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Application of *Bacillus amyloliquefaciens* as probiotic for *Litopenaeus vannamei* (Boone) cultivated in a biofloc system

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Abstract

Probiotics can improve growth, survival and resistance to pathogenic organisms of the cultivated species in aquaculture systems with water recirculation. However, their possible benefits on biofloc systems have been less studied. In this study, the benefits of *Bacillus amyloliquefaciens* bacterium, on a biofloc culture of *Litopenaeus vannamei* were evaluated. *B. amyloliquefaciens* was applied as dissolved in water. To our knowledge, no previous assays on biofloc systems have been published, and on recirculation systems it has only been tested mixed with feed. The objective of the present study was to evaluate the effect of *B. amyloliquefaciens* on water quality, growth parameters and the immune system of shrimp. Three concentrations of probiotic were tested in triplicate (9.48×10^4, 1.90×10^5, and 3.79×10^5 cfu ml^{-1}) and were compared with the control (without probiotics). Water quality parameters such as nutrients and suspended solids were monitored. In *L. vannamei*, growth, survival and their immune system parameters (total protein concentration, cell number with apoptosis and percentage of granular and hyaline hemocytes) were studied. The results showed that the application of *B. amyloliquefaciens* did not produce significant differences in water quality or shrimp growth. However, it showed significant improvements in the immune system. As compared with the control treatment, an increase in the total protein concentration and granular hemocytes, and a decrease in the cell number with apoptosis in the hempolymph were observed. Thus, we can conclude that *B. amyloliquefaciens* provides greater resistance to shrimp against the attack of pathogens in biofloc systems.

Keywords: Growth parameters, Immunological parameters, Water quality, White shrimp

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Introduction

Biofloc technology systems (BFT) is based on the development of macroaggregates composed of heterotrophic bacteria, phytoplankton, food debris, organic matter and other organisms, capable of maintaining water quality at adequate values for shrimp and fish farming (Crab *et al.*, 2012; Emerenciano *et al.*, 2013). When heterotrophic bacteria present in the BFT have an appropriate C:N ratio, they oxidize the total ammonia nitrogen, excreted by cultivated species (Crab *et al.*, 2012; Emerenciano *et al.*, 2013). This nitrification process leads to an increase in microbial biomass (Schyrve *et al.*, 2008; Kuhn *et al.*, 2009), which is rich in protein and is consumed as a food supplement by cultivated species, improving their growth (Schyrve *et al.*, 2008; Kuhn *et al.*, 2009).

BFT can have a probiotic effect on shrimp and fishes (Crab *et al.*, 2012; Emerenciano *et al.*, 2013). However, it has been observed that the extra addition of probiotics can enhance the beneficial effects of BFT (Souza *et al.*, 2012). Probiotics are live microorganisms, usually bacteria, the effects of which vary depending on method of application, dosage and bacteria species (Van Hai and Fotedar, 2010). There are three application methods: through larval inmersion in the probiotic culture (Van Hai and Fotedar, 2010), mixed with feeds or dissolved in water. Immersion in probiotic cultures is only feasible for very small specimens due to its difficult handling (Van Hai and Fotedar, 2010). The application in feeds is the most usual and is usually done during the manufacturing process (Wang *et al.*, 2008). However, the administration of probiotics in commercial feeds prevents producers from selecting separately the most appropriate feed and probiotic for their cultures. Probiotics dissolved in water are used in a wide dosage range, depending on the species and culture conditions, doses from 10^3 to 10^8 cfu mL^{-1} being the most usual (Zhou *et al.*, 2009; Van Hai and Fotedar, 2010; Souza *et al.*, 2012; Ramezani-Fard *et al.*, 2014).

In aquaculture systems, with water recirculation, it has been observed that the application of probiotics can produce the following positive effects: (i) decrease occurrence of diseases, by direct competition with pathogenic organisms or by secretion of bactericidal substances (Pandiyan *et al.*, 2013), (ii) consumption of nitrogenous compounds in water, which produces an improvement in water quality (Dalmin *et al.*, 2001), (iii) enzyme secretion in the intestine of the cultivated species that help food digestion (Zhou *et al.*, 2009), (iv) stimulation of the immune system, increasing the protection of the host against pathogens (Rengpipat *et al.*, 2000).

In BFT, some studies have been conducted on the effect of probiotics composed of a combination of different genera or species of bacteria. Krummenauer *et al.* (2014) observed that by applying probiotics composed of different genera of bacteria (*Bacillus* sp., *Enterococcus* sp., *Thiobacillus* sp.,

Paracoccus sp. and *Lactobacillus* sp.) the effects of a *Vibrio parahemolyticus* infection on *L. vannamei* were reduced. Rengpipat *et al.* (2000) also observed, the benefitial effects of *Bacillus* sp. against *Vibrio* sp. Vita (2008) observed improvements in the size of white shrimp *L. vannamei* by using a probiotic composed by two species of *Bacillus* sp. (*Bacillus licheniformis* and *B. subtilis*). Souza *et al.* (2012) tested three probiotics, a *Bacillus* sp. mix (*B. subtilis*, *B. licheniformis* and *B. pumilus*), a multistrain probiotic (*Bacillus* sp., *Enterococcus* sp. and *Lactobacillus* sp.) and a monoespecific probiotic with *Bacillus cereus* var. *toyoi*, and observed that different probiotic bacteria improved growth, survival and the immune system of *Farfantepenaeus brasiliensis*. In this experiment, Souza *et al.* (2012), observed that a combination of *Bacillus* sp., had better results than other bacteria combinations. However, the common technique of studying the effect of combined probiotics prevents the determination of the role of each genus or species of probiotic in aquaculture systems in general, and in BFT in particular. *B. amyloliquefaciens* is a probiotic bacterium, which has been applied to feed *Litopenaeus vannamei*, *C. carpio* and *Oreochromis niloticus*, cultured in water recirculation systems, by Camacho (2012), Nuez-Ortín (2013), Huang *et al.* (2015) and Saputra *et al.* (2016). Their results showed the following advantages of applying *B. amyloliquefaciens* as a probiotic in water recirculation systems: 1) it produces digestive enzymes which improve the growth of the cultured species (Nuez-Ortín, 2013); 2) it has bactericidal characteristics produced by the secretion of barnase and lactic acid (Cao *et al.*, 2011; Nuéz-Ortín, 2013); 3) it improves the immune system and survival of guests (Huang *et al.*, 2015; Saputra *et al.*, 2016); 4) it has bactericidal characteristics against *Vibrio alguinolyticus*. Camacho (2012); and 5) it has good properties to promote biofloc creation because it has higher protein levels and grows faster than other bacteria (Bao, 2014). In spite of these characteristics, there are no references to the application of *B. amyloliquefaciens* in biofloc systems and to its application directly in water.

In this study, we assessed the effect of a monospecific probiotic, *B. amyloliquefaciens*, dissolved in water. The objective was to evaluate the effect of *B. amyloliquefaciens* on water quality, zootechnical development and the immune system of white shrimp in a biofloc system.

Materials and methods

Shrimp

The shrimp post larvae (PLs) were bought from a comercial laboratory (Aquatec), which certificated that PLs were free of phatogens, and moved to Universidade Federal do Rio Grande (FURG) instalations. After acclimation, the PLs underwent an intermediate nursery phase. This phase was carried out in a greenhouse, in 35 m^3 tank, provided with mature bioflocs at a temperature of 28 ºC and a salinity of 30 g L^{-1}. The larvae were cultivated at a density of 1500 shrimps m^{-2} and were

fed 4 times a day with feed (38% protein), they remained in the nursery until reaching 2.0±0.7 g of weight. Then, the shrimp were moved to experimental tanks to begin the experiment, which lasted 42 days.

The shrimp were distributed in twelve 500 L square tanks (1,25 m^2 each one). Each tank was inoculated with 50 L of heterotrophic biofloc from a previous culture of *L. vannamei*, and filled with 450 L of disinfected marine water (final salinity 17.33±0.59 g L^{-1}). The tanks were constantly individually aerated. Shrimp density was 300 shrimp per cubic metre.

Ecobiol Plus®, a probiotic made up of *B. amyloliquefaciens* was tested as follows. Before applying Ecobiol Plus® to the culture water, five samples of probiotic were seeded on soy agar, during 24 hours at 30°C, to determine the accurate concentration of *B. amyloliquefaciens* CECT-5940. Four treatments were essayed in triplicate: 1) control treatment (CO) without probiotic; 2) treatment A (TA) with Ecobiol Plus® in a dose of 9.48×10^4 cfu mL^{-1}; 3) treatment B (TB) with Ecobiol Plus® in a dose of 1.90×10^5 cfu mL^{-1} (twice the recommended dose of probiotics); and 4) treatment C (TC) with Ecobiol Plus® in a dose of 3.79×10^5 cfu mL^{-1} (four times the recommended dose). The recommended dose is the average recommended dose for other probiotics applied in water, since there are no previous studies of the application of *B. amyloliquefaciens*.
The shrimp were fed daily with commercial feed (Guabi – Active 38) specifically designed to encourage growth in *L. vannamei*. The quantity of feed was calculated according to shrimp biomass (Jory *et al.*, 2001). The feed was provided twice per day, 40% in the morning and 60% in the afternoon, and distributed on feeding trays. Water renewal during the experiment was minimal, and limited to avoid surpass of 8 mg L^{-1} nitrite. Levels above that threshold value can cause mortality in the shrimp *L. vannamei*, as indicated by Lin and Chen (2003).

The maintenance of the biofloc system was carried out following the methodology proposed by Avnimelech (1999) and Ebeling *et al.* (2006). The system was fertilized with molasses of sugar cane. Molasses were administered when total ammonia nitrogen reached a concentration greater than 1 mg L^{-1}, to mantain a carbon:nitrogen relationship of 15:1.

Water quality

pH, dissolved oxygen, salinity and temperature were monitored in situ, using a multi-parameter probe (YSI Professional Plus), twice a day (morning and afternoon).

Every two days, a water alliquot was collected to determine the concentration of the following nutrients: 1) total ammonia nitrogen (N-TA) using the methodology described by UNESCO (1983); 2) nitrites ($N\text{-}NO_2^-$) using the methodology of Bendschneider and Robinson (1952) described in Baumgarten *et al.* (2010); 3) nitrates ($N\text{-}NO_3^-$) were analyzed using the methodology described by Grasshoff (1976); and 4) phosphates ($P\text{-}PO_4^{3-}$)

were analyzed following Murphy and Riley (1962).

The biofloc volume (BV) was monitored weekly by placing one liter of water in an Inhoff cone, following the methodology described by Avnimelech (2009). Total suspended solids (TSS) were determined as described by Baumgarten *et al.* (2010), an aliquot of 50 mL from each tank was filtered (0.45 μm) and filters were dried for approximately 24 hours at 105ºC. Then, non volatile suspended solids (NVSS) and volatile suspended solids (VSS), were calculated according to Baumgarten *et al.* (2010) after calcination in a muffle. Water alkalinity was monitored at the beginning, in the middle and at the end of the experiment, using the trimetric method of APHA (1998).

Growth parameters

30 shrimp per tank were measured using a 0.1 g precision digital scale (Marte Slim). These measurements were done at the beginning of the experiment and every 10 days during the study period, to monitor weight growth of the shrimp (g) and to re-adjust the feed amount. Once the experiment ended, survival, weight gain, weekly weight gain, biomass gain, feed conversion rate and productivity were determined following the equations described by Furtado *et al.* (2011) and Macias-Sancho *et al.* (2014).

Survival=(final shrimps amount/initial shrimps amount)×100

Weight gain=(final wet weight–initial wet weight).

Weekly weight gain (WWG)=[(final wet weight–initial wet weight)/week number].

Biomass gain (BG)=(final biomass–initial biomass)

Feed conversion rate (FCR)=(dry feed consumption/biomass gain)

Productivity (P)=(biomass gain/m^3)

Immunologic parameters

To study the shrimp immunological system the following parameters were determined, after 42 days: granular hemocyte (GH) and hyaline hemocyte (HH) percentage, total protein concentration in hemolymph (TPC), and the apoptotic cell number in hemolymph. A hemolymph sample was extracted from the hearts of 5 shrimp per tank, using a 50 μl Hamilton syringe. The samples were transferred to polyethylene tubes containing heparin to avoid the coagulation of the samples (Maggioni *et al.*, 2004).

The percentage of granulate and hyaline hemocyte present was determined by microscope observation following Weibel (1980) from one drop of hemolymph spread on a microscope slide. A microscope lens with integration Disc.1- @5 points G49 (Carl Zeis) connected to a Zeiss Primo Star microscope was used. The TPC in the shrimp serum, was determined according to the Bradford (1976) method, using a 10 μL hemolymph aliquot.

The apoptotic cell number in hemolymph was evaluated by the TUNEL method using the ApopTag® Plus Peroxidase In Situ Apoptose Detection kit (Millipore) according to

Charriaut-Marlangue and Ben-Ari (1995) and Wang and Zhang (2008). A 5 µL hemolymph aliquot was placed in histological sheets positively charged to enable the identification and counting of cells with apoptose using an optic microscope (Carl Zeis).

Statistic analysis

A non parametric one-way analysis of variance (Kruskal-Wallis) was used to test differences in physico-chemical variables between probiotic treatments (CO, TA, TB and TC). An analysis of variance (ANOVA) was used to test differences in growth parameters and immunologic parameters between probiotic treatments (CO, TA, TB and TC). The software Statgraphics® Centurion XVII was used.

Results

Water quality

Temperature remained stable in the greenhouse, although small variations were observed between 23.6 and 33.0ºC. Dissolved oxygen values were kept above 5 mg L^{-1} in all treatments. pH range had small variations in all treatments between 6.9 and 9.1. Salinity was kept between 16.5 and 18.6. The alkalinity decreased during the experiment in all treatments, it decreased from 255 (day 1) to 45 (day 42) mg $CaCO_3$ L^{-1}.

The evolution of N-TA was similar in all treatments (Fig. 1a), N-TA values were not statistically different between treatments (p>0.05). N-TA concentration was under 0.20 mg L^{-1} during all the study period. For the first 20 days, the maximum values of N-TA detected reached maximum values of 0.84, 0.70, 0.71 and 1.19 mg L^{-1} in CO, TA, TB and TC treatments respectively. After the first 20 days, N- TA values dropped to nearly 0 mg L^{-1} in all the treatments. The maximum N-NO_2^- concentration was 5.88, 6.33, 6.83 and 6.49 mg L^{-1} in CO, TA, TB and TC treatments respectively, these values were detected between days 11 and 26. To avoid toxic levels, water was renewed depending on the needs of each tank, 30%, 8.33% 21.67% and 38.33% of total water volume in CO, TA, TB and TC treatments respectively. Nitrite concentrations for the rest of the study period were always below 5 mg L^{-1} (Fig. 1b). There were no significant differences (p>0.05) in nitrite concentrations among the treatments.

N-NO_3^- evolution was different to the observed for N-NO_2^- and N-TA (Fig. 1c). N-NO_3^- remained below 10 mg L^{-1} during the first 19 days, after that N-NO_3^- started increasing. The maximum nitrates values were reached on the last study day, these values were 53.02, 51.89, 53.00, and 45.27 mg L^{-1} in treatments CO, TA, TB and TC (Fig. 1c). No statistical differences were observed in nitrates levels between treatments (p>0.05). A rising trend in P-PO_4^{3-} was observed in all treatments. Maximum P-PO_4^{3-} values were 10.43, 11.38, 11.10 and 8.23 mg L^{-1} in treatments CO, TA, TB and TC. No statistical difference was observed between treatments (p>0.05). However, TC showed lower values of phosphates in the last days of the experiment, as shown in Fig. 1d.

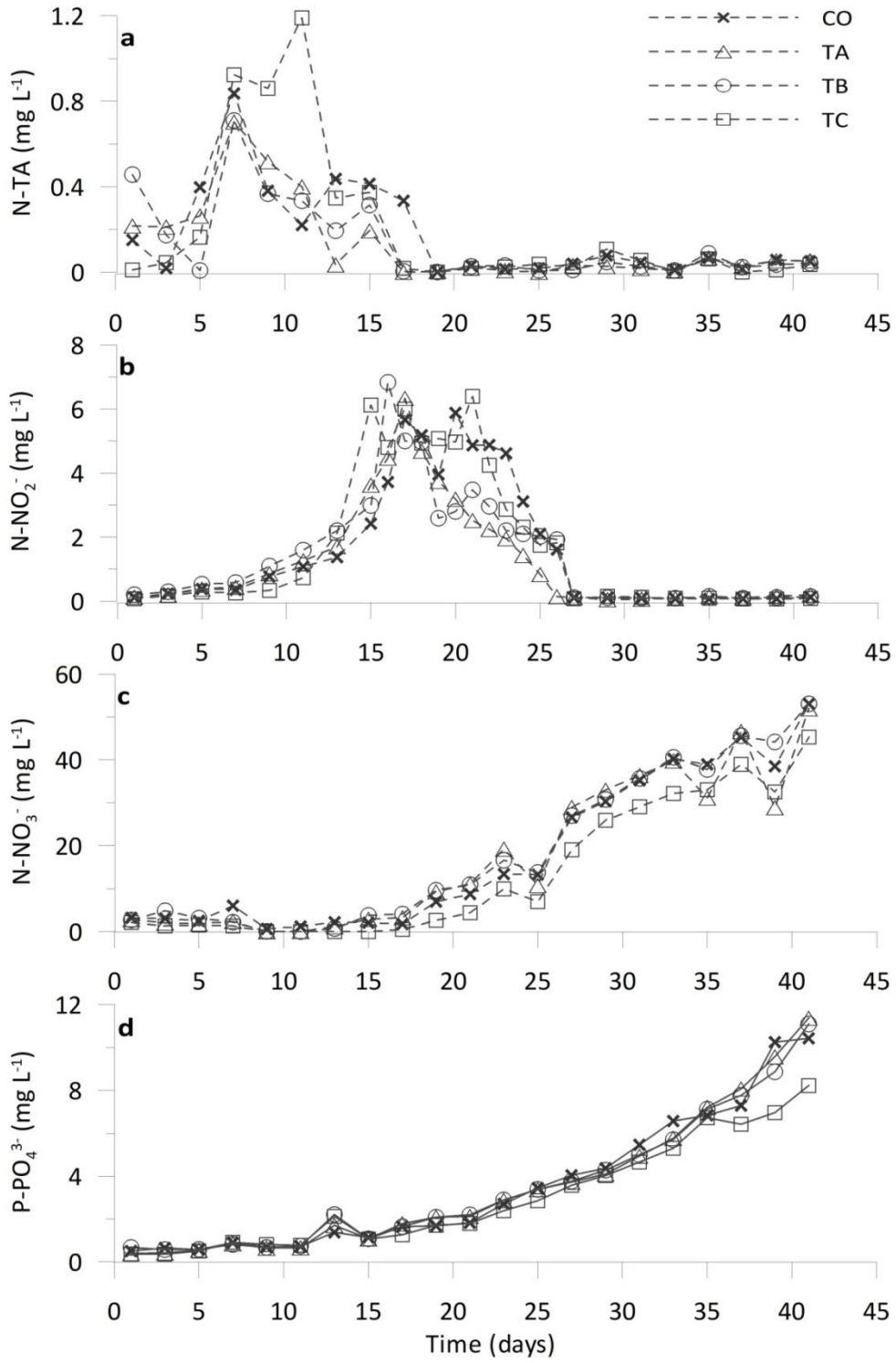

Figure 1: Evolution of total ammonia nitrogen, nitrite, nitrate and phosphate concentration. Control treatment (CO) contains 0 cfu mL^{-1}, treatment A (TA) 9.48×10^{4} cfu mL^{-1}, treatment B (TB) 1.90×10^{5} cfu mL^{-1}, and treatment C (TC) 3.79×10^{5} cfu mL^{-1}. Kruskal-Wallis analysis was applied for comparison of treatments. No statistical differences between treatments were observed (p>0.05).

TSS and BV showed an increasing trend as shown in Fig. 2. Table 1 shows the mean and range of biofloc volume, total suspended solids, volatile suspended solids and non-volatile suspended solids for each treatment.

According to Kruskal-Wallis test, there was no significant difference between treatments in these parameters (p>0.05).

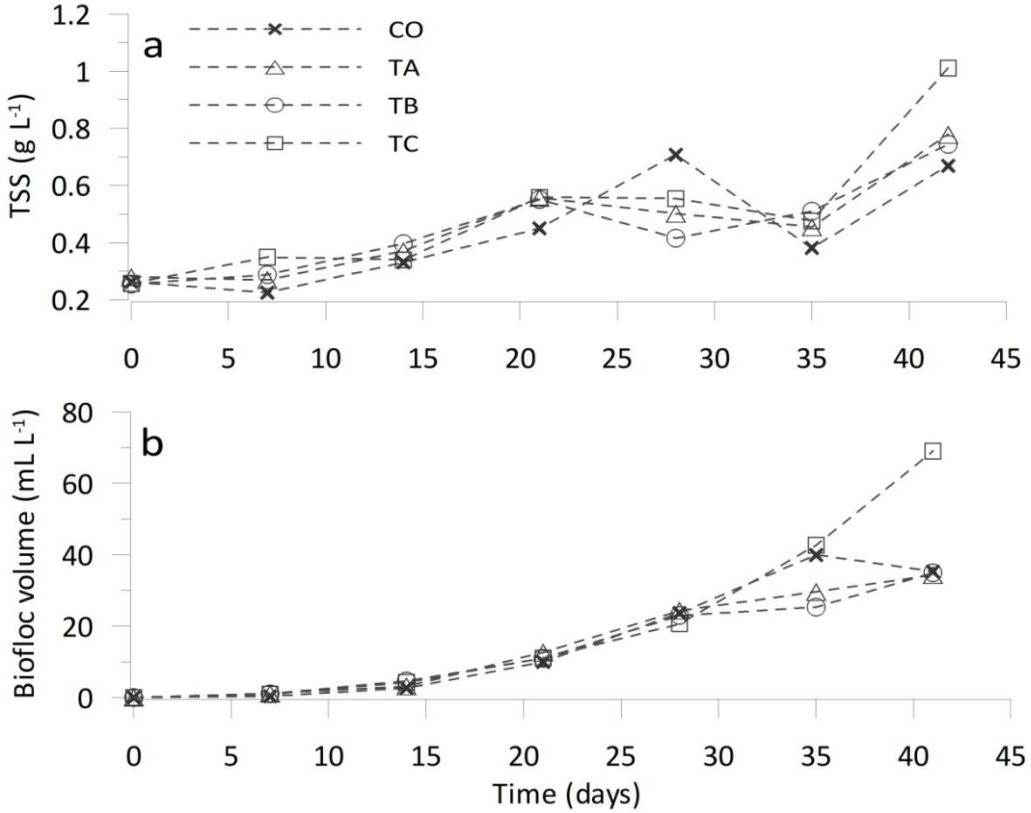

Figure 2: Evolution of total suspended solids and biofloc volume. Control treatment (CO) contains 0 cfu mL^{-1}, treatment A (TA) 9.48×10^4 cfu mL^{-1}, treatment B (TB) 1.90×10^5 cfu mL^{-1}, and treatment C (TC) 3.79×10^5 cfu mL^{-1}. Kruskal-Wallis analysis was applied for comparison of treatments. No statistical differences between treatments were observed (p>0.05).

Table 1: Values of biofloc volume (BV), total suspended solids (TSS), volatile suspended solids (VSS) and non-volatile suspended solids (NVSS) (mean and range). Control treatment (CO) contains 0 cfu mL^{-1}, treatment A (TA) 9.48×10^4 cfu mL^{-1}, treatment B (TB) 1.90×10^5 cfu mL^{-1}, and treatment C (TC) 3.79×10^5 cfu mL^{-1}.

	CO	TA	TB	TC
BV	16.0	15.0	14.3	21.2
(mL L^{-1})	(0.0 – 40.0)	(0.0 – 34.3)	(0.0 – 35.0)	(0.0 – 69.0)
TSS	0.4321	0.4585	0.4512	0.5064
(g L^{-1})	(0.2247 – 0.7073)	(0.2687 – 0.7780)	(0.2547 – 0.7440)	(0.2567 – 1.0113)
VSS	0.2193	0.2265	0.2164	0.1958
(g L^{-1})	(0.0694 – 0.4902)	(0.0947 – 0.4984)	(0.0977 – 0.4207)	(0.0609 – 0.5066)
NVSS	0.2088	0,2320	0.2348	0.3106
(g L^{-1})	(0.1358 – 0.3961)	(0.1591 – 0.3262)	(0.1214 – 0.3397)	(0.1742 – 0.5048)

Kruskal-Wallis analysis was applied for comparison of treatments. No statistical differences between treatments were observed (p>0.05).

Growth parameters

The results of survival, final weight, weight gain, weekly weight gain, biomass gain, FCR and productivity are presented in Table 2. According to the ANOVA analysis, there were no significant differences between treatments in growth parameters (p>0.05). At the end of the experiment, average shrimp weight ranged between 9.2 and 10.2 g, average survival was 99.30, 99.56, 98.45 and 96.21% in treatments CO, TA, TB and TC respectively, and average feed

conversion rate ranged between 1.2 (treatments CO, TA and TB) and 1.3 (treatment TC). During the experiment, the shrimp grew around 1.3 g per week and the average productivity was 2.315, 2.383, 2.055 and 2.118 g m^{-3} in treatments CO, TA, TB and TC, respectively.

Table 2: Probiotic effect on growth parameters as survival, final weight, weight gain, weekly weight gain (WWG), biomass gain (BG), feed conversion rate (FCR) and productivity (P). Control treatment (CO) contains 0 cfu mL^{-1}, treatment A (TA) 9.48×10^4 cfu mL^{-1}, treatment B (TB) 1.90×10^5 cfu mL^{-1}, and treatment C (TC) 3.79×10^5 cfu mL^{-1}. The table shows the average and standard deviation.

	CO	TA	TB	TC
Survival (%)	99.33 ± 1.15	99.56 ± 0.77	98.45 ± 1.93	96.21 ± 3.35
Final weight (g)	9.9 ± 0.5	10.1 ± 0.9	9.2 ± 1.2	10.2 ± 0.6
Weight gain (g)	8.1 ± 0.6	8. 3 ± 0.6	7.3 ± 1.1	7.8 ± 0.6
WWG (g)	1.3 ± 0.1	1.4 ± 0.1	1.2 ± 0.2	1.3 ± 0.1
BG (kg)	1.199 ± 0.083	1.235 ± 0.106	1.074 ± 0.170	1.111 ± 0.033
FCR	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	1.3 ± 0.1
P (kg m^{-3})	2.351 ± 0.166	2.383 ± 0.217	2.055 ± 0.328	2.118 ± 0.062

ANOVA analysis was applied for comparison of treatments. No statistical differences between treatments were observed (p>0.05).

Immunological system

The results of TPC, HG, HH and number of cells with apopotose are presented in Table 3. The TPC analysis indicated that TA, TB and TC had significantly higher levels of TPC in the hemolymph (128 and 124 mg mL^{-1}) than CO (104 mg mL^{-1}, respectively) (p<0.05) (Table 3). The percentage of GH was also significantly higher in TA, TB and TC shrimps (79, 81 and 77 % respectively) than CO (51 %) (p<0.05). Conversely, HH had significantly lower percentage in TA, TB and TC (21, 19, 23 %) than CO (49 %) (p<0.05). The number of cells with apoptose in the hemolymph was 3 in CO treatment which was significantly higher than in probiotic treatments (only 1 or 2 cells with apoptose) (p<0.05).

Table 3: Total protein concentration (TPC), percentage of granular hemocytes (GH), hyaline hemocytes (HH) and number of the cells with apoptose. Control treatment (CO) contains 0 cfu mL^{-1}, treatment A (TA) 9.48×10^4 cfu mL^{-1}, treatment B (TB) 1.90×10^5 cfu mL^{-1}, and treatment C (TC) 3.79×10^5 cfu mL^{-1}. The table shows the average and standard deviation.

	CO	TA	TB	TC
TPC (mg mL^{-1})	104 ± 7^a	128 ± 4^b	128 ± 4^b	124 ± 6^b
GH (%)	51 ± 7^a	79 ± 5^b	81 ± 5^b	77 ± 5^b
HH (%)	49 ± 7^a	21 ± 5^b	19 ± 5^b	23 ± 5^b
Cell number with apoptose	3 ± 1^a	1 ± 1^b	1 ± 1^b	2 ± 1^b

Means with the same letter in the row are not significantly different as showed by ANOVA analysis (p<0.05).

Discussion

The physical parameters such as temperature, dissolved oxygen, pH, salinity and alkalinity were maintained during all the study period at the optimum value for shrimp cultivation

(Van Wyk and Scarpa, 1999). The levels of N-TA and $N\text{-}NO_2^-$ were maintained within the limits of safety determined by Li and Chen (2001 and 2003). For this reason it can be stated that the water quality was at optimal values for white shrimp production. Concentrations of N-TA, $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ in all the treatments during the experiment followed the dynamic observed by Avnimelech (2009). During the first two weeks, the N-TA was accumulated in the system. The N-TA peak was replaced by a second peak of $N\text{-}NO_2^-$ when the oxidation processes by the heterotrophic bacteria began. Two weeks later, nitrification was completed, the $N\text{-}NO_2^-$ peak disappeared and an accumulation of $N\text{-}NO_3^-$ was observed in the system during the rest of the experiment. Some authors have observed that probiotics are able to eliminate nitrogen compounds from traditional aquaculture systems, helping to maintain water quality (Rengpipat *et al.*, 1998; Vaseeharan and Ramasamy, 2003; Balcázar *et al.*, 2007). However, in the biofloc system of our study, no significant differences in water quality were observed between the control treatment and the treatments with *B. amyloliquefaciens*. This may be due to the high efficiency of the heterotrophic bacteria of the BFT in the elimination of nitrogen compounds. Due to the high transformation rate by heterotrophic bacteria, the addition of probiotic bacteria does not produce a significant enhancement in this process. Other authors observed no improvement in water quality with the addition of probiotics applied in water (Vita, 2008; Souza *et al.*, 2012; Krummenauer *et al.*, 2014), but no studies had been done with *B. amyloliquefaciens* dissolved in water.

TSS and BV were inside the range recommended by Avnimelech (2009) and Ray *et al.* (2010). It has been demonstrated that *B. amyloliquefaciens* shows a high growth rate in vitro (Bao, 2014). The average value of total bacteria in a BFT according to Emerenciano *et al.* (2012) and Kim *et al.* (2014) is of the order of 10^7 cfu mL^{-1}. However, the daily addition of 9.48×10^4, 1.90×10^5 or 3.79×10^5 cfu mL^{-1} of *B. amyloliquefaciens* in the BFT did not produce an increase in suspended solids, neither in weight or in volume (Table 3). This indicates that the probiotic bacteria did not colonized water.

In this experiment, survival rate and growth of shrimp were those characteristic of BFT (Krummenauer *et al.*, 2011; Baloi *et al.*, 2013). Our results showed no improvement in the probiotic treatments as compared to the control. Other authors observed the benefitial effects of *B. amyloliquefaciens* probiotics in recirculation systems (Nuez-Ortín, 2013), which include improved survival rates, weekly gains in weight and FCR. The nutritional benefits of BFT as compared to recirculation systems have been already studied (Crab *et al.*, 2012; Emerenciano *et al.*, 2013). It seems that the addition of *B. amyloliquefaciens* did not produce a significant enhancement of the BFT benefits. However, other authors observed growth benefits in

BFT with other probiotic bacteria appplied in feeds (Vita, 2008; Souza *et al.*, 2012). Then, the positive effect of probiotics on growth parameters in BFT must not be disregarded, but should be better studied to unveil the specific role of each species.

The TPC levels in the hemolymph obtained were within the range detected by Cheng *et al.* (2002), Li *et al.* (2008), Macias-Sancho *et al.* (2014) and Souza *et al.* (2016) in white shrimp. Our results showed that *B. amyloliquefaciens* increased the TPC of the hemolymph (TA, TB and TC were higher than CO). Previous studies in BFT with other probiotics did not show this effect on shrimp (Souza *et al.*, 2012). The proteins in the hemolymph are the mechanism of the shrimp to identify pathogens and their morphology, furthermore the proteins regulate the union of pathogens with the hemocytes (Johansson *et al.*, 1999) and the phagocytosis capacity of hemocytes (Wang and Zhang, 2008). Then, an increase in TPC is key for an enhanced immunological system.

The results of our experiment showed that all treatments had a high percentage of GH to the detriment of HH, similar to that observed by Macias-Sancho *et al.* (2014) and Souza *et al.* (2016) in white shrimp, cultivated in biofloc systems. The percentage of GH was significantly higher in the treatments with *B. amyloliquefaciens* (TA, TB and TC) than in the control (CO). The effect of *B. amyloliquefaciens* on the percentage of GH, has already been observed by Camacho (2012) in a recirculation system. However, other probiotics tested in shrimp in BFT did not produce this effect (Souza *et al.*, 2012). The higher percentage of GH increases the response capacity against pathogens (Xu and Pan, 2013). The GH have different ways to counter pathogens, such as phagocytosis, encapsulation, cytotoxicity, storage and release into the proenoloxidase system; while the hyaline hemocytes only can fight against pathogens through phagocytosis (Johansson *et al.*, 2000).

The number of cells with apoptose observed in our control treatment was similar to thate observed by Macias-Sancho *et al.* (2014) in white shrimp cultivated in biofloc system. But, in our experiment the number of cells with apoptose was significantly lower for all tested doses (TA, TB and TC) than in the control. Apoptose, also known as programmed cell death, is a mechanism that normally occurs in cells of all tissues in normal physiological situations. In pathological situations, apoptose is produced to avoid the replication or dispersion of pathogens which are fundamentally viruses (Everett and McFadden, 1999). This mechanism is used by shrimp to avoid the replication of white spot syndrome virus (Khanobdee *et al.*, 2002) and the yellow head virus among others (Wongprasert *et al.*, 2003). The smaller number of cells with apoptosis in treatments with probiotic is related with an increase in the percentage of GH and TPC. That means that *B. amyloliquefaciens* reinforces the immune system so it can fight pathogens through other mechanisms

without resorting to cell death. (Khanobdee *et al.*, 2002; Wongprasert *et al.*, 2003; Wang and Zhang, 2008).

To conclude, the application of *B. amyloliquefaciens* dissolved in water in a biofloc system strengthened the immune system of shrimp: increased the percentage of granular hemocytes and the concentration of total protein in the hemolymph, and decreased the number of cells with apoptosis. Thus, *B. amyloliquefaciens* improved the ability of detection and performance of the immune system to combat pathogens. Its application can be very useful in the prevention of diseases in shrimp farming. No positive effect of *B. amyloliquefaciens* on growth parameters in BFT was observed, but it must not be disregarded if applied combined with other probiotic species. Future studies should better study effects on shrimp growth to unveil the specific role of each species, as well as the minimal dose for observing effects.

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