

DIETARY SUPPLEMENTATION WITH PROBIOTIC AND BUTYRATE IN THE SHRIMP NURSERY IN BIOFLOC

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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 (1.0×10^7 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.

SUPLEMENTAÇÃO DIETÉTICA COM PROBIÓTICO E BUTIRATO NO BERÇÁRIO DO CAMARÃO EM BIOFLOCOS

RESUMO

O objetivo deste trabalho foi avaliar o efeito conjunto e isolado do sal orgânico butirato de sódio e do probiótico *Lactobacillus plantarum* para pós-larvas do camarão *Litopenaeus vannamei* cultivado em tecnologia de bioflocos sobre os parâmetros zootécnicos, microbiológicos e histológicos, além da qualidade da água do sistema. Os aditivos alimentares foram adicionados nas concentrações de 200 mL de probiótico ($1,0 \times 10^7$ UFC mL⁻¹) e 2,0% do sal orgânico (p/p) na ração, com o seguinte delineamento: 1) Probiótico; 2) Butirato; 3) Probiótico+Butirato; 4) Controle, e cada tratamento foi composto por três réplicas. Biometrias foram realizadas uma vez por semana e, da mesma forma, foram feitas análises dos parâmetros de qualidade de água das unidades experimentais. Ao final do experimento, os resultados mostraram diferença estatística nas contagens de bactérias ácido lácticas do trato intestinal dos camarões alimentados com as dietas contendo probiótico. Conclui-se que a adição de probiótico e o butirato de sódio não influenciaram nos parâmetros produtivos dos camarões nem os parâmetros de qualidade de água, mas a inclusão do probiótico *L. plantarum* na dieta aumentou as contagens de bactérias ácido lácticas no intestino do camarão *L. vannamei*, contudo sem alterar as contagens de *Vibrio* spp. e bactérias heterotróficas totais no intestino.

Palavras-chave: *Litopenaeus vannamei*; sistema BFT; *Lactobacillus plantarum*; ácido orgânico.

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

(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 some 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 (1.0x10⁷ 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	48.4
Soybean meal ²	23.7	23.7
Wheat flour ³	10	10
Rice sauce ⁴	1.8	1.8
Cod liver oil ⁵	1.4	1.4
Vitaminic Premix ⁶	0.4	0.4
Vitamin C ⁷	0.1	0.1
Macro-Mineral Premix ⁸	6.6	6.6
Micro-mineral Premix ⁹	1.6	1.6
Lecithin ¹⁰	2.0	2.0
Carboxymethylcellulose ¹¹	2.0	2.0
Kaolin ¹²	2.0	-
Sodium Butyrate ¹³	-	2.0

¹Tectron Nutrição Animal (Paraná, Brazil); ²Nicoluzzi Rações Ltda (Santa Catarina, Brazil); ³Dona Benta (Santa Catarina, Brazil); ⁴Rice Sauce (Rio Grande do Sul, Brazil); ⁵Hollandand & Barrett; ⁶In 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 70,000mg; pantothenic acid 40,000 mg; vit. B6 33,000; riboflavin 20,000 mg; thiamine 30,000 mg; ⁷Labsynth Produtos para Laboratórios Ltda (São Paulo, Brazil); ⁸Labsynth (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; ⁹In Vivo Nutrição e Saúde Animal, guarantee levels per kg of product: coper 23,330 mg; manganese 6,500 mg; selenium 125 mg; zinc 100,000 mg, iodine 1,000 mg; cobalt 50 mg, magnesium 20 mg; potassium 6.1 mg; ¹⁰Quimidrol Produtos Químicos Ltda. (Santa Catarina, Brazil); ¹¹Diprolab Comércio de Materiais para Laboratório (Santa Catarina, Brazil); ¹²Mineração Riaj Ltda (São Paulo, Brazil); ¹³Laboratório Sovereign (São 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 homoscedasticity 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 CaCO ₃ L ⁻¹)	Ammonia N-NH ₃ (mg L ⁻¹)	Nitrite N-NO ₂ (mg L ⁻¹)	Nitrate N-NO ₃ (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	<i>Vibrio</i> spp.	Total Heterotrophic Bacteria	Lactic Acid Bacteria
Probiotic	3.43±3.06 ^a	7.19±0.64 ^{a*}	5.87±0.85 ^a
Butyrate	3.73±0.20 ^a	7.72±0.42 ^a	1.34±2.33 ^b
Probiotic+ Butyrate	2.66±2.33 ^a	8.11±1.17 ^a	4.39±0.37 ^a
Control	4.17±0.43 ^a	7.27±0.59 ^a	2.36±2.07 ^b

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 1×10^6 CFU mL⁻¹, they observed that larvae survival increased, as well as counts of total bacteria and *Vibrio* spp.

(FAO, 2016). 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 (MUÑOZ-ATIENZA *et al.*, 2013).

The counts of total heterotrophic bacteria and *Vibrio* spp. in the midgut of the shrimps fed with sodium butyrate, *L. plantarum*, and the control group did not present statistical differences. To account for the failure of statistical difference to appear as a result of culture, we point to the fact that the experiment was carried out in a biofloc system where a heterotrophic bacterial community is omnipresent, using the available organic carbon as an energy source and assimilating nitrogen to form cellular proteins. In contrast, the shrimps that received diets containing the probiotic *L. plantarum* had higher counts of lactic acid bacteria in their intestinal tract, showing the effectiveness of probiotics in colonizing the intestinal tract of shrimp after the ingestion of feed. According to FEČKANINOVÁ *et al.* (2017), the ability of probiotic microorganisms to survive and multiply in the host is directly correlated with its overall effect, meaning that bacteria must be metabolically stable and active in the diet provided and then survive during passage through the intestinal tract in large numbers.

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 BOLÍVAR RAMÍREZ *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.

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