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The Role of *Bacillus amyloliquefaciens* on *Litopenaeus vannamei* During the Maturation of a Biofloc System

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Abstract: Biofloc technology is a sustainable aquaculture production system which uses microorganisms to maintain water quality and to increase productivity. In this system, probiotics can enhance the positive effects of bioflocs on the cultured species. The objective of this research is to study the role of the probiotic bacterium *Bacillus amyloliquefaciens* during the formation of a biofloc system for the culture of *Litopenaeus vannamei*. Two doses of probiotic were assayed and applied directly to the water. The experiment was developed in nine tanks distributed as follows: Three control tanks with no probiotic, three tanks with a probiotic dose of 10^3 cfu/mL, and three tanks with a dose of 10^4 cfu/mL. Water quality, microbial activity, growth parameters and the immune system state of shrimps were monitored throughout the maturation process. The results indicate a positive effect upon the shrimp immune system throughout the study period, where specifically there was an increase in granular hemocytes in the shrimp hemolymph. During the immature biofloc phase, granular hemocytes were 5% higher in tanks supplemented with the probiotic. During the mature biofloc phase, granular hemocytes were 7% higher in those same tanks. During the maturation of the biofloc, environmental conditions are more unfavorable for shrimp growth, due to the accumulation of nitrites. So, the effect of the probiotic is especially important during this stage when the shrimp are stressed and are more vulnerable to diseases. However, the effects on microbial activity, water quality and *Litopenaeus vannamei* growth did not increase the benefits of the biofloc system.

Keywords: growth parameters; immune system; microbiological activity; probiotic; shrimp; water quality; white shrimp

1. Introduction

Biofloc technology (BFT) is considered a sustainable aquaculture production system because it reduces water renewal needs thanks to the activity of microorganisms [1,2]. In BFT systems, we can find different microorganisms, such as bacteria, phytoplankton, rotifers, protozoans and copepods [3], which interact with the nutrients and organic matter present in water and form bioflocs [3,4]. The bioflocs are formed because the organic matter (unconsumed feed, dead organisms, old exoskeletons of shrimp, etc.) and the microorganisms (bacteria, phytoplankton, rotifers, etc.) tend to agglutinate. Bacteria are the most important microorganisms for the maintenance of water quality because they eliminate the majority of nitrogen compounds present in the water column [1,3,4]. The most abundant bacteria in aquaculture systems are heterotrophic and autotrophic bacteria [1–3].

On the one hand, heterotrophic bacteria remove nitrogen compounds dissolved in water more efficiently than autotrophic bacteria [1]. Nitrogen compounds are converted into microbial protein

which can be consumed by the cultured species, such as shrimps [1–3]. On the other hand, autotrophic bacteria oxidize nitrogen compounds [1–3]. The bacteria are responsible for the maturation process, because they are in charge of controlling the nitrogen compounds at safe levels for shrimp [5,6]. The maturation process of BFT systems has two stages. First, the immature state is characterized by no bacteria community and the accumulation of ammonium and nitrite. Second, when the bacteria community develops, the system is mature, and ammonium and nitrite are oxidized quickly. In this later stage nitrate accumulates in the water [5,6]. Elimination of the nitrogen compounds, fundamentally ammonia and nitrite, is the main reason why BFT systems have been considered as an environmentally friendly aquaculture technique, because the heterotrophic bacteria maintain these nutrients under low concentration [1]. In addition, these bacteria compete for resources and space with pathogens, and have a probiotic effect upon shrimps. Probiotics are any live microorganisms that, when administered in adequate amounts, confer a health benefit upon the host [7]. BFT systems, also allow increasing the farming density, being more productive than traditional aquaculture systems [4].

Probiotics are live microorganisms, which may reduce the probability of some pathogens to affect the shrimps [8]. Currently, some farmers frequently apply medicines and antibiotics as preventive treatments [8] with no dose assays, which can end in overdoses. The use of probiotics can be an alternative to preventive medicine [8]. The abundance of heterotrophic bacteria in BFT systems decreases the probability of pathogens proliferation and it affects positively the immune system of the shrimps [3,9]. The addition of probiotics are usually used preventively [8] or for the treatment of pathogens [10], and it can help the cultured species to better resist the stress produced by adverse conditions, such as those produced during the maturation of biofloc systems [3,5,11]. Probiotics are characterized by distinctive action mechanisms [8]: (1) Enzyme production in the host intestine, which increases the digestibility of the food and improves the growth of the host [12,13]; (2) the production of bactericidal compounds inside the host [14]; (3) a stimulation of the host immune system, which makes it more resistant to pathogens [15,16]; (4) competition with the rest of the bacteria for resources, which can diminish the presence of pathogens [17,18]; and (5) a maintenance of water quality; probiotics consume nitrogen compounds, such as nitrites and ammonia, which are toxic to cultured species [19,20]. In cultured shrimp, the dose of the probiotic dissolved in water depends on the species and culture conditions. Doses from 10^3 to 10^8 cfu mL^{-1} are the most usual [8,9,12,21].

Biofloc technology is being developed mostly in farms in North America, South America and Asia [1], where there is an important tradition of shrimp farming. In Europe, this technology has started to develop recently. One of the main disadvantages of shrimp farms in the European Union (EU) is the limited choice of any products marketed, specifically to develop the aquaculture activity, such as feed and probiotics. The low number of BFT shrimp farms in this region has discouraged European manufacturers. Moreover, importing these products from other world regions such as America or Asia is difficult. European Union legislation is strict, especially for probiotics, because they are live microorganisms, and their importation requires different controls and certificates to avoid introducing foreign diseases. This demands expensive administrative procedures, which are not profitable for distribution companies, due to the small sales volume in the European Union.

The probiotic Ecobiol Plus® is composed of the bacterium *Bacillus amyloliquefaciens*, and it is marketed in the European Union, to be used in chickens and pigs [22–25]. Different experiences have shown that it also has positive effects in aquaculture water recirculation systems [13,26–29]. However, BFT aquaculture systems conditions are strongly different, and the Ecobiol Plus® has not been tested during the maturation of a biofloc system.

The objective of this research is to study the role of the probiotic bacterium *B. amyloliquefaciens* during the maturation process of a biofloc system. We studied its effect on the biofloc system (water quality dynamics, trophic state development and microbial activity evolution), and on cultured shrimps (growth parameters and the immune system).

2. Materials and Methods

2.1. Location and Shrimp Culture System

The experiment was developed in the Universitat Politècnica de València facilities (Grau de Gandia, Spain), during 169 days, between 11 May (end of spring) and 16 October (early autumn) 2016. Postlarvae white shrimp (PL) were purchased from a commercial laboratory (Shrimp Improvement Systems, FL, USA), and they were certificated as free of pathogens. This certificate guarantees that the shrimp are not infected by the principal diseases as Taura syndrome, white spot disease and yellow head disease, among others [30,31]. PLs of 0.07 ± 0.04 g weight were distributed within nine square tanks filled with 2250 L of water and with a surface area of 3.2 m^2 for each tank. The shrimp density was 200 individuals/m^2. Each tank was filled with a mixture of seawater and freshwater which had a salinity level of 22.5 g/L. The water was disinfected with 10 mg/L of chlorine, which was subsequently eliminated by adding 1 mg/L of ascorbic acid to the tanks [10]. The tanks were located in a greenhouse and were constantly individually aerated. Every day the shrimp were fed with commercial feed (Le Gouessant) specifically designed for *L. vannamei*. Fortnightly, this feed amount was calculated according to the shrimp estimated biomass (calculated each 15 days) and the daily water temperature, according to Jory et al. [31]. Feeding was provided twice a day, 40% in the morning and 60% in the afternoon, and it was placed into feed trays.

The following water quality parameters were monitored: Salinity, temperature, pH, alkalinity, dissolved oxygen, nutrients and total suspended solids (TSS). Dissolved oxygen (DO), salinity and temperature were monitored in situ, using two parameter probes (YSI ProODO and WTW Multi 340i respectively), twice a day. The pH was measured once a day using pHMeter BASIC 20+, the Crison. Alkalinity was analyzed after the first month of culture, every two weeks, by titration with hydrochloric acid [31]. Every two days an aliquot of water was collected to determine the concentration of the total dissolved ammonia (N-TA mg/L) using the methodology described by Baumgarten et al. [32], nitrites (N-NO_2^- mg/L), using the methodology of Bendschneider and Robinson described in Baumgarten et al. [32] and the nitrates (N-NO_3^- mg/L) were analyzed following the methodology described by Grasshof [33]. In addtion, phosphates (P-PO_4^{3-} mg/L) were analyzed following the colorimetric reaction described by Murphy and Riley [34]. The TSS was analyzed once a week as described by Baumgarten et al. [32].

Due to evaporation, salinity can increase above to 22.5 g/L, so in this case freshwater was added to keep salinity under this threshold. Different shade cloths were used to control the greenhouse temperature, in order to keep the water temperature within the optimal range for shrimp culture according to Van Wyk and Scarpa [35]. At the beginning of the study period (5 May) the greenhouse roof was covered with a white shade cloth. On day 59 (8 July), the shade cloth was substituted by a black one. Finally, on day 136 (23 September), the black shade cloth was removed, due to a lower environmental temperature at the end of summer and the beginning of autumn. The evolution of maximum and minimum daily environmental temperature was recorded from the data of the weather station of the Reial Club Nàutic de Gandia [36]. The pH was maintained between 7 and 9 (slightly alkali) [35]. We added 0.15 mg/L of calcium hydroxide when pH values below 7.50 were detected [37]. Alkalinity was maintained above 50 mg $CaCO_3$/L [34]. Also, we added 0.20 g/L of sodium bicarbonate when alkalinity below 120 mg $CaCO_3$/L was observed [38].

The initial fertilization of the system was done with sucrose (15 mg/L of carbon), to facilitate the initial development of heterotrophic bacteria. During the experiment, sucrose was added, in a ratio C:N-TA of 15:1, when the ammonia, produced by the shrimps and organic matter, reached a concentration greater than 1 mg/L [2,39]. Eventual renewal of the water during the experiment was minimal, and was performed when the nitrite level reached 15 mg/L, in order to avoid the toxic effects of nitrites [40]. When we detected a toxic concentration of nitrite (higher than 15 mg/L) in a tank, we renewed between 10% and 15% of the water (between days 56 and 80). At the end of experimental

period the total renewal water was 25% in all of the tanks. Between days 35 and 100, the feed was reduced by 40% in order to minimize the contribution of nitrogen to the system.

2.2. Probiotic Treatments

During the experiment, the probiotic Ecobiol Plus® was used, with a certified content by the manufacturer of 1.3×10^{10} cfu/g of viable spores of *B. amyloliquefaciens* in powder form. The experiment was developed in nine tanks distributed as follows: Three control tanks with no probiotic (control treatment), three tanks with a probiotic dose of 10^3 cfu/mL (low bacteria treatment) and three tanks with a dose of 10^4 cfu/mL (high bacteria treatment). The probiotic was applied directly to the water every day.

2.3. Chlorophyll a and Microbial Activity

To determine chlorophyll *a* (Chl*a*), an aliquot of water was filtered on a glass fiber filter (25 mm Ø) and ultra-frozen (−86 °C). Subsequentl the pigments were extracted with acetone high-performance liquid chromatography (HPLC) grade. Chlorophyll *a* was measured by HPLC, according to the method of Wright et al. [41], modified by Hooker et al. [42]. Microbial activity was analyzed once a week during the whole experiment. The method of dark and transparent bottles of Strickland adapted by Schveitzer et al. [43] was used. Six bottles of 100 mL (3 dark and 3 transparent) were placed in each tank five centimeters below the water surface. To keep the bioflocs in suspension inside the bottles, they were shaken manually every 20 min, following the methodology of Schveitzer et al. [43]. The bottles were incubated for approximately 8 h. Dissolved oxygen was measured at the beginning and end of each incubation. Water column respiration (WCR), net primary productivity (NPP) and gross primary productivity (GPP) were calculated using Equations (1)–(3) following Dodds and Cole [44].

$$\text{WCR}\ (\text{mg}\ O_2/(\text{L}{\cdot}\text{h})) = \frac{\text{initial}\ O_2\ \text{of dark bottle} - \text{final}\ O_2\ \text{of dark bottle}}{\text{time}\ (\text{h})} \quad (1)$$

$$\text{NPP}\ (\text{mg}\ O_2/(\text{L}{\cdot}\text{h})) = \frac{\text{final}\ O_2\ \text{of light bottle} - \text{initial}\ O_2\ \text{of light bottle}}{\text{time}\ (\text{h})} \quad (2)$$

$$\text{GPP}\ (\text{mg}\ O_2/(\text{L}{\cdot}\text{h})) = \text{WCR} + \text{NPP} \quad (3)$$

2.4. Growth Parameters

An initial biometry was performed on 100 PLs with a balance (Kern ABT 220-4M; ±0.0001). Biometry of 30 shrimps per tank was done every two weeks with a balance (Kern EW600-2M; ±0.01) to monitor growth and adjust the dose of feed required. At the end of the experiment, the number of shrimps was counted, and 50 shrimps per tank were weighed. We calculated weight gain, weekly weight gain, final biomass, biomass increase, feed conversion rate (FCR) and survival with the following equations (Equations (4)–(9)).

$$\text{Weight gain}\ (\text{g}) = \text{final wet weight} - \text{initial wet weight} \quad (4)$$

$$\text{Weekly weight gain}\ (\text{g/week}) = \frac{\text{weight gain}}{\text{number of weeks}} \quad (5)$$

$$\text{Biomass}\left(\frac{\text{g}}{\text{m}^2}\right) = \frac{\text{wet weight shrimp} \times \text{number of shrimp}}{\text{m}^2} \quad (6)$$

$$\text{Biomass gain}\left(\text{g}/\text{m}^2\right) = \text{final biomass} - \text{initial biomass} \quad (7)$$

$$\text{Feed conversion rate} = \frac{\text{dry feed consumption}}{\text{weight gain}} \quad (8)$$

$$\text{Survival } (\%) = \frac{\text{final shrimp amount}}{\text{initial shrimp amount}} \times 100 \quad (9)$$

2.5. Immune Parameters

The state of the shrimp immune system was determined in the middle of the experiment (day 86), when the system was immature, and at the end of the experiment (day 169), when the system had completed its maturation process. For the analysis of the immune system, hemolymph was collected from five shrimps per tank with a sterile syringe BD Plastipak®, which is the minimum number of samples recommended for this analysis (three to eight shrimp per tank) [45–49]. The sample was divided into two aliquots. The first aliquot of 20 µL was mixed with 80 µL of Alsever solution, to determine the percentage of granular (GH) and hyaline (HH) hemocytes (with an approximate diameter of 10–15 µm), using a Bürker chamber and a Leica DM 2500 microscope [50]. The second aliquot of 500 µL was allowed to coagulate for 2 h at 4 °C. Then it was centrifuged at 2000× *g* to extract the serum, which was frozen [50]. Subsequently, the total protein concentration (TPC) was analyzed using the method described by Lowry et al. [51].

2.6. Statistical Analysis

The statistical analysis was carried out using Statgraphics® Centurion XVI.I. First, the normality and homoscedasticity of all the parameters were analyzed. A univariate analysis was performed to detect significant differences according to the probiotic treatments (high bacteria, low bacteria or control) (Tables 1 and 2) in all of the variables studied. An Analysis of Variance (ANOVA) test was used for variables that followed a normal distribution, or were transformed to a normal distribution through a $\log_{10}$ or $\sqrt[2]{x}$ conversion (WCR, Chl*a*, growth parameters and immune system parameters). The non-parametric Kruskal-Wallis test was applied to the variables with non-normal distribution (nutrients, TSS, GPP and NPP). Also, the results of the immune system parameters were compared during the different stages of maturation of the Biofloc technology (BFT) system (Table 3). The results are expressed as the average ± standard deviation.

Table 1. Average and standard deviation of pH, alkalinity, salinity, dissolved oxygen concentration and dissolved oxygen saturation percentage for high bacteria treatment (2.8×10^4 cfu/mL), low bacteria treatment (2.8×10^3 cfu/mL) and control treatment. The *n* values are indicated for each treatment.

	High Bacteria Treatment	Low Bacteria Treatment	Control Treatment
pH	7.77 ± 0.39 (n = 450)	7.74 ± 0.43 (n = 450)	7.74 ± 0.40 (n = 450)
Alkalinity (mg $CaCO_3$/L)	108.89 ± 30.05 (n = 42)	96.70 ± 27.29 (n = 42)	101.56 ± 27.92 (n = 42)
Dissolved oxygen (mg/L)	5.88 ± 0.48 (n = 507)	5.97 ± 0.37 (n = 507)	5.95 ± 0.38 (n = 507)
Dissolved oxygen (%)	91.4 ± 8.38 (n = 507)	93.0 ± 6.66 (n = 507)	93.7 ± 7.73 (n = 507)
Salinity (g/L)	22.5 ± 0.08 (n = 498)	22.5 ± 0.08 (n = 498)	22.5 ± 0.07 (n = 498)

Table 2. Mean and standard deviation of the growth parameters for high bacteria treatment (2.8×10^4 cfu/mL), low bacteria treatment (2.8×10^3 cfu/mL) and control treatment. The *n* values are indicated for each treatment.

	High Bacteria Treatment	Low Bacteria Treatment	Control Treatment	*p*
Weight gain (g)	16.59 ± 1.15 (*n* = 3)	17.41 ± 1.42 (*n* = 3)	17.76 ± 0.65 (*n* = 3)	0.33
Weekly weight gain (g/week)	0.69 ± 0.01 (*n* = 3)	0.72 ± 0.06 (*n* = 3)	0.73 ± 0.03 (*n* = 3)	0.33
Biomass production (ton/m^2)	21.74 ± 1.83 (*n* = 3)	23.00 ± 2.67 (*n* = 3)	21.07 ± 2.03 (*n* = 3)	0.58
FCR	1.91 ± 0.11 (*n* = 3)	1.81 ± 0.15 (*n* = 3)	1.96 ± 0.21 (*n* = 3)	0.53
Survival (%)	65.77 ± 4.97 (*n* = 3)	66.19 ± 3.48 (*n* = 3)	59.53 ± 4.92 (*n* = 3)	0.21

Table 3. Average and standard deviation of immune system parameters (total protein concentration (TPC), granular hemocytes (GH) and hyaline hemocytes (HH)) in different stages of Biofloc technology (BFT) evolution (immature and mature BFT) for high bacteria treatment (2.8×10^4 cfu/mL), low bacteria treatment (2.8×10^3 cfu/mL) and control treatment. The *n* values are indicated for each treatment.

Immature Biofloc System (day 86)			
	High Bacteria Treatment	**Low Bacteria Treatment**	**Control Treatment**
TPC (mg/mL)	93.18 ± 3.99 [a] (*n* = 3)	89.49 ± 21.16 [a] (*n* = 3)	82.58 ± 10.10 [a] (*n* = 3)
GH (%)	30.00 ± 1.40 [a] (*n* = 3)	31.33 ± 3.58 [a] (*n* = 3)	25.13 ± 0.99 [b] (*n* = 3)
HH (%)	70.00 ± 1.40 [a] (*n* = 3)	68.67 ± 3.58 [a] (*n* = 3)	74.87 ± 0.99 [b] (*n* = 3)
Mature Biofloc System (day 169)			
	High Bacteria Treatment	**Low Bacteria Treatment**	**Control Treatment**
TPC (mg/mL)	123.14 ± 8.82 [a] (*n* = 3)	120.62 ± 12.26 [a] (*n* = 3)	117.75 ± 25.60 [a] (*n* = 3)
GH (%)	46.66 ± 2.80 [a] (*n* = 3)	46.28 ± 2.89 [a] (*n* = 3)	39.03 ± 3.89 [b] (*n* = 3)
HH (%)	53.34 ± 2.80 [a] (*n* = 3)	53.72 ± 2.89 [a] (*n* = 3)	60.97 ± 2.89 [b] (*n* = 3)

[a] and [b] superscript indicate Analysis of Variance (ANOVA) test results. Those treatments that do not share the same letter show statistically significant differences for the variable shown ($p < 0.05$).

3. Results

3.1. Water Quality

Average water temperature, as well as minimum and maximum ambient temperature values are shown along the study period (Figure 1). The change in water temperature was due to the ambient temperature variation. The water temperature ranged between an average maximum temperature of 31.0 °C (day 35) and a minimum of 22.5 °C (day 165). The greenhouse shade cloths were handled to avoid excessively high temperatures. Table 1 shows pH, alkalinity, salinity and dissolved oxygen (concentration and saturation percentage) average values and the standard deviation for each treatment. The average of pH and alkalinity was around 7.75 and 102.38 mg $CaCO_3$/L, respectively. Dissolved oxygen and salinity were stable with an average around of 5.93 mg/L and 22.5 g/L, respectively.

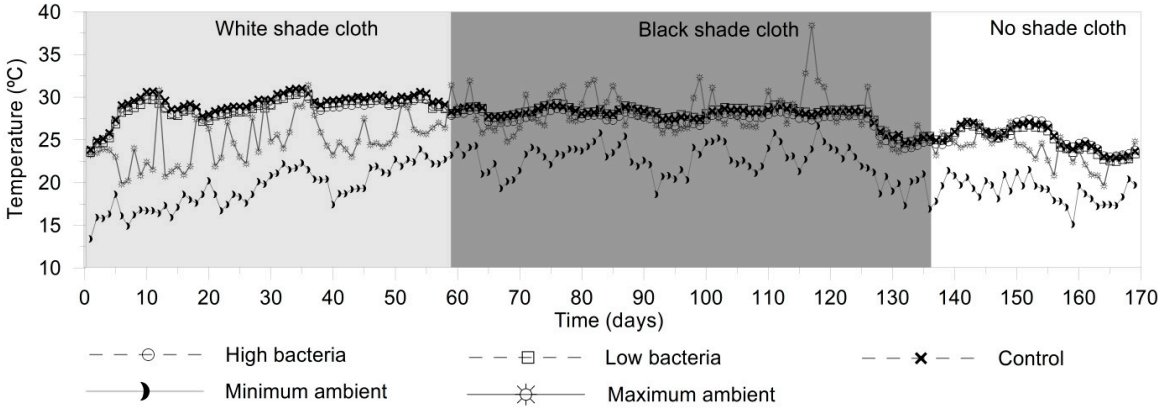

Figure 1. Evolution of water temperature, minimum and maximum ambient temperature values are shown along the study period. Each data represents the average of the three experimental units for high bacteria treatment (2.8×10^4 cfu/mL), low bacteria treatment (2.8×10^3 cfu/mL) and control treatment.

In water, N-TA was accumulated during the first 45 days (Figure 2a), registering maximum values of 1.44 mg/L in the low bacteria treatment and 0.89 mg/L in the high bacteria treatment and control treatment. $N\text{-}NO_2^-$ remained at very low values until day 35, when it began to increase until reaching a maximum of 19.08 mg/L in the high bacteria treatment, 20.39 mg/L in the low bacteria treatment and 21.10 mg/L in our control treatment. Around day 100, the concentrations of $N\text{-}NO_2^-$ were progressively reduced, reaching very low levels since day 118 (Figure 2b). $N\text{-}NO_3^-$ was first detected on day 90, and increased progressively reaching its maximum value at the end of the culture (Figure 2c), when concentrations of 65.18, 77.72 and 74.54 mg/L were measured in high bacteria, low bacteria and control treatments, respectively. $P\text{-}PO_4^{3-}$ was accumulated in water during the entire culture (Figure 2d). The highest values were observed at the end of the culture, being 17.91, 15.34 and 13.45 mg/L in high bacteria, low bacteria and control treatment, respectively. No statistically different levels of nutrients were observed between treatments (N-TA, $p = 0.11$; $N\text{-}NO_2^-$, $p = 0.06$; $N\text{-}NO_3^-$, $p = 0.53$; $P\text{-}PO_4^{3-}$, $p = 0.95$). However, a trend close to significance ($p = 0.06$) was observed for $N\text{-}NO_2^-$ with higher values in the control treatment.

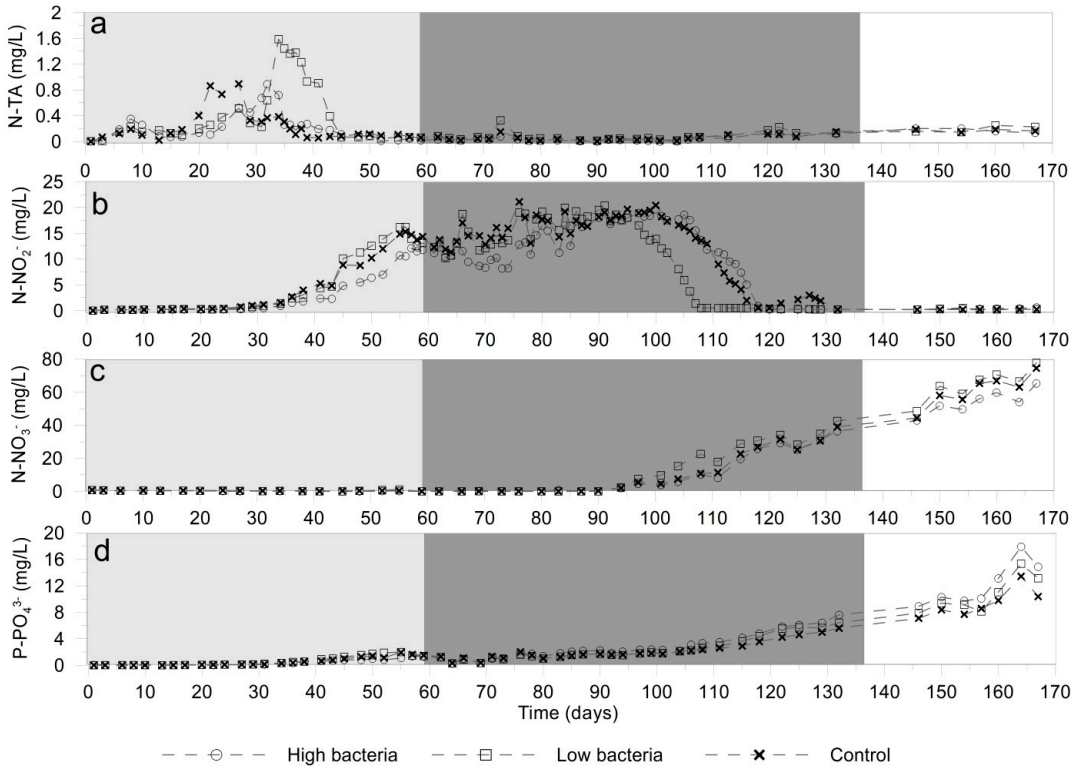

Figure 2. Evolution of nutrients, (**a**) total dissolved ammonia, (**b**) nitrites, (**c**) nitrates and (**d**) phosphates. Each data represents the average of the three experimental units for high bacteria treatment (2.8×10^4 cfu/mL), low bacteria treatment (2.8×10^3 cfu/mL) and control treatment. Between days 56 and 80 approximately 10 to 15% of water was renewed to avoid any toxic concentration of nitrite.

TSS was accumulated in the system during the culture period (Figure 3). The maximum average values obtained were 1.19, 0.73 and 0.67 g/L in the high bacteria, low bacteria and control treatments, respectively. Between day 60 and day 130, small decreases were observed in TSS concentration, which were produced due to water renewal, to control the $N\text{-}NO_2^-$ levels in the system.

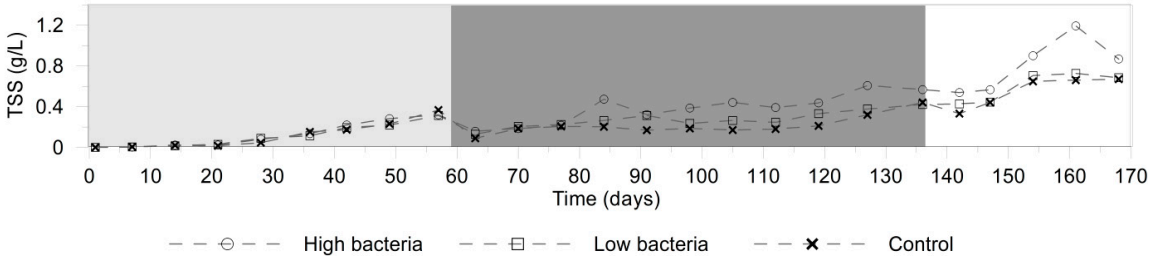

Figure 3. Evolution of total solid suspends. Each data represents the average of the three experimental units for high bacteria treatment (2.8×10^4 cfu/mL), low bacteria treatment (2.8×10^3 cfu/mL) and control treatment.

3.2. Microbial Activity

GPP was maximum during the first culture phase, where values of 1.15, 0.93 and 0.74 mg O_2/(L·h) were reached in high bacteria, low bacteria and control treatments, respectively; after day 58 it remained below 0.5 mg O_2/(L·h) in all treatments (Figure 4a).

WCR increased during the first weeks until day 35, and then remained stable throughout the experiment, reaching its maximum on day 135, with values of 0.44, 0.45 and 0.51 mg O_2/(L·h) in high

bacteria, low bacteria and control treatments, respectively (Figure 4b). The NPP was positive during the first weeks, observing a maximum of 0.82, 0.72 and 0.61 mg O_2/(L·h) in high bacteria, low bacteria and control treatments, respectively. On day 58, the first NPP negative values were recorded, which slowly decreased until the end of the experiment (Figure 4c). No statistically significant differences were observed between the different treatments for NPP (p = 0.55), GPP (p = 0.29) nor WCR (p = 0.30). Chl*a* (Figure 4d) was below the detection limit in the first two weeks, increasing later to reach a maximum of 573.82, 460.16 and 703.58 µg/L in high bacteria, low bacteria and control treatments, respectively (between days 38 and 52). When the greenhouse was covered with the black shade cloth the concentration of Chl*a* was stabilized around 150 µg/L. When the black shade cloth was removed, day 136, a second Chl*a* rise was observed. There were no statistically significant differences between treatments (p = 0.07) for this variable.

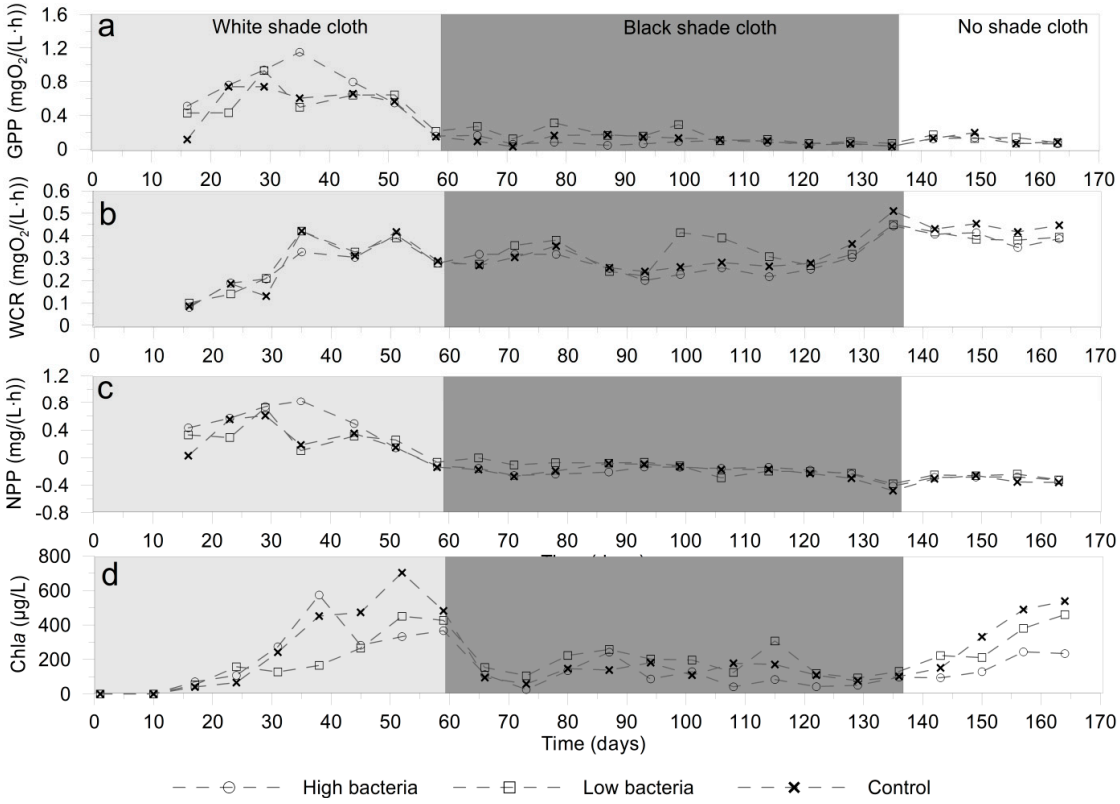

Figure 4. Evolution of microbial activity, (**a**) gross primary production, (**b**) water column respiration, (**c**) net primary production and (**d**) chlorophyll *a*. Each data represents the average of the three experimental units for high bacteria treatment (2.8×10^4 cfu/mL), low bacteria treatment (2.8×10^3 cfu/mL) and control treatment.

3.3. Growth Parameters

Shrimp growth was equal for all of the treatments (Figure 5). At the beginning of the experiment the shrimps weighed 0.0675 ± 0.0433 g (initial biomass 13.5074 g/m^2). They grew up to 16.76 ± 0.15, 17.58 ± 1.41 and 17.94 ± 0.65 g in high bacteria, low bacteria and control treatments, respectively.

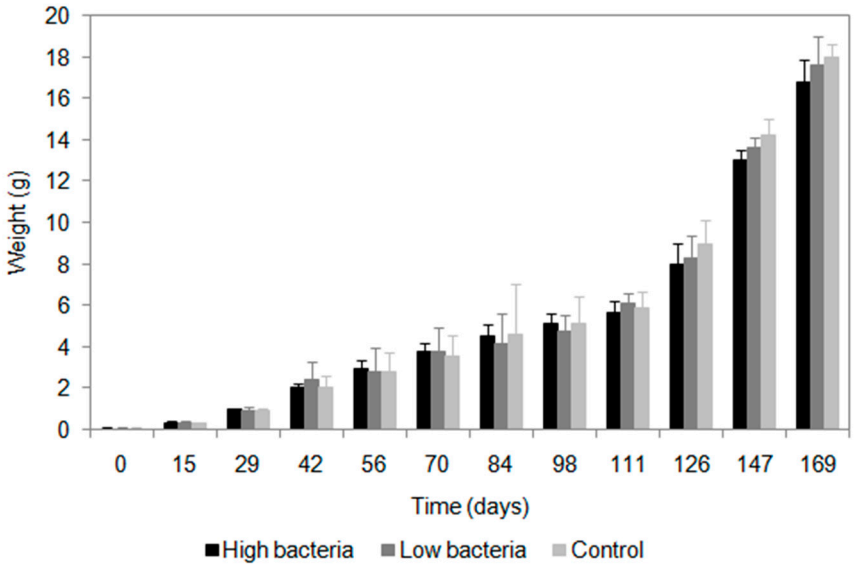

Figure 5. The shrimp weight gain. Each bar represents the average of the three experimental units for the high bacteria treatment (2.8×10^4 cfu/mL), low bacteria treatment (2.8×10^3 cfu/mL) and control treatment.

Weight gain, weekly weight gain, biomass gain, FCR and survival results are included in Table 2. ANOVA showed no statistically significant differences between the different treatments in these growth parameters.

3.4. Immune System Parameters

On day 86, within the immature system, our TPC was around 80-90 mg/mL in the shrimp hemolymph. Statistical analysis showed no significant differences between treatments ($p = 0.65$) (Table 3). GH percentage was around 30 and HH was around 70% in the treatments with *B. amyloliquefaciens*. In the control treatment values were close to 25 and 75% for GH and HH, respectively. This difference (5%) between control and treatments with the probiotic is statistically significant ($p = 0.04$).

On day 169, within the mature system, TPC in the hemolymph was around 120 mg/mL in all treatments ($p = 0.93$). The GH percentage was around 46 and the HH was around 54% in the treatments with *B. amyloliquefaciens*. In the control treatment, values were close to 39 and 61% for GH and HH, respectively. This difference (7%) between the control and treatments with the probiotic is statistically significant ($p = 0.04$).

4. Discussion

4.1. Effects on the Biofloc System

The experiment was carried out between spring and autumn in a Mediterranean climate area. In temperate climates, greenhouses are a good strategy to maintain water temperature, facilitating shrimp culture during the coldest months [52–54]. During the experiment the high environmental temperatures forced us to use a white shade cloth in spring and a black shade cloth in summer for temperature control inside the greenhouse. At the end of the experiment (autumn), the shade cloths were removed to avoid water cooling. Using different shade cloths to control the effect of environmental temperatures inside the greenhouse is usual [55], and thanks to them the water temperature was maintained within the adequate range for the cultivation of *L. vannamei* [35] in this experiment.

Dissolved oxygen remained above 5 mg/L and 85% saturation throughout the experiment, which are the recommended values by Cheng et al. [56]. Both the pH and the alkalinity decreased throughout the experiment, and this trend is common in BFT, due to the high metabolic rate of bacteria present in

the system [39]. In spite of the fact that low alkalinity can negatively affect the nitrification process [57], the lowest levels were observed at the end of the experiment, just when the system was mature. In this moment, TAN and nitrites did not accumulate in the water column. This suggests that alkalinity did not affect the nitrification processes. Moreover, the addition of sodium bicarbonate and calcium hydroxide helped to maintain pH and alkalinity respectively within the optimal values determined by Van Wyk and Scarpa [35].

The nitrogen compounds (N-TA, $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$) followed the usual evolution in BFT described by Avnimelech [2]. At the beginning of the experiment, N-TA was accumulated, which is typical of the immature BFT phase. When the bacterial nitrification started, N-TA was oxidized to $N\text{-}NO_2^-$, and then N-TA descended and an $N\text{-}NO_2^-$ peak appeared. Since day 115, the peak of $N\text{-}NO_2^-$ disappeared and $N\text{-}NO_3^-$ was accumulated in water, indicating a mature BFT characterized by low levels of N-TA and $N\text{-}NO_2^-$ [5]. N-TA did not exceed the safety level recommended by Lin and Chen [58] for the cultivation of *L. vannamei*. On the contrary, the $N\text{-}NO_2^-$ levels did exceed safety limits. According to Lin and Chen [40], exceeding the safety level (15 mg/L $N\text{-}NO_2^-$) does not cause shrimp mortality, but prolonged exposure to high levels could cause it. In this experiment, the 15 mg/L limit of $N\text{-}NO_2^-$ was exceeded for approximately 30–40 days, with a maximum value of 21.10 mg/L. Xie et al. [27] observed under laboratory conditions, that germinated *B. amyloliquefaciens* could eliminate up to 10 mg/L of $N\text{-}NO_2^-$ in 24 hours. Previous studies with mature biofloc systems did not find significant nitrite reductions, when the probiotic was applied directly to the water column. In this research we studied the effect during the maturation process of the biofloc, but we neither found a significant reduction of nitrites with respect to the control treatment ($p = 0.05$). $P\text{-}PO_4^{3-}$ and $N\text{-}NO_3^-$ were accumulated in water throughout the culture, following normal BFT dynamics and reaching levels similar to those observed in other experiments [38,59,60].

The evolution of TSS was similar to that observed by Gaona et al. [52] and Ray et al. [59]. In the final two weeks of the experiment, the TSS levels were slightly higher than the optimal value of 0.5 g/L established by Samocha et al. [61] for the cultivation of *L. vannamei.* TSS reduction would reduce the amount of bacteria present in the system, and therefore reduce the consumption of dissolved oxygen [52]. In this experiment, since dissolved oxygen concentrations did not drop below 5 mg/L, it was not necessary to use any solids removal techniques, which simplified culture management. The results obtained show that, as other probiotics [9], the application of the spores of *B. amyloliquefaciens* does not affect the dynamics of N-TA, $N\text{-}NO_2^-$, $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$ and TSS in biofloc systems.

Average Chl*a* values were around 150 µg/L, similar to those observed by other authors such as Liu et al. [62] and Martins et al. [63]. Maximum Chl*a* concentration(703.58 µg/L) was higher than that found by Gaona et al. [52] and Emerenciano et al. [64] (500 µg/L), although it was below that observed by Schrader et al. [65]. Chl*a* concentration in BFT is highly variable, and depends on environmental conditions and changes that occur in the BFT itself [65,66]. In this experiment, two Chl*a* peaks were observed at the beginning and at the end of the experiment due to shade cloths management. The first peak was observed with the white shade cloth and the second when the shade cloths were removed. NPP, WCR and GPP dynamics are in agreement with those observed by Vinatea et al. [67], where the WCR increased and the GPP decreased, mainly during the first two months of culture. WCR values were similar to those observed by Vilani et al. [68], around 0.45 mg O_2/(L·h) and lower than those observed by Schveitzer et al. [42] and Vinatea et al. [67], who obtained values above 1 mg O_2/(L·h). NPP evolution shows the predominance of autotrophic processes in the BFT during the first culture weeks, characterized by lower oxygen consumption than production. Since day 58 a change of trophic state to a dominance of heterotrophic processes was observed. Chl*a* evolution shows a second peak at the end of the experiment. This increase in Chl*a* did not lead to an increase in GPP and NPP. Thus, the change in the trophic status of the BFT is attributed, not to the evolution of Chl*a*, but to the evolution of the bacterial population.

The beginning of nitrification processes by bacteria begins to generate a significant bacterial mass that causes heterotrophic processes to predominate in the culture system. WCR increases slowly over the weeks, at the same time as the TSS increases, which consume any water-dissolved oxygen [52].

4.2. Effects on Shrimps

The results showed a survival and a weekly weight increase around 60% and 0.7 g/week respectively, in all of the treatments, which was similar to those observed by other authors [43,69–71]. These low survival values are related to the increase of N-NO_2^- during an important phase of the culture. The long exposure of shrimps to high nitrites values forced us to reduce their feed to 60%, which decreased the shrimp growth between days 70 and 110, as observed in Figure 5. During the period of peak N-NO_2^-, a few dead shrimps were observed in the feeders. This mortality could be a consequence of prolonged exposure to high levels of N-NO_2^- [40], which affected the final survival in all of the treatments equally. Authors such as Camacho [26] and Reda and Selim [72], applied *B. amyloliquefaciens* in a recirculation system, and observed improvements in the growth parameters due to the production of enzymes in the shrimp digestive system [13]. However, we have not observed this effect when applying this probiotic in BFT. It might be because the protein intake from the consumption of bioflocs [3] provides more nutritional benefits than the generation of digestive enzymes in the digestive tract of shrimp by *B. amyloliquefaciens*, not showing any difference between treatments.

During the experiment the immune system was analyzed when the BFT was in two different states, immature (day 86) and mature (day 169) biofloc. The percentages of granular and hyaline hemocytes in the hemolymph were within the range observed by different authors for *L. vannamei* [26,45–47]. In the first sampling, it was observed that the percentage of GH was 5% higher in both treatments with *B. amyloliquefaciens*. In the second sampling, the difference between treatments with probiotic and control treatment was 7%. These results show that the application of *B. amyloliquefaciens* increases the percentage of GH with respect to that of HH. GH have different mechanisms of action against pathogens such as phagocytosis, encapsulation, cytotoxicity and the storage and release of prophenoloxidase into the system [73,74]. HH only fight pathogens by phagocytosis, making them less effective against pathogens [73,74]. When the biofloc system was immature, the level of nitrite was higher than safety.

The application of *B. amyloliquefaciens* significantly increased GH proportion in the hemolymph, and thereforeimproved the immune response capacity of the shrimp in high and low bacteria treatments. This effect was significant during the period when the biofloc was immature and the nitrite levels were high and produced stress to shrimp [40]. It is extremely valuable that this immune response could be achieved at the time of highest stress exposure amongst the shrimp, and might alleviate the negative effects of culture conditions, because shrimp were very sensitive to stress situations [73,75].

A 7% increase in GH is comparable to that caused by other probiotics or food components [9,69]. In fact, Souza et al. [9] observed a similar increase in the percentage of granular hemocytes when they used a multi-species probiotic with a similar concentration (between 10^3 and 10^4 cfu/mL). So, *B. amyloliquefaciens* has a better effect upon the immune system than other probiotics, and combining *B. amyloliquefaciens* with other probiotic strains may extend the benefits over shrimp cultured in BFT. *B. amyloliquefaciens* has the appropriate characteristics to be considered as a probiotic for the culture of shrimps in BFT, as a preventive measure to the appearance of possible diseases.

The TPC observed was similar to that observed in *L. vannamei* by other researchers [26,45–47]. In this variable no statistically significant differences were observed between the treatments in any of the samples. Previously unpublished research by F. Llario observed an improvement in TPC when applying *B. amyloliquefaciens* in a mature BFT. The stress produced during the maturation of the BFT could have affected to a greater extent the TPC than the probiotic, masking the effects of *B. amyloliquefaciens* on this variable. Among the functions of the proteins in the hemolymph is the recognition of pathogens, their inhibition and their agglutination, so that they can be eliminated by the hemocytes [74], being a variable widely used to monitor the state of the immune system [46,47].

If we compare the results in the immune system between the mature and immature stage of BFT (obviating the dose of probiotic received), it is appreciated that the GH values increased 15% with the maturation of the BFT ($p = 0.00$). Also, TPC increased 34 mg/mL ($p = 0.00$) from day 86 to 169. Although shrimp growth could positively affect TPC levels [75], the maturation of BFT and the elimination of N-NO_2^- by BFT bacteria causes improvements in the shrimp immune system [48,49,75]. In this experiment, it is shown that the effect of probiotics does complement that of BFT. The role of *B. amyloliquefaciens* is of great importance mainly during the maturation of BFT, because it provides a reinforcement of the immune system that could help the shrimps to fight against possible pathogens.

The different doses of *B. amyloliquefaciens* tested (high bacteria treatment (2.8×10^4 cfu/mL) and low bacteria treatment (2.8×10^3 cfu/mL)), did not produce significant differences. This shows that *B. amyloliquefaciens* is effective, when applied to water, at a dose of at least 10^3 cfu/mL, doses clearly lower than those used by other authors in shrimp culture [8,9].

5. Conclusions

Despite the good characteristics of *B. amyloliquefaciens* as a biofloc promoter bacterium, its direct application in the water column did not have a significant effect upon water quality. The addition of *B. amyloliquefaciens* to the water column, during the maturation process of the biofloc, did not produce significant changes on nutrient dynamics, nor a significant reduction of nitrites with respect to our control treatment. Further studies are necessary to analyze whether improving the application technique in water can enhance their effect. The addition of the probiotic also failed to alter the trophic state of the system, or to influence the Chl*a* levels.

There was also no significant effect on the growth parameters when applying the probiotic bacteria. These results seem to indicate that the nutritional improvements of the biofloc system surpass those that *B. amyloliquefaciens* could have. However, where this bacterium can play a very important role, it is in the reinforcement of the shrimp immune system, mainly under unfavorable culture conditions, such as an immature biofloc system. *B. amyloliquefaciens* manages to increase the percentage of GH, both in mature and immature BFT, although the increase in GH that a mature BFT provides is more important than that produced by the probiotic. In addition, it has been observed that *B. amyloliquefaciens* is effective for the immune system at a dose of 10^3 cfu/mL, a dose lower than that recommended for other probiotics, reducing costs and maintaining the benefits on the system. However, we cannot exclude that higher doses could have an effect on water quality.

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