Dietary Supplementation with Probiotic and Butyrate in the Shrimp Nursery in Biofloc

ABSTRACT

This study aimed to evaluate the combined and isolated effect of sodium butyrate and the probiotic *Lactobacillus plantarum* on the performance and midgut microbiological parameters of *Litopenaeus vannamei* post-larvae reared on biofloc technology, as well as the water quality of the system. Feed additives were added at the concentrations of 200 mL of probiotic (1.0x10^7 CFU mL^-1) and 2.0% of organic salt (w/w) in the diet, as follows: 1) Probiotic; 2) Butyrate; 3) Probiotic+Butyrate; 4) Control. Each treatment was composed of three replicates. Biometric measurements were performed once a week, as well as analysis of water quality. At the end of the experiment, statistical difference was observed in the counts of lactic acid bacteria from the intestinal tract of shrimp fed diets containing probiotic. Therefore, while the addition of probiotic and sodium butyrate had no effect on the productive parameters of shrimp or water quality, the inclusion of the probiotic *L. plantarum* in the diet did increase the counts of lactic acid bacteria in the intestine of *L. vannamei* without altering the counts of *Vibrio* spp. or total heterotrophic bacteria in the intestine. Key words: *Litopenaeus vannamei*; BFT system; *Lactobacillus plantarum*; organic acid.

Introduction

Fisheries and aquaculture provide employment and support for an estimated 54.8 million people involved in the primary production sector (FAO, 2014). Brazilian shrimp farming has demonstrated significant technical, economic, social and environmental viability, while generating shrimp production and the resultant net profit and job creation (TAHIM and ARAÚJO JUNIOR, 2014; BARBIERI et al., 2016). In recent years, some problems faced by shrimp farmers, mainly related to diseases, have stimulated the generation of new production practices. Most of these cultures aim to reduce the amount of water used, while promoting biosecurity, to produce organisms

Received: December 01, 2017
Approved: March 29, 2018
(KRUMMENAUER et al., 2010). In this context, the Biofloc Technology System (BFT) is an alternative to much more wasteful production systems.

The biofloc system, according to WASIELESKY et al. (2006), is based on the formation of microbial flakes that serve as a food supplement for the organisms. This system is characterized by high stock density (above 300 shrimp m⁻³) and the production of high biomasses, reaching 10 kg m⁻³ (SAMOCHA et al., 2007). BFT works with reduced, or even zero, water exchange, and it is possible to reduce the discharge of effluents, as well as prevent the entry of any viral pathogens into the culture (TACON et al., 2002). In addition, the bacterial community established in the system with microbial flakes can inhibit the proliferation of pathogens by competitive exclusion of food and space (CRAB et al., 2012). In the shrimp production process, nursery is a vital phase. This stage contributes to the rapid growth of animals, tolerates a higher density of organisms, reduces the costs of production and allows a greater number of production cycles per year (EMERENCIANO et al., 2013; WASIELESKY et al., 2006). When combined with BFT, the nursery stage may result in shrimps with better growth potential and health, which will later affect growth performance (KRUMMENAUER et al., 2010; WASIELESKY et al., 2006). The most common response of an organism to conditions of environmental imbalance is the appearance of opportunistic diseases caused by microorganisms already present in the organism itself or in the culture environment (NICOLAS et al., 2008). Consequently, research has found that dietary supplementation in commercial feed will promote animal growth and health by using such compounds as probiotics and organic acids (SILVA et al., 2013).

Probiotics are live microbial supplements which, by modifying their associated microbial community and the environmental culture, will have beneficial effects on the host, increasing the quality of feed and its nutritional value, improving the host response to diseases and also the quality of the environment (VERSCHUERE et al., 2000). Among the probiotics currently used in aquaculture, lactic bacteria stand out since they are used in the manufacture of human foods and are, therefore, safe for human consumption. Moreover, such bacteria tolerate the activity of gastric acids and bile, converting lactose into lactic acid, decreasing the pH of the gastrointestinal tract, and reducing bacterial colonization by pathogens (CARR et al., 2002). With all the possible advantages that lactic acid bacteria can bring, this supplement can potentially stimulate the immune system and possibly improve the nutrition of penaeid shrimps (SILVA et al., 2013).

Research investigating organic acid dietary supplementation for aquaculture is receiving global attention for its ability to promote growth, as well as its use as a prophylactic against such pathogenic bacteria as *Vibrio* spp. (ADAMS and BOOPATHY, 2013; SU et al., 2014; ROMANO et al., 2015). The combined use of organic acids and probiotics in aquaculture is recent, and few studies are found in the literature. In fact, few studies have reported on the combined use of probiotics and organic salts, and these reports mostly involve terrestrial animals (WOLFENDEN et al., 2007; BOZKURT et al., 2009).

It is important that techniques related to management and production in biofloc systems be improved to obtain a greater use of the microbial community as a source of supplementary food, in addition to controlling disease in shrimp. Since most studies have focused on the use of probiotics, research that explores the use of organic salts, either alone or combined with probiotics, would be of commercial interest, in particular crustacean farming in the early stages of culture when organisms are more susceptible to disease.

Therefore, this work aims to evaluate the effect of both combined and isolated inclusion of organic sodium butyrate and the probiotic *L. plantarum* on the diet of the shrimp *Litopenaeus vannamei* reared in the nursery phase in a biofloc system.

**METHODS**

**Experimental design**

The experiment, which entailed 35 days of culture, was conducted in twelve polyethylene tanks (500 L) containing salt water and was equipped with constant heating and aeration. The stocking density was 2,250 shrimp m⁻³, and post-larvae reached stage thirty (PL₃₀), with the average weight of 0.03±0.001 g. The experimental design was completely randomized and was composed of the following four treatments: 1) Probiotic; 2) Butyrate; 3) Probiotic + Butyrate and 4) Control, with three replicates for each treatment.

**Biofloc system**

The water used for the formation of the biofloc inoculum initially had a concentration of 575 mg L⁻¹ of total suspended solids (TSS). An inoculum corresponding to 35% (140 L) of each tank’s useful volume (400 L) was transferred from a laboratory shrimp culture to the experimental units, and the remaining volume was filled with salt water. The initial TSS concentration was 152 mg L⁻¹. According to the methodology described by AVNIMELECH (1999) and EBELING et al. (2006), organic fertilization was carried out, using 20 grams of carbohydrate for the neutralization of each gram of ammonia, when necessary, to balance the biofloc system. Sugar was used as a source of carbohydrate, and the feed provided to the shrimp served as a source of nitrogen for the formation of the microbial aggregates of the bioflocs.

**Diet preparation and food management**

Two basic diets were used, and their composition is described in Table 1. Sodium butyrate was added in the proportion of 2.0% of organic salt (w/w) in the diet, according to the methodology described by SILVA et al. (2013).

After homogenization, the mixture received approximately 20% of water in its composition, which was pelletized and dried at approximately 25 °C for 24 hours. Probiotics were added in the proportion of 200 mL of probiotic (1.0x10⁶ CFU mL⁻¹) per kilogram of feed. The bacterial strain used to form the probiotic (*L. plantarum*) was obtained from the microbiology collection of the LCM-UFSC Laboratory (VIEIRA et al., 2008). The probiotic formulation was performed in MRS broth (Man, Rogosa and
Table 1. Diet composition used in the experiment.

| Ingredients                      | g.100g⁻¹ |
|----------------------------------|----------|
| Salmon by-product meal           | 48.4     |
| Soybean meal²                    | 23.7     |
| Wheat flour³                     | 10       |
| Rice flour⁴                      | 1.8      |
| Cod liver oil⁵                   | 1.4      |
| Vitaminic Premix⁶                 | 0.4      |
| Vitamin C⁷                        | 0.1      |
| Macro-Mineral Premix⁸             | 6.6      |
| Micro-mineral Premix⁹             | 1.6      |
| Lecithin¹⁰                       | 2.0      |
| Carboxymethylcellulose¹¹         | 2.0      |
| Kaolin¹²                         | 2.0      |
| Sodium Butyrate¹³                 | 2.0      |

¹ Tectron Nutrição Animal (Paraná, Brazil); ² Nicoluzzi Rações Ltda (Santa Catarina, Brazil); ³ Dona Benta (Santa Catarina, Brazil); ⁴ Rice Sauce (Rio Grande do Sul, Brazil); ⁵ Holland & Barrett; ⁶ In Vivo Nutrição e Saúde Animal, guarantee levels per kg of product: vit. A 900 mg; vit.D 25 mg; vit.E 46,900 mg; vit.K 14,000 mg; vit. B12 50 mg; biotin 750 mg; folic acid 3,000 mg; niacin 70,000 mg; pantothenic acid 40,000 mg; vit. B6 33,000; riboflavin 20,000 mg; thiamine 30,000 mg; ⁷ Labsynth Produtos para Laboratórios Ltda (São Paulo, Brazil); ⁸ Labsynth (Diadema, SP, Brazil). Macro mineral mixture composition (g kg⁻¹): dicalcium phosphate, 454 g; potassium sulphate, 297 g; sodium chloride, 174 g; magnesium sulphate, 75 g; ⁹ In Vivo Nutrição e Saúde Animal, guarantee levels per kg of product: coper B12 50 mg; biotin 750 mg; folic acid 3,000 mg; niacin 70,000 mg; pantothenic acid 40,000 mg; vit. B6 33,000; riboflavin 20,000 mg; thiamine 30,000 mg; ¹⁰ Labsynth Produtos para Laboratórios Ltda (São Paulo, Brazil); ¹¹ Seminova Produtos para Ração Animal Ltda (São Paulo, Brazil); ¹² Diprolab Comércio de Materiais para Laboratório (Santa Catarina, Brazil); ¹³ Laboratório Sovereign (Santa Paulo, Brazil).

Sharpe) with the addition of 3.0% salt (NaCl), incubated for 24h at 35 °C for growth of the bacterial strain, and then transferred to a solution containing milk at 1.0x10⁷ CFU mL⁻¹ to obtain the final volume added to the experimental feeds. The addition of probiotic was performed daily.

During the experimental period, the shrimps were fed four times a day (08:00, 11:00 14:00 and 17:00 hours). The amount of feed supplied followed the methodology suggested by VAN-WYK (1999).

Water quality parameters

During the experiment, the dissolved oxygen was maintained at 5.0±1.0 mg L⁻¹ and water temperature at 28.0±1.0 °C, respectively (digital oximeter model YSI Pro20). Once a week, samples were collected to measure pH (Thermo Scientific Orion Star A211 digital pH meter), salinity (EcoSense EC300A digital salinometer), TSS, volatile suspended solids (VSS), fixed suspended solids (FSS) (APHA, 1995), nitrite (STRICKLAND and PARSONS, 1972) and nitrate (Hach commercial kit ACA01). Alkalinity (APHA, 1995) and ammonia (STRICKLAND and PARSONS, 1972) were analyzed twice a week.

Growth performance

Weekly biometric measurements were performed with the shrimp in the proportion of 5.0% of the stocking density in each experimental unit. Average shrimp weights were subsequently estimated. These biometric measurements formed the basis for determining the amount of feed supplied to the shrimp, or adjusted, during the experimental period. In the final biometry, all shrimps of each replicate were selected, weighed and counted. The performance of shrimps was evaluated by survival (%), feed conversion rate (FCR), total weight gain (g), final weight (g), biomass production (kg) and final productivity (kg m⁻³).

Microbiological analysis

At the end of the experiment, thirty shrimps were sampled from each tank, followed by removal of midguts. Subsequently, the samples were macerated, homogenized, serially diluted in saline solution (3.0%) and seeded in Marine Agar, TCBS and MRS Agar (total heterotrophic bacteria, total *Vibrio* spp. and total lactic acid bacteria). The samples were incubated at 30 °C during 24 hours in the Agar Marine and TCBS Agar cultures and 48 hours in the MRS agar. Afterwards, total counts of colony forming units (CFU) were performed.

Statistical analysis

Statistical Analysis of Factorial Variance (ANOVA) was used, and the assumptions of homocedasticity and normality of the statistical data were first determined by the Levene’s test and Shapiro-Wilk test, respectively. The initial verification of differences between the replicates was done; if these were not significant, data were collected and analyzed for observation and detection of differences between treatments. When significant differences between the treatments were detected, the Tukey test of means separation with a significance level of 5.0% was used.

RESULTS

Water quality parameters

Water quality parameters remained relatively stable throughout the experiment (Table 2). The values of salinity (31.80±1.45 g L⁻¹), pH (8.30±0.12) and alkalinity (168.69±7.25 mg CaCO₃ L⁻¹) presented no significant differences, and all values were within acceptable standards for marine shrimp (DIAZ and ROSENBERG, 1995; VAN WYK and SCARPA, 1999). Ammonia (N-NH₃) did not present significant differences among the treatments. Similarly, nitrite (N-NO₂⁻) and nitrate (N-NO₃⁻) also remained stable throughout the experiment. TSS, VSS and FSS showed no significant differences, and all parameters remained within the levels recommended for this shrimp species (RAY et al., 2010).

Growth performance

Table 3 summarizes the parameters (± standard deviations) of *L. vannamei* at the end of 35 days of culture. No differences were found for final weight (g), total weight gain (g), survival (%), final biomass (kg), final productivity (kg m⁻³) or feed conversion rate (FCR)
Table 2. Water quality parameters of *L. vannamei* cultivated in a biofloc system and fed diets supplemented with probiotic (*Lactobacillus plantarum*), sodium butyrate (2.0%), probiotic + sodium butyrate and control (no supplementation) during 35 days.

| Treatment          | Alcalinity (mg CaCO₃ L⁻¹) | Ammonia N-NH₄ (mg L⁻¹) | Nitrate N-NO₃⁻ (mg L⁻¹) | Nitrate N-NO₂⁻ (mg L⁻¹) | TSS (mg L⁻¹) | VSS (mg L⁻¹) | FSS (mg L⁻¹) |
|--------------------|---------------------------|------------------------|-------------------------|-------------------------|--------------|--------------|--------------|
| Probiotic          | 175.24±4.33               | 0.96±1.23              | 0.38±0.65               | 13.35±2.57              | 356.66±17.72 | 169.06±26.33 | 187.60±16.59 |
| Butyrate           | 169.30±7.45               | 1.02±1.44              | 0.46±0.83               | 12.37±2.66              | 357.33±13.52 | 175.73±31.07 | 181.60±29.99 |
| Probiotic + Butyrate| 167.26±6.19               | 1.04±1.22              | 0.93±1.07               | 14.62±2.48              | 378.86±13.30 | 154.86±29.54 | 224.01±28.91 |
| Control            | 158.98±8.44               | 1.00±1.35              | 0.58±0.80               | 10.57±3.33              | 351.01±13.21 | 164.53±36.13 | 187.13±25.57 |

Table 3. Growth parameters of *L. vannamei* cultivated in a biofloc system and fed diets supplemented with probiotic (*Lactobacillus plantarum*), sodium butyrate (2.0%), probiotic + sodium butyrate and control (no supplementation) during 35 days.

| Treatment          | Final Weight (g) | Total Weight Gain (g) | Survival (%) | Final Biomass (kg) | Final Productivity (kg.m⁻³) | Feed Conversion Rate (FCR) |
|--------------------|------------------|-----------------------|--------------|--------------------|-----------------------------|---------------------------|
| Probiotic          | 0.90±0.01        | 0.87±0.06             | 97.15±2.57   | 0.75±0.06          | 1.95±0.07                   | 1.22±0.05                 |
| Butyrate           | 0.60±0.16        | 0.57±0.18             | 96.96±1.89   | 0.52±0.14          | 1.31±0.35                   | 1.62±0.27                 |
| Probiotic + Butyrate| 0.77±0.18       | 0.74±0.14             | 95.74±2.68   | 0.66±0.16          | 1.66±0.40                   | 1.43±0.43                 |
| Control            | 0.78±0.17        | 0.75±0.17             | 96.19±0.46   | 0.67±0.14          | 1.68±0.36                   | 1.41±0.42                 |

Table 4. Microbiological parameters (log value) of *L. vannamei* cultivated in a biofloc system and fed a diet supplemented with probiotic (*Lactobacillus plantarum*), sodium butyrate (2%), probiotic + sodium butyrate and control (no supplementation) during 35 days.

| Treatment          | *Vibrio* spp. | Total Heterotrophic Bacteria | Lactic Acid Bacteria |
|--------------------|---------------|------------------------------|---------------------|
| Probiotic          | 3.43±3.06     | 7.19±0.64                    | 5.87±0.85           |
| Butyrate           | 3.73±0.02     | 7.72±0.04                    | 1.34±2.33           |
| Probiotic + Butyrate| 2.66±2.33    | 8.11±1.17                    | 4.39±0.37           |
| Control            | 4.17±0.43     | 7.27±0.59                    | 2.36±2.07           |

Different letters indicate statistical differences by the Tukey test.

Microbiological parameters

No significant differences were found in total heterotrophic bacteria and *Vibrio* spp. (Table 4). The lactic acid bacteria values found in the probiotic and probiotic + sodium butyrate treatments were significantly higher (p>0.05) than those found for control and butyrate treatments, respectively.

DISCUSSION

Water quality, as determined from the results of this study, indicates that environmental factors likely played no role in the development of *L. vannamei* shrimps. Rather, the use of BFT presupposes that water quality will be maintained in good condition and that microbiota will maintain water quality in the *L. vannamei* post-larvae culture. Bioflocs are also considered as a food supplement for shrimp by converting nitrogen compounds produced by animals into microbial protein (AVNIMELECH, 1999; EMERENCIANO et al., 2013; KHATOON et al., 2016). Although the parameters were within acceptable standards for Pacific white shrimp culture, the ammonia levels in this study were, on average, 1.0±0.03 mg L⁻¹, probably owing to the high protein content used in post-larvae feed, which resulted in a higher nitrogen uptake and accumulation in the system. In general, the water quality parameters in this experiment showed that BFT can be a viable alternative for maintaining water quality in shrimp farming.

No significant differences were observed among the growth parameters in this study. The experiment was performed in triplicate, so it is possible that the inclusion of more experimental units could have evinced statistical differences in the probiotic treatment. However, different studies have demonstrated good results with the use of probiotic for *L. vannamei* when using it as a dietary supplement, aiding in growth, food efficiency and survival of the species (CHIU et al., 2007; VIEIRA et al., 2010; KONGNUM and HONGPATTARAKERE, 2012; DASH et al., 2014; VAN DOAN et al., 2014; ZHENG et al., 2016).

In a study using *L. plantarum* as a probiotic, TALPUR et al. (2013) tested three levels of inclusion of this lactic acid bacterium in diets for larvae of *Portunus pelagicus*. Adding probiotic in the concentration of 1x10⁶ CFU mL⁻¹, they observed that larvae survival increased, as well as counts of total bacteria and *Vibrio* spp.
The results showed that probiotic action in diets may have the desired effect when the level of concentration is at least $10^6$ or $10^7$ CFU mL$^{-1}$ of live probiotic bacteria in the diet (FAO, 2016). In addition, administering small concentrations of probiotics or decreasing the number of days of use may cause a low, or insufficient, colonization of the intestinal tract (MUNOZ-ATIENZA et al., 2013). They observed that the microflora of tilapia (NG et al., 1996), as well as increased nutrient digestibility and intestinal growth of Atlantic salmon (BAEVERFJORD and KROGDAHL, 2004), supplementation in fish diets have shown good results for increased numbers. The use of sodium butyrate in the diet of shrimp, when experimentally challenged with Vibrion harveyi, and the best results were observed in shrimp fed diets that included organic salts at 2.0% kg$^{-1}$ of feed.

Some studies demonstrate the beneficial effects of probiotics as immunostimulants in shrimps. When challenged with different species of the genus Vibrio, probiotics stimulate the maintenance of the active defense system, increasing resistance to viral challenge or maintaining control of pathogenic bacteria (GULLIAN et al., 2004; LI et al., 2007; CHAI et al., 2016). Although no such viral challenge was mounted in this experiment, it is thought that probiotics would improve the survival of shrimp during culture, even to the point of improving animal health and contributing to good performance in the productive stages.

In the present study, no significant differences were found in treatments using sodium butyrate. These results corroborate the findings of BOLIVAR RAMIREZ et al. (2017) who reported that the use of sodium butyrate in the diet of Litopenaeus vannamei in clear water did not show any differences in growth parameters when compared to the control and probiotic groups analyzed in that study. The use of organic acids in shrimp farming and the knowledge of their effects as an additive are still tentative, but they are already showing some positive results, as evidenced in current research. In terrestrial animals, such as pigs and chickens, for example, studies already show that the inclusion of butyrate in the diet results in increased weight gain, feed conversion, and the growth of intestinal microbiota in these animals (KOTUNIA et al., 2004; HU and GUO, 2007). Furthermore, some studies of organic acid supplementation in fish diets have shown good results for increased growth of Atlantic salmon (BAEVERFJORD and KROGDAHL, 1996), as well as increased nutrient digestibility and intestinal microflora of tilapia (NG et al., 2009; ZHOU et al., 2009).

SILVA et al. (2013) tested different organic salts (sodium formate, sodium acetate, sodium lactate, sodium propionate, sodium butyrate, sodium fumarate, sodium succinate or sodium citrate) to supplement the diet of L. vannamei. They observed significantly better growth for animals fed diets treated with sodium propionate, sodium butyrate, sodium fumarate and sodium succinate. Similarly, ROMANO et al. (2015) used a combination of four organic salts (formic, lactic, malic and citric) to supplement the diet of L. vannamei. They observed that the use of these salts significantly increased the resistance of shrimps when experimentally challenged with Vibrio harveyi, and the best results were observed in shrimp fed diets that included organic salts at 2.0% kg$^{-1}$ of feed.

These beneficial results can likely be attributed to the acidifying effect of these compounds, which reduces and accelerates the conversion of pepsinogen to pepsin, which, in turn, improves the absorption of amino acids and minerals (BARUAH et al., 2007; SILVA et al., 2016). Histological samples in experiments using butyrate have shown that this organic acid is also capable of exerting positive effects on a range of cellular functions relevant to intestinal health, such as inhibition of inflammation and carcinogenesis (HAMER et al., 2008), aiding in the formation of muscular layers of fish intestine (RIMOLDI et al., 2016), and may also increase the absorptive function of the gut by enhancing the proliferation and differentiation of intestinal epithelial cells (TOPPING and CLIFTON, 2001; WONG et al., 2006).

**CONCLUSION**

Use of the probiotic L. plantarum and sodium butyrate did not change the growth performance of Pacific white shrimp reared in a biofloc system at nursery stage. The addition of probiotic did, however, increase the counts of lactic acid bacteria in the shrimp midgut, albeit without influencing the counts of Vibrio spp. and total heterotrophic bacteria in shrimps.

**ACKNOWLEDGEMENTS**

The authors recognize the financial support of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), corresponding to the PVE/2014 project, and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Felipe Vieira and Walter Seiffert received a productivity research fellowship from CNPq (protocol nº PQ 309868 / 2014-9 and 304277/2015-0). The authors thank Mr. Sérgio Pitz (Atlântico Sul Maricultura LTDA) for the supply of L. vannamei post-larvae used in this study.

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