prevalence of *Vibrio* sp. in the water when cultures were supplemented with the probiotic.

Different factors affect the effectiveness of probiotic bacteria. For example, *Bacillus* enzymes are active in living and dead cells (Tan and Qian, 1996). In this context, most of the viability analyses and antagonism tests are performed with bacteria grown in culture medium. However, this technique may present some methodological difficulties, since the assessment of *Bacillus* and *Vibrio* growth is affected by the culture medium and can cause false negative results, according the review by Letchumanan et al. (2014).

Many culture-independent molecular biology techniques are based on the amplification of nucleic acids with qualitative or semi-quantitative results. In these cases, it is not possible to visualize each individual and to evaluate the spatial distribution of aggregated or non-aggregated microorganisms. However, the FISH technique allows the quantification of individual microorganism cells. It is an efficient tool to visualize and quantify the specifically labeled bacteria in this study. This technique has been applied in aquaculture to characterize the microbiota and bacterial diversity of water and waste water (Garcia and Olmos, 2007; Paungfoo et al., 2007; Payne et al., 2007; Pereira et al., 2011) and the intestinal tract of fish (Asfie et al., 2003; Balcázar et al., 2010; Huber et al., 2004) and oysters (Hernandez-Zarate and Olmos-Soto, 2006). Additionally, Del'Duca et al. (2013) used the FISH technique to evaluate the efficiency of *Bacillus* as a probiotic and its ability to control putative pathogenic bacteria in the gut of tilapia. The authors suggest that the FISH technique can quantify and follow changes in the number of probiotic and pathogenic microorganisms. Thus, the microbial community structure (i.e., the taxa and number of each taxa of bacteria) allows an assessment of the probiotic efficiency (Del'Duca et al., 2013).

The total bacterial abundance quantified in the present study was higher in the BFT system in the presence of the probiotic mixture (BFT + P). However, the results also indicate that *Bacillus* can colonize the gut more effectively when L. *vannamei* is cultured in clear water than in a BFT system, which can possibly be attributed to the composition of the biofloc system in which a large diversity of microorganisms take part (De Schryver et al., 2008). On the other hand, the proportion of *Vibrio* related to the total microorganisms was lower in the treatment containing the probiotic mixture, which can be associated with the role of the biofloc in controlling or encouraging potential pathogenic bacteria, especially *Vibrio* (Hargreaves, 2013).

Hence, these observations indicate that adding exogenous bacteria to an existing bacterial community can affect the dynamics and interactions between the various groups of organisms that compose the biofloc system. These indications can be further reflected in different approaches to managing the biofloc or the probiotic application. Thus, it is possible to reinforce the importance of probiotic supplementation, especially in conventional clear water culture systems.

5. Conclusions

The commercial probiotic mixture composed of *Bacillus subtilis* and *B. licheniformes* colonized the intestinal tract of L. *vannamei* and controlled the putative pathogenic populations of *Vibrio*.

The difference in efficiency of ProW® between the two culture systems was in proportion of *Bacillus* to the total microorganisms in the gut of the shrimp. Higher probiotic colonization occurred in CW + P. The intestinal tract of the shrimp farmed in BFT + P had a greater number of microorganisms in the presence of the probiotic.

The addition of the probiotic did not modify the water quality in either system. Likewise, it promoted a decrease in the density of *Vibrio* and an increase of *Bacillus* in the shrimp gut in both systems.

The number of pathogenic and probiotic individual cells as well as the proportion are important in shrimp farming. The FISH technique is a tool that allows for the quantification and monitoring of a probiotic along with the detection of putative pathogens or any other type of bacteria present in the digestive tract of shrimp and in the water.

Additionally, the results obtained in this study can contribute to our understanding of the microbial ecology of a biofloc. It is currently considered a key issue of this culture system.

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