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EFEITO DA FREQUÊNCIA DE ADIÇÃO DE *Brachionus plicatilis* NA FASE BERÇÁRIO DE *Litopenaeus vannamei* EM SISTEMA DE BIOFLOCOS

RILDO JOSÉ VASCONCELOS DE ANDRADE

Dissertação apresentada ao Programa de Pós-Graduação em Recursos Pesqueiros e Aquicultura da Universidade Federal Rural de Pernambuco como exigência para obtenção do título de Mestre.

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RILDO JOSÉ VASCONCELOS DE ANDRADE

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Dedico este trabalho a minha família, principalmente aos meus pais: Rildo e Ana.

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Resumo

O sistema de BFT pode proporcionar um aumento nos índices zootécnicos em comparação com o sistema convencional, pois permite trabalhar com altas densidades, menor troca de água e, aproveitamento da biomassa microbiana, embora, em termos nutricionais, o bioflocos possua deficiência em metionina, PUFA e HUFA. Uma forma de melhorar a qualidade nutricional deste floco microbiano e, consequentemente, o desempenho dos organismos cultivados é a adição suplementar de microrganismos nutricionalmente ricos em aminoácidos e ácidos graxos ao sistema, principalmente nas fases iniciais de cultivo. O rotífero *B. plicatilis* se enquadra nestes requisitos, pois tem bom conteúdo nutricional, além da capacidade de tolerar uma ampla faixa de salinidade, mobilidade lenta e rápida reprodução, sendo estas características cruciais a sua utilização como alimento suplementar para as fases iniciais. O objetivo deste trabalho foi avaliar o efeito da frequência de adição do *B. plicatilis* sobre o cultivo de pós-larvas de *L. vannamei* no sistema de BFT. O experimento teve duração de 42 dias com quatro tratamentos: BFT (bioflocos); BFT-1F (frequência de adição de *B. plicatilis* no 1º dia de cultivo); BFT-2F (frequência de adição de *B. plicatilis* no 1º e 10º dia de cultivo) e BFT-4F (frequência de adição de *B. plicatilis* no 1º, 10º, 20º e 30º dia de cultivo) em triplicata. O experimento foi realizado em unidades experimentais com 40 litros de volume útil e densidade de 3000 animais m^{-3}. As variáveis de qualidade de água não apresentaram diferenças significativas entre tratamentos. Entretanto, o desempenho zootécnico no tratamento BFT-4F demonstrou um melhor peso final (0,73g), produtividade (1,88kg/m^3) e taxa de crescimento específico (14,31% dia). Em relação à comunidade planctônica, os filos que apresentaram uma maior abundância foram Chlorophyta e Protozoa. Para as amostras microbiológicas, ocorreu um aumento na quantidade de *Bacillus* sp., diminuição da contagem de fungos presentes no camarão, além de 100% de prevalência de colônias sacarose positiva em detrimento da sacarose negativa ao final do ciclo de cultivo. Conclui-se que a adição de rotífero em quatro frequências durante a fase de berçário de 42 dias no sistema de bioflocos se torna uma importante fonte nutricional para *L. vannamei*.

Palavras Chave: Berçário; sistema intensivo; alimento vivo; comunidade planctônica; análise bacteriológica

Abstract

The BFT system can provide an increase in the zootechnical indexes in comparison with the conventional culture system, since it allows working with high stocking densities, and with less water exchange. In addition, this system provides shrimp with better immune response and better control of water quality, but in terms of nutrition, bioflocs are deficient in methionine, PUFA and HUFA. One way to improve the nutritional quality of this microbial floc and consequently the performance of the cultured organisms, is the supplementary addition of microorganisms nutritionally rich in amino acids and fatty acids to the system. The rotifer *B. plicatilis* fits these requirements, as it has the capacity to tolerate a wide range of salinity, it has slow mobility and rapid reproduction, and these characteristics contribute to the use of this organism as supplementary food for the initial stages. The objective of this work was to evaluate the effect of adding *B. plicatilis* on *L. vannamei* postlarvae culture in biofloc system. The experiment lasted 42 days with four treatments: BFT (biofloc no addition of *B. plicatilis*); BFT-1F (frequency of adding *B. plicatilis* on the 1st day of culture); BFT-2F (frequency of adding *B. plicatilis* on the 1st and 10th day of culture) and BFT-4F (frequency of adding *B. plicatilis* on the 1st, 10th, 20th and 30th day of culture) each in triplicate. The experimental units with 40 L of useful volume and stocking density of 3000 animals m^{-3} in experimental units. Water quality variables are not shown differences between treatments. However, the variables of zootechnical performance in BFT-4F treatment show the best final weight (0.73g), yield (1.88kg/m^3) and specific growth rate (14.31% day). In relation to the plankton community, the phylum that had the greatest growth were Chlorophyte and Protozoa. For Vibrio density, there was a reduction at the final time in relation at initial experimental time, the BFT-2F treatment with the largest number of colonies at the end. Therefore, the addition of rotifers in four frequencies in the biofloc system becomes a good option for the nursery of *L. vannamei*.

Key words: Nursery; intensive system; live food; planktonic community; bacteriological analysis

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1. Introdução

O camarão marinho *Litopenaeus vannamei* é a espécie de crustáceo mais cultivada no mundo, onde no ano de 2018 representou 52,9 % da produção total deste grupo, com 4,9 milhões de toneladas (FAO, 2020). No Brasil, sua produção foi de 46 mil toneladas em 2018, sendo a região nordeste responsável por quase a totalidade da produção nacional (98,8%) (IBGE, 2019).

No entanto, nos últimos anos é notória a queda de produtividade em sistemas tradicionais de cultivo de camarões marinhos, devido ao efeito dos organismos patogênicos, que é um dos maiores desafios para esta atividade (BECERRA-DÓRAME et al., 2012). Dentre as bacterioses, as causadas pelo gênero *Vibrio* (RUANGPAN e KITAO, 1991), são as mais patogênicas para o camarão, provocando sinais clínicos como: musculatura opaca, expansão dos cromatóforos e necrose (SONG et al., 1993). Neste sentido, a busca por sistemas de cultivo como maior biosseguridade e com possibilidade de convivência com os patógenos se torna necessário.

Os sistemas de mínima troca de água como bioflocos e mixótroficos são compostos por agregados orgânicos (flocos) tendo uma variedade de organismos como: microalgas, bactérias, rotíferos, protozoários entre outros (AVNIMELECH, 2012). Este floco pode proporcionar um aumento nos índices zootécnicos em comparação com o sistema convencional de cultivo, pois permite trabalhar com altas densidades de estocagem, e com uma menor troca de água, desde que alguns requisitos sejam respeitados, tais como: sistema de aeração eficiente, controle da alcalinidade e pH, estímulo a comunidade heterotrófica e nitrificante (AVNIMELECH, 2015).

Para estimular a formação microbiana saudável no ambiente de cultivo pode-se utilizar probióticos e prebióticos (GATESOUPE, 2008). Os probióticos são microrganismos que afetam beneficamente os hospedeiros (camarões, peixes e etc), melhorando a microbiota intestinal, além da produção de ácido lático (COLLINS e GIBSON, 1999). Já um prebiótico, é um ingrediente alimentar não digerível ou parcialmente digerível que serve de substrato para as bactérias no cólon (GIBSON e ROBERFROID, 1995).

A combinação de probiótico e prebiótico (fonte de carbono orgânico - farelo de arroz, trigo e/ou soja) após o processo fermentativo ou respiração microbiana é uma ferramenta bastante interessante em sistema de mínima troca de água como os bioflocos, pois os microrganismos podem melhorar a solubilidade do teor de fibras, proteínas, lípideos e carboidratos em água, aumentando seu uso potencial como fonte de carbono neste sistema (ROMANO et al., 2018).

Esta combinação é benéfica para o hospedeiro, melhorando a sobrevivência e a proliferação de microrganismos no trato gastrointestinal, estimulando o crescimento ou ativando o metabolismo de bactérias que são promotoras de saúde (GIBSON e ROBERFROID, 1995). Segundo Li et al. (2009), esta combinação pode melhorar significativamente a resistência às doenças porque aumenta o *status* imune do animal.

Além do aumento da resposta imune em camarões e controle da qualidade da água, os flocos microbianos do ponto de vista nutricional, possuem altas concentrações de proteínas e aminoácidos, mas apresentam deficiência de metionina, PUFA (ácidos graxos poli-insaturados) e HUFA (ácidos graxos altamente insaturados) (ABREU et al., 2019; VALLE et al., 2015)

Esta composição nutricional está fortemente relacionada aos microrganismos que compõem o floco, além de fatores, como: intensidade luminosa e quantidade de nutrientes disponíveis (EMERENCIANO et al., 2011). Uma forma de melhorar a qualidade nutricional deste floco microbiano e, consequentemente, o desempenho dos organismos cultivados é a adição suplementar de microrganismos nutricionalmente ricos em aminoácidos e ácidos graxos ao sistema (CRAB et al., 2012; EKASARI et al.,2014; MALIWAT et al., 2017; SHAH et al., 2018).

Diferentes fontes de alimento vivo ou inerte já foram testadas nas fases iniciais de peixes e crustáceos, com o objetivo de melhorar a qualidade nutricional. Para selecionar um alimento vivo adequado tem que ser considerado o tamanho, facilidade no cultivo e o valor nutricional, em termos de aminoácidos e ácidos graxos (LAVENS et al., 2000; BARROS e VALENTI, 2003). O rotífero *Brachionus plicatilis* se enquadra nestes requisitos e tem sido utilizado como fonte de alimento vivo para as fases iniciais dos peixes e camarões (SEIXAS-FILHO et al., 2000).

O rotífero tem capacidade de tolerar uma ampla faixa de salinidade, apresenta mobilidade lenta e rápida reprodução, sendo estas características que contribuem para a utilização deste organismo como alimento suplementar para as fases iniciais (HOFF e SNELL, 2001; SORGELOOS e LAVEN, 1996; LUBZENS et al., 1997; LUBZENS e ZMORA, 2003). O *B. plicatilis* possui em sua composição entre 480–590 g de proteína bruta e 61–142 g de lipídios por quilograma de matéria seca, sendo o perfil de PUFA entre 25-35 g de EPA e entre 63–311 g de DHA para cada quilograma de ácidos graxos totais quando alimentando com as microalgas *Nannochloropsis* sp. e *Isochrysis galbana* na concentração de $7x10^6$ células ml^{-1} (DEMIR e DIKEN, 2011; JEEJA et al., 2011).

O valor nutricional do *B. plicatilis* também está altamente correlacionado com o tipo de alimento que é ofertado (LUBZENS et al., 1995). As microalgas são uma fonte de alimento para rotíferos e larvas (FERREIRA et al., 2008; MATSUNARI et al., 2012), e a alga *Nannochloropsis oculata* é muito utilizada na alimentação de rotíferos principalmente devido ao seu alto valor nutricional, e por ser bastante rica em ácidos graxos insaturados (HUFA-n-3) e vitamina B_{12} (WATANABE et al., 1983; GALVÃO et al., 1996).

Alguns estudos mostraram que a adição de plâncton no sistema de berçários tem contribuído para a melhora do desempenho zootécnico do camarão. Brito et al. (2016) demonstraram que pós-larvas de *L. vannamei* em sistema berçário com bioflocos e adição do *B. plicatilis* mais a microalga *Navicula* sp. obtiveram um peso médio final superior ao sistema sem adição destes microrganismos. Segundo Najmi et al. (2018), pós-larvas de camarão alimentadas com *B. plicatilis* enriquecida com probióticos apresentaram um melhor crescimento e sobrevivência.

Entretanto, apesar das contribuições do sistema de mínima troca de água como bioflocos à produção do camarão marinho nas últimas décadas (AVNIMELECH, 2012; HASLUN et al., 2012), informações sobre o efeito da frequência da adição de zooplâncton, em relação ao desempenho zootécnico e crescimento microbiano (*Bacillus Vibrio* e fungos) ainda não estão disponíveis.

1.2 Objetivos

1.2.1 Objetivo geral

Avaliar o efeito da frequência de adição do *Brachionus plicatilis* sobre o cultivo de pós-larvas de *L. vannamei* em sistema de bioflocos.

1.2.2 Objetivos específicos

- Determinar a melhor frequência de inoculação da *B. plicatilis* no cultivo;
- Avaliar os parâmetros físico-químicos da qualidade de água ao longo do cultivo;
- Identificar e quantificar a comunidade fitoplanctônica e zooplanctônica ao longo do cultivo em sistema de bioflocos com adição em diferentes frequências de inoculação do zooplâncton *B. plicatilis;*
- Avaliar o desempenho zootécnico dos camarões;
- Determinar a contagem bacteriana (*Vibrio* e *Bacillus*) e de fungos presente na água e nos camarões;
- Determinar a contagem total de hemócitos nos camarões.

2 - Artigo Científico

Parte dos resultados obtidos durante o trabalho experimental desta dissertação está apresentada no artigo intitulado “Effect of different frequencies of the addition of *Brachionus plicatilis* on the performance of *Litopenaeus vannamei* in a nursery biofloc system with rice bran (anaerobic and aerobic fermentation) as an organic carbon source” (manuscrito), que se encontra anexado.

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Effect of different frequencies of the addition of *Brachionus plicatilis* on the performance of *Litopenaeus vannamei* in a nursery biofloc system with rice bran (anaerobic and aerobic fermentation) as an organic carbon source

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ABSTRACT

The aim of the present study was to evaluate the effect of the different frequencies of the addition of *Brachionus plicatilis* on growth, water quality, plankton composition, *Vibrio, Bacillus,* yeast and total hemocyte count in a nursery biofloc system with rice bran (anaerobic and aerobic fermentation) as an organic carbon source for *Litopenaeus vannamei*. Four treatments were used: BFT (biofloc); BFT-1F (addition of 30 org ml^{-1} of *B. plicatilis* on the 1st day); BFT-2F (addition of 30 org ml^{-1} of *B. plicatilis* on the 1st and 10th days) and BFT-4F (addition of 30 org ml^{-1} of *B. plicatilis* on the 1st, 10th, 20th and 30th days). All experiments were performed in triplicate. The shrimp (PL_{10} 3.0 ± 0.02 mg) were stocked at a density of 3,000 m^{-3} and fed a commercial feed (45% crude protein and 8% lipids). The biofloc system was fertilized every three days for 42 days with anaerobic and aerobic rice bran using a microbial mix with 6.5 X 10^7 CFUs g^{-1} *Bacillus subtills,* 2.1 X 10^7 CFUs g^{-1}*B. Licheniformes* and 3.7 X 10^7 CFUs g^{-1} *Bacillus* strains. The BFT-4F treatment led to a higher final weight (0.73 g), yield (1.88 kg/m^3) and specific growth rate (14.31% day). No significant differences ($p \geq 0.05$) in water quality parameters were found among the four treatments. The main plankton groups were Chlorophyta and Protozoa. The microbiological analysis revealed an increase in the amount of *Bacillus* sp., a decrease in the count of fungi in the shrimp and a decrease in sucrose-negative colonies at the end of the experiment compared to the initial culture in both the water and shrimp. The addition of rotifers four times to nursery biofloc system led to better performance variables, indicating the benefit of these organisms as a natural food source for *L. vannamei* postlarvae.

Keywords: Nursery; intensive system; live food; planktonic community; bacteriological analysis

1. Introduction

The production of the whiteleg shrimp, *Litopenaeus vannamei*, in Brazil was approximately 46 thousand tons in 2018 (IBGE, 2019) and most of this production was conducted in semi-intensive systems with lower biosecurity. However, this type of shrimp farming has experienced diminished production in recent years due to problems related to pathogenic organisms (Becerra-Dórame et al. 2012), such as the outbreak of viruses and bacterial diseases (Lightner et al. 2012; Thitamadee et al. 2016). The main diseases are White spot syndrome disease (WSSD) and bacterial diseases cause by *Vibrio* spp. (Sánchez-Paz, 2010; Tran et al. 2013; Kondo et al. 2015).

The use of a production system with greater biosecurity has become important in this scenario of disease outbreaks. Biofloc technology (BFT) is an organic aggregate composed of bacteria, phytoplankton and zooplankton that develop stimulated by the addition of organic carbon and with the minimal exchange of water (Avnimelech, 2009; De Schryveret al. 2012; Samocha et al. 2017). Moreover, the combination of prebiotic and prebiotic (source of organic carbon – rice bran and soybean meal) after the anaerobic and/or aerobic process is useful in a minimal water exchange system, such as BFT, as the microorganisms improve the solubility of the bran-based carbon source in water (Romano et al. 2018).

These microbial aggregates favor better productive outcomes, such as a greater final weight, greater growth rate and smaller feed conversion rate, in comparison to traditional culture systems (Avnimelech, 2009; Pérez-Fuentes et al. 2013; Zhao et al. 2012). Besides the enhancement of the immune response in the shrimp and the control of water quality, microbial flocs have high concentrations of proteins and amino acids, but are deficient in methionine, polyunsaturated fatty acids (PUFAs) and highly unsaturated fatty acids (HUFAs) (Abreu et al. 2019; Valle et al. 2015).

This nutritional quality is strongly associated with the microorganisms that compose the floc (Emerenciano, 2011). Thus, the addition of microorganisms (phytoplankton and zooplankton) in the initial phases of shrimp culture can help improve the nutritional composition of microbial flocs. Abreu et al. (2019) found greater concentrations of PUFAs in microbial flocs with the addition of *Navicula* sp. during the culture cycle. *Brachionus plicatilis* has 480 to 590 g of crude protein and 61 to 142 g

of lipids per kilogram of dry matter, with a PUFA profile of 25 to 35 g of EPA and 63 to 311 g of DHA per kilogram of total fatty acids when feeding on the microalgae *Nannochloropsis* sp. and *Isochrysis galbana* at a concentration of 7 x 10^6 cells ml^{-1} (Demir and Diken, 2011; Jeeja et al. 2011).

Besides their nutritional characteristics, rotifers are able to tolerate a broad salinity range, have low mobility and fast reproduction, which are essential characteristics for a live supplementary feed (Hoff and Snell, 2001; Sorgeloos and Laven, 1996; Lubzens et al. 1997; Lubzens and Zmora, 2003). Moreover, *B. plicatilis* may have an antibacterial effect, as microalgae commonly used with rotifer feeds produce metabolites that can potentially be converted into bioactive antibacterial compounds (Farisa et al. 2019).

Despite the contributions of BFT to marine shrimp production in recent decades, information on the effect of different frequencies of the addition of zooplankton on shrimp performance are not yet available. Therefore, the aim of the present study was to evaluate the effect of the different frequencies the addition of *Brachionus plicatilis* on growth, water quality, plankton composition, *Vibrio, Bacillus,* yeast and total hemocyte count in a nursery biofloc system with rice bran (anaerobic and aerobic fermentation) as an organic carbon source for *Litopenaeus vannamei*.

2. Materials and Methods

2.1 Experimental conditions

An indoor trial was conducted for 42 days at the Sustainable Mariculture Laboratory (LAMARSU) of the Fisheries and Aquaculture Department (DEPAq) of the Rural Federal University of Pernambuco (UFRPE), Recife, Brazil. The experimental design was completely randomized with four treatments: BFT (biofloc); BFT-1F (addition of 30 org ml^{-1} of *B. plicatilis* on the 1^{st} day); BFT-2F (addition of 30 org ml^{-1} of *B. plicatilis* on the 1^{st} and 10^{th} days); and BFT-4F (addition of 30 org ml^{-1} of *B. plicatilis* in 1^{st}, 10^{th}, 20^{th} and 30^{th}). All experiments were conducted in triplicate.

A matrix tank with water salinity of 30 g L^{-1} was chlorinated with 13 mg L^{-1} (chlorine). After 72 hours of aeration, organic fertilization (24 h in an anaerobic phase followed by 24 h of an aerobic

phase) was initiated with six applications. The organic fertilizer was composed of rice bran < 200 µm (20 g m^{-3}), molasses (2 g m^{-3}), sodium bicarbonate (4 g m^{-3}), a bacterial product (0.05 g m^{-3}) containing 6.5 x 10^7 CFUs/g of *Bacillus subtilis*, 2.1 x 10^7 CFUs/g of *Bacillus licheniformis* and 3.7 x 10^7 CFUs/g of *Bacillus sp* (Kayros Ambiental e Agrícola, SP, Brazil) and seawater (10 ppm chlorine) at ten times the amount of rice bran. The fertilizer was added with a three-day interval between applications.

At the end of the organic fertilizer process, the water from a matrix (0.45 mg L^{-1} total ammonia nitrogen, 0.3 mg L^{-1} $N\text{-}NO_2$, 1.44 mg L^{-1} $N\text{-}NO_3$, 140 mg alkalinity $CaCO_3$ L^{-1}, 20.51 mg L^{-1} orthophosphate and pH 8.4) was mixed and equally distributed to fill twelve black plastic tanks (40 L). The organic fertilizer was added at different quantities from week 0 to 2 (rice bran < 200 µm 20 g m^{-3}, molasses 2 g m^{-3} and sodium bicarbonate 4 g m^{-3}), week 2 to 4 (rice bran < 200 µm 15 g m^{-3}, molasses 1.5 g m^{-3} and sodium bicarbonate 3 g m^{-3}) and week 4 to 6 (rice bran < 200 µm 10 g m^{-3}, molasses 1 g m^{-3} and sodium bicarbonate 2 g m^{-3}), with the composition described above and application three times a week.

The experimental units were constantly aerated (> 5.0 mg L^{-1}); temperature was controlled (30 to 32°C) with a thermostat (100 W/40 L) and light intensity was kept at 27 µmol m^{-2} s^{-1} with a natural photoperiod. No water exchange occurred out during the experimental period, although dechlorinated freshwater was added to compensate evaporation. Sodium bicarbonate (relative total neutralization power of 56%) was added to maintain alkalinity > 150 mg L^{-1} and pH > 7.5 (Furtado et al. 2011).

2.2. Production and addition of *Brachionus plicatilis*

Rotifers (*Brachionus plicatili*s "large" strain [average size: 198 µm]) were obtained from the Live Food Production Laboratory (LAPAVI) – DEPAq – UFRPE. The rotifers were cultured in a transparent glass conical cylinder with a volume of 150 L and light intensity maintained at 40 µmol m^{-2} s^{-1} using a fluorescent lamp with a constant photoperiod. Culture water with 35 g L^{-1} salinity was initially chlorinated with 15 ppm chlorine, with constant aeration, pH 7.5 and temperature 30 ± 1°C. A total of 0.5 mL L^{-1} of vitamins (Citoneurin 500 - thiamine hydrochloride [vitamin B1] and

pyridoxine hydrochloride [vitamin B6]) and 0.1 g L^{-1} of fish oil (360 mg of EPA and 240 mg of DHA)) were added weekly and suspended three days before use in the experimental units. The rotifers were with microalgae *Nannochloropsis* sp. (50 x 10^4 cell mL^{-1}) in the exponential growth phase. The organisms were filtered using a 50-micro mesh (with approximate body sizes between 150 and 250 µm) and density was estimated in a Sedgewick-Rafter chamber to enable the addition of the organisms to the experimental units. Rotifers (30 org mL^{-1}) were added on the 1st, 10th, 20th and 30th days to the treatments, corresponding to an addition of approximately 1 L in each experimental tank, based on Brito et al. (2016).

2.3. Water quality

Dissolved oxygen, temperature, salinity and pH were monitored (YSI model 556, Yellow Springs, Ohio, USA) twice a day (08:00 a.m. and 04:00 p.m.). Settleable solids (SS) (Imhoff cone) (Avnimelech, 2009) were monitored three times a week. Total ammonia nitrogen (TAN) (APHA, 2012), N-nitrite (N-NO_2) (Fries, 1971) and alkalinity (mg L^{-1} $CaCO_3$) (APHA, 2012) were monitored once a week. N-nitrate (N-NO_3) (APHA, 2012) and orthophosphate (PO_4^3) (APHA, 2012) were monitored every fifteen days.

2.4. Shrimp stocking, feeding and monitoring

L. vannamei postlarvae (3.0 ± 0.02 mg) were obtained from a commercial shrimp hatchery (Aquatec LTDA, RN, Brazil) and stocked at a density of 3,000 individuals m^{-3} (120 shrimp per 40-L experimental unit) for 42 days. The postlarvae were fed four times a day (08:00 a.m., 11:00 a.m., 02:00 p.m. and 04:00 p.m.) with a commercial shrimp feed containing 45% crude protein, 9.5% lipids, 9.5% fiber, 4% ash and 13% moisture (In vivo Animal Nutrition and Health). The daily feeding rate of 35% of body weight used at the onset of the culture was gradually reduced to 10% of body weight after 42 days based on the Van Wyk (1999) table and adjusted daily according to estimated shrimp consumption and mortality rates.

Shrimp weight was monitored weekly after 21 days of culture to determine growth and adjust the amount of feed offered. At the end of the experiment, biomass gain, mean final weight, feed conversion ratio (FCR), survival and yield were determined based on the following equations: Biomass gain (g) = final biomass (g) − initial biomass (g); Final weight (g) = final biomass (g)/number of individuals at end of evaluation period; FCR = feed supplied/biomass gain; Survival (%) = (number of individuals at end of evaluation period/initial number of individuals) × 100; Yield (kg m^{-3}) = final biomass (kg)/volume of experimental unit (m^3) and PER = total weight gain/total protein intake.

2.5. Phytoplankton and zooplankton community

Phytoplankton and zooplankton were recorded on Days 1, 7, 14, 21, 28, 35 and 42 of culture using 500-mL plastic bottles. The water was filtered through a cylindrical-conical 250-, 125- and 70-µm mesh net with the purpose of reducing the amount of suspended solids in the sample and was then filtered with a 50-µm mesh for zooplankton retention and 15-µm mesh for phytoplankton retention, with concentration in 25-mL containers. Subsequently, a 2.5-mL aliquot was fixed in 4% formalin and stored for further analysis. A Sedgewick-Rafter chamber and binocular optical microscope (Olympus CH30) with magnification of 400x (Pereira-Neto et al. 2008) were used for identification at the genus level with the aid of identification keys for phytoplankton (Hoek et al., 1995; Stanford, 1999; Bicudo and Menezes, 2006) and zooplankton (Boltovskoy, 1999; Bradford-Grieve et al. 1999; Foissner et al. 1999). Phytoplankton was expressed in cells per milliliter (cell mL^{-1}) following the method described by Hötzel and Croome (1999) and zooplankton was expressed in individuals per liter (ind L^{-1}), following the method described in APHA (2012).

Absolute (cells. mL^{-1}) and relative (%) abundances were calculated for each taxon. Relative abundance was calculated with a dominant species defined as accounting for more than 50% of the total number of organisms in the sample (Lobo and Leighton, 1986).

2.6. Microbiological samples and analyses.

Bacteriological analyses were performed at the Aquatic Animal Health Laboratory (LASAq)/Fishery and Aquaculture Department (DEPAq) of the Rural Federal University of Pernambuco (UFRPE) for the quantification of the colony-forming units (CFUs) of *Vibrio* sp., *Bacillus* sp. and fungi in both the water and shrimp samples.

Samples from each treatment were collected at the beginning and end of the trial. The surface water was sampled using sterile Falcon tubes (4.5 mL) and 500 µL were diluted in 4.5 mL of 1% alkaline peptone solution (pH 8.6) (Silva et al. 2019). Shrimp samples were collected at the beginning (whole body of postlarvae) and end (hepatopancreas) of the trial (Vandenberghe et al. 1999), washed in a 70% alcohol solution for 15 seconds, immersed in 1.5% sodium hypochlorite solution with 0.1% Tween-80 for 15 minutes and rinsed three times with sterile water. After disinfection, the material was weighed (0.5 g) and macerated using an alkaline peptone solution.

After homogenization, the samples (water and shrimp) were serially diluted (10^{-1} to 10^{-5}) and inoculated in triplicate by the spread-plate method (Silva et al. 2019) using thiosulfate citrate bile salt sucrose agar (TCBS) for the *Vibrio* sp. count, mannitol egg yolk polymyxine (MYP) agar for the *Bacillus sp*. count and Sabouraud dextrose agar for the fungal count. The plates were incubated at 30°C for 24 h for the quantification of *Bacillus* and *Vibrio* sp. and incubated at 36°C for 72 h for the quantification of fungi. After incubation, total colonies were counted using a colony counter. For the TCBS medium, non-sucrose-fermenting (green *Vibrio*-like) bacteria and sucrose-fermenting (yellow *Vibrio*-like) bacteria were identified and counted (Samocha, 2019). For Sabouraud dextrose agar, CFUs were classified as yeast or filamentous fungi.

The conversion to CFUs/g for the shrimp samples and CFUs/mL for the water sample was performed using the following formula:

CFUs/g or CFUs/mL = number of colonies x dilution factor / weight (shrimp) or volume (mL) (water) of the sample.

2.7. Total hemocyte count

At the end of nursery phase, hemolymphs were sampled following the method described by Guertler et al. (2013) using a 1-ml syringe containing 200 µl of precooled anticoagulant modified Alsever solution (MAS) (336 mmol/L of NaCl, 115 mmol/L of glucose, 27 mmol/L of sodium citrate, 9 mmol/L of EDTA, pH 7.2) at a proportion of 1:2 (v:v). For the total hemocyte count (THC), the number of hemocytes was determined in triplicate in 100 µl of diluted hemolymph with 4% formalin using a hemocytometer under a light microscope.

2.8. Statistical analyses

The data were checked for the homogeneity of variances and normality using the Cochran test ($\alpha \leq 0.05$) and the Shapiro-Wilk test ($\alpha \leq 0.05$), respectively. Water quality variables were analyzed using Friedman's test ($p < 0.05$) to compare and rank median results of the three treatments and control. One-way ANOVA was used to analyze production variables and total hemocyte count. Duncan's test ($p < 0.05$) was used to compare and rank mean results of the three treatments and the control. Statistical analyses were performed using Statistica version 12.0 (StatSoft).

The plankton composition data were previously logarithm transformed (log(x+1) for analysis of similarity (ANOSIM) ($p < 0.05$) with 999 permutations to identify differences within and between groups (Clarke, 1993). Cluster analysis (Bray-Curtis similarity) and non-metric multidimensional scaling (NMS) were used to determine similarities in the phytoplankton on the temporal and spatial scales and identify the possible formation of groups. Similarity percentage (SIMPER) analysis was performed to determine the main typifying species of the groups, for which the highest values of the similarity/standard deviation (Sim/SD) ratio were prioritized. This analysis was performed with aid of the PRIMER 6.0 program. For nonparametric statistical data (plankton densities), the Kruskal–Wallis test ($\alpha < 0.05$) and Dunn' test ($\alpha < 0.05$) were used to compare and rank median results of the three treatments and the control.

The results for both water and shrimp microbial counts were tested for normality (Shapiro-Wilk's test) and homogeneity of variance (Levene's test) ($p < 0.05$). Mean values were analyzed using Welch's ANOVA (allowing for unequal variance) and the Games-Howell post hoc test to determine significant differences ($p < 0.05$) between treatments (Zaar, 2013). All statistical analyses were performed with the SPSS® software, version 26 (IBM®, USA).

3. Results

The water quality variables in the tanks are presented in Table 1. The main nitrogen compounds were $N\text{-}NO_3$, which ranged from 5.9 to 7.9 mg L^{-1}, followed by $N\text{-}NO_2$ (0.71 to 0.80 mg L^{-1}) and TAN (0.17 to 0.21 mg L^{-1}). No significant differences ($p \geq 0.05$) among treatments were detected in water quality regarding temperature, dissolved oxygen, pH, salinity, TAN, $N\text{-}NO_2$, $N\text{-}NO_3$, PO_4^3 and SS.

The outcomes in BFT-4F (final weight: 0.73 ± 0.01 g; yield: 1.88 ± 0.10 kgm^{-3}; specific growth rate [SGR]: 14.31% day^{-1}) were higher than those in the BFT treatment (final weight: 0.61 ± 0.02 g; yield: 1.60 ± 0.14 kgm^{-3}; SGR: 13.92% day^{-1}) ($p < 0.05$). Survival rates were all 86 to 92% during the 42-day experimental period and did not differ significantly ($p \geq 0.05$) among treatments. The FCR and PER did not differ significantly ($p \geq 0.05$) among treatments (Table 2).

In the present study, the phytoplankton community was represented by ten genera distributed among Chlorophyta (n = 4), Heterokontophyta (n = 3), Cyanophyta (n = 2) and Dinophyta (n = 1). Chlorophyta was dominant, with relative abundances higher than 95%. No significant differences in phytoplankton density were found among the treatments ($p \geq 0.05$). However, significant differences were found among evaluation days ($p < 0.05$), with Day 0 differing from Days 28 and 35. Minimum density was 16,007 cells mL^{-1} on Day 14 in the BFT-2F treatment and maximum density was 74,369 cells mL^{-1} on Day 21 in the BFT treatment (Figure 1). ANOSIM revealed no significant differences in the phytoplankton community among the treatments (global R = -0.106). However, dissimilarities were found among evaluation days (global R = 0.818) (Table 3). The cluster analysis of the phytoplankton community enabled the identification of three groups at a cutoff of 75%: Day 0 (Group

I), Day 7 (Group II) and Days 14, 21, 28, 35 and 42 (Group III). The MDS highlighted the distances among these groups (Figure 2). SIMPER analysis demonstrated the variation in the contribution of the species throughout the culture days. *Nannochloropsis* sp. and *Mychonastes* sp. were the main contributors and were present in all samples. In contrast, *Chaetoceros* sp. and *Pyrophacus* sp. contributed significantly only in the initial phase of culture (Day 0) and *Thalassiosira* sp. contributed significantly only on the last day (Day 42) (Figure 3A).

The zooplankton community was represented by nine genera distributed among Ciliophora (n = 4), Sarcodina (n = 1), Rotifera (n = 2), Amoebozoa (n = 1) and Nematoda (n = 1). Ciliophora was dominant, with relative abundances higher than 70%. No significant differences in zooplankton density were found among the treatments ($p \geq 0.05$). However, significant differences were found among evaluation days ($p < 0.05$), with Days 14 and 21 differing from Day 35. Minimum zooplankton density was 47 ind mL^{-1} in the BFT-2F treatment on Day 7 and maximum density was 185 ind mL^{-1} in the BFT treatment on Day 42 (Figure 4). The ANOSIM revealed no significant differences in the zooplankton community among the treatments (global R = -0.106). However, dissimilarities were found among evaluation days (global R = 0.875) (Table 4). The community in the initial phase (Day 0) and on Day 7 differed most from the community on the other days. Cluster analysis and MDS of the zooplankton revealed the formation of two groups at a cutoff of 80%: Day 0 (Grupo I) and Days 7, 14, 21, 28, 35 and 42 (Group II), with MDS highlighting the distance between these groups (Figure 5). The SIMPER revealed the species that most contributed to similarity throughout the culture days, highlighting *Paramecium* sp. and *Arcella*, with rare contributions from *B. plicatilis, Asplanchna sp.* and *Leprotintinnus sp.* during the 42 days (Figure 2B).

Vibrio-like bacterial colonies were determined in the water samples at the beginning of the trial, with a bacterial count of 366.7 x 10^4 CFUs mL^{-1} (85.04% sucrose-fermenting [yellow] colonies and 14.96% non-sucrose-fermenting [green] colonies). The samples at the end of the trial had mean values ranging from 60.9 to 571.6 x 10^4 CFUs mL^{-1} (100% sucrose-fermenting colonies), with a significant difference ($p < 0.05$) between the BFT-4F treatment and the other treatments. BFT-4F was

the only treatment with a higher total count than that found at the beginning of the trial. The count of *Vibrio*-like bacterial colonies in the animals (postlarvae) at the beginning of the trial was 288.9 x 10^4 CFUs g^{-1} (28.49% sucrose-fermenting colonies and 71.51% non-sucrose-fermenting colonies). At the end of the trial, the mean values among the treatments ranged from 2.07 to 5.91 x 10^4 CFUs g^{-1} (100% sucrose-fermenting [yellow] colonies). Although the BFT-4F treatment had the highest mean and differed significantly from the other treatments ($p < 0.05$), the counts in all treatments were lower at the end of the trial compared to the counts in the initial samples (Table 5).

The mean total count of *Bacillus* sp. colonies in the water samples at the beginning of the trial was 192.2 x 10^4 CFU, whereas the mean values among the treatments at the end of the trial ranged from 416.7 x 10^4 to 456.7 x 10^4, with no significant differences ($p \geq 0.05$). In the shrimp, the count found at the beginning of the experiment (1.39 x 10^4 CFUs g^{-1}) was lower than that found at the end of the trial, with values among the treatments ranging from 1.7 x 10^4 to 6.9 x 10^4 CFUs. The BFT and BFT-4F treatments did not differ significantly from each other (2.07 ± 0.36 x 10^4 and 2.71 ± 1.67 x 10^4 CFUs, respectively), but had significantly lower values ($p < 0.05$) to that found for BFT-1F (5.91 ± 0.946 x 10^4 CFUs), whereas no significant differences ($p \geq 0.05$) were found between BFT-2F (4.87 ± 1.69 x 10^4 CFU) and the other treatments (Table 6).

The mean fungal count in the water samples was 5.17 x 10^4 CFUs ml^{-1} at the beginning of the trial and no significant difference was found among the treatments at the end of the trial, with the counts ranging from 5.20 to 6.80 x 10^4 CFUs ml^{-1}. However, when classifying CFUs into yeasts and filamentous fungi, the percentage of filamentous fungi in the BFT treatment was significantly higher than that found in the other treatments. The mean fungal count in the animal samples was 0.24 x 10^4 CFUs g^{-1} at the beginning of the experiment, whereas values at the end of the trial ranged from 0.04 to 2.02 x 10^4 CFUs g^{-1}, with the highest mean found in the BFT treatment, differing significantly from the other treatments ($p < 0.05$). No significant differences among treatments were found when evaluating the percentages of yeasts and filamentous fungi in the animal samples (Table 7).

The THC ranged from 598.58 to 778.14 $x10^4$ cells mL^{-1}, with no significant differences ($p \geq 0.05$) among treatments at the end of the nursery phase (Table 8).

4. Discussion

The water quality variables (dissolved oxygen, temperature, pH and salinity) remained within the ideal range for *L. vannamei* (Van Wyk and Scarpa, 1999). Ammonia nitrogen is one of the main factors that can cause mortality and immunological depression in shrimp, increasing the susceptibility to pathogens, such as WSSV, when the animals are excessively exposed to high concentrations (Jiang, 2004). In the present study, TAN values remained between 0.17 and 0.21 mg L^{-1}, which is within the ideal range for shrimp farming in an intensive system with minimal water exchange (Samocha et al. 2017). $N\text{-}NO_2$ concentrations ranged from 0.71 to 0.75 mg L^{-1}, which is considered safe for the farming of *L. vannamei* under the salinity conditions found in the present study. Lin and Chen (2003) report that a concentration of N-nitrite up to 25.7 mg L^{-1} is safe for shrimp in salinity of 35 gL^{-1}. A greater concentration of $N\text{-}NO_3$ in comparison to TAN and $N\text{-}NO_2$ in an intensive system indicates that nitrifying bacteria are present in the environment (Xu and Pan, 2014). Thus, organic fertilization using rice bran-based anaerobic and aerobic processes as the bacterial mix favored nitrification processes in the culture units. Moreover, the addition of *B. plicatillis* seems not to cause any changes in the heterotrophic and/or nitrifying bacterial community in a biofloc system, which could change the dynamics of nitrogen compounds. Similar results have been found with the addition of *B. plicatillis* to shrimp nurseries in a previous study (Brito et al. 2016).

The pH and alkalinity of the water behaved similarly, with a tendency toward a reduction throughout the culture cycle due to the nitrification process stemming from the use of inorganic carbon (Ebeling et al. 2006). However, the weekly addition of sodium bicarbonate to the experimental units maintained these values within the range recommended by Van Wyk and Scarpa (1999) (pH from 7.0 to 8.3 and alkalinity > 100 mg $CaCO_3L^{-1}$). Moreover, the addition of the rotifer had no effect on the reduction in these variables. Settleable solids (< 3.00 $ml.L^{-1}$) were low in comparison to a

biofloc system with the addition of molasses (Samocha et al. 2017) and were not influenced by the addition of the rotifer.

Phosphate enters shrimp farming systems mainly through the offer of feed and accumulates over time (Eaton et al. 1995). According to Samocha et al. (2017), there are no available data on the toxicity of phosphate to shrimp and concentrations higher than 32 mg L^{-1} in growth systems were reported to have no apparent impact. In the present study, the concentration of orthophosphate accumulated throughout the culture cycle in all treatments, with the mean ranging from 33.36 to 44.23 mg L^{-1}, indicating that the addition of rotifers had no effect on this variable.

Studies involving the addition of live feed (phytoplankton and zooplankton) for *L. vannamei* in the nursery phase have demonstrated an increase in growth performance. Using *Artemia* enriched with L-lysine and DL-methionine as supplementary feed, Bahabadi et al. (2018) obtained a greater final weight in comparison to shrimp fed *Artemia* enriched with only with DHA. With the combined addition of diatoms (*Navicula* spp.) and rotifers (*B. plicatilis*) every five days, Brito et al. (2016) obtained a greater final weight and an increase in the body protein content of shrimp in comparison to a biofloc system without the addition of plankton. Moreover, Jamali et al. (2015) found that the combination of *Artemia nauplii* and *B. plicatilis* resulted in better shrimp growth compared to animals fed *Artemia* alone.

Essential amino acids and highly unsaturated fatty acids assist in shrimp growth performance (Martins et al. 2016) and rotifers have good quantities of these nutrients (Demir and Diken, 2011; Jeeja et al. 2011), which is one of the possible explanations for the better performance with the BFT-4F treatment in comparison to the other treatments. The BFT-4F treatment led to a better final weight (0.73 ± 0.01 g), SGR (14.31 ± 0.08%/day) and productivity (1.88 ± 0.10 kg m^{-3}) compared to the BFT treatment. In contrast, the FCR, PER and survival were similar among the treatments. The greater final weight in BFT-4F demonstrates the potential use of *B. plicatillis* as a supplemental source for the nursery phase of *L vannamei*, likely due to the composition (DHA, EPA and amino acids) in rotifers fed *Nannochloropsis* sp. and the addition of a fatty acid source, since rotifers are used as

bioencapsulation vehicles. Thus, the addition of *B. plicatillis* at a frequency of four times (BFT-4F) during the nursery phase is a feeding strategy that contributes to improving the productivity of *L. vannamei* in a biofloc system. The frequency of the addition of rotifers in the present study (four times) was half the frequency used by Brito et al. (2016) (eight times in 35 days in the nursery phase), but productivity was similar in the two studies, indicating the possibility of reducing production costs with live feed.

Even in a biofloc system, where bacteria dominate, phytoplankton and zooplankton are found. However, Cyanophyta generally predominates among phytoplankton (Marinho et al. 2014; 2017; Manan et al., 2016; Campos et al., 2019), whereas Rotifera (Marinho et al., 2014) and Nematoda (Rajkumar et al., 2015) generally predominate among zooplankton. Cyanophytes are undesirable, as these organisms produce toxins that can cause a reduction in growth, the loss of immunocompetence, abnormal behavior and even the death of shrimp (Hwang et al., 1990; Perez-Linares et al., 2003). Chlorophyta dominated in the present study and this group was not a problem for the shrimp, as these microalgae do not have the capacity to produce toxins (Graham and Wilcox, 2000) and have good nutritional quality (Muller-Feuga et al., 2003). Moreover, we found an increase in the cell density of *Heterokontophyta* throughout the culture cycle, which was likely related to the form of organic fertilizer, as a reduction in *Heterokontophyta* occurs in the final composition of the phytoplankton in bioflocs fertilized with sugarcane molasses *in natura* (Ray et al., 2010; Marinho et al., 2017). This increase in *Heterokontophyta* throughout the culture cycle may contribute to shrimp growth due to its good nutritional composition (Khatoon et al., 2009; Abreu et al., 2019).

In the present study involving rice bran (anaerobic and aerobic) as an organic carbon source and a bacterial mix, the mean concentration of zooplankton (≥ 80 ind mL) was very high compared to the systems involving the use of sugarcane molasses *in natura* (~3.35 ind mL^{-1} [Marinho et al., 2014]; ≤ 10 ind mL^{-1} [Manan et al., 2016]). Zooplankton plays an important role in shrimp farming, as it transfers nutrients through the consumption of algae and bacteria, significantly contributing to the nutritional needs of shrimp (Focken et al., 1998). Ciliates are used as a nutritional source in the

early stages by shrimp, which can ingest ~ 4000 ciliates per individual per day (Nagano and Decamp, 2004). Ciliates feed on bacteria, algae, fungi and soluble organic substances (Fedonenko et al., 2017). According to Boechat et al. (2005), *C. paramecium* has EPA and DHA contents of 5.9 ± 1.2 µg mg^{-1} and 0.94 ± 0.53 µg mg^{-1}, respectively. Thus, ciliates are a source of natural feed in biofloc systems.

A biofloc system is usually dominated by bacteria, which participate in the complex interaction among organic matter, the physical substrate and a large range of microorganisms, such as phytoplankton and zooplankton (Emerenciano et al., 2013). The taxonomic composition varies among systems and even within the same system over time. Several factors can alter the floc composition, such as temperature, salinity, pH, photoperiod, the intensity of vertical mixing and the type of organic carbon available for bacterial metabolism. Some strains of bacteria, such as *Bacillus* sp., serve as a natural probiotic, inhibiting the growth of pathogenic bacteria (Emerenciano et al., 2013; Samocha, 2019), which makes these species an important component of the system.

The regular application of the bacteria-based product composed of *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus* sp. during the experiment period resulted in similarity among the treatments regarding the count of *Bacillus* sp. in the water samples. The animal samples revealed a different scenario, as treatments with the rotifer *B. plicatilis* had higher CFU g^{-1} counts compared to the BFT treatment. An explanation for this result is that by grazing on the biofloc particles (Ray et al., 2010) and being immersed in water inoculated with the bacteria-based product, the rotifer served as a vector for probiotic bacteria, assisting the transportation of the microorganisms in the water for colonization of the hepatopancreas. Similar results were described in a study by Jamali et al. (2015), in which *B. plicatilis* immersed in an enrichment solution with 1 x 10^6 CFUs mL^{-1} of *Bacillus* sp. was able to carry the bacteria to the digestive tract of *Litopenaeus vannamei* larvae.

Probiotics, such as *Bacillus* sp., can help control *Vibrio* outbreaks by competing for chemicals, nutrients and adhesion sites, producing antibiotic compounds and enhancing the immune response of the shrimp (Van Hai and Fotedar, 2010). Moreover, an extract of rotifers fed with different microalgae was found to have an inhibitory effect on the growth of *Vibrio harveyi* when tested *in vitro* (Farisa et

al., 2019). For the present study, it is plausible that both the presence of the probiotic and the possible inhibitory aspect of the rotifer had a causal effect on the reduction in the count of *Vibrio*-like bacteria in the animal samples compared to the initial analysis. Analyzing the water samples, although the BFT-2F and BFT-4F treatments had the highest CFU counts of *Vibrio*-like bacteria, all treatments remained within the range recommended by Cuéllar-Anjel et al. (2014) on TCBS agar for the hepatopancreas ($\leq 1 \times 10^5$ CFUs g^{-1}), indicating a positive effect of the addition of the rotifer.

Regarding the characteristics of the colonies identified in the TCBS medium, pathogenic *Vibrio* spp., such as *V. harveyi*, *V. vulnificus* and *V. parahaemolyticus*, usually appear as green (non-sucrose-fermenting) colonies, whereas non-pathogenic strains are identified by the yellow (sucrose-fermenting) appearance (Samocha, 2019). In this trial, non-sucrose-fermenting *Vibrio*-like bacteria were found only in the initial analyses, whereas 100% of the colonies at the end of the trial were yellow (sucrose-fermenting bacteria). These results confirm that sucrose-fermenting *Vibrio* is more abundant in systems in which bioremediation and/or probiotics are applied (Moriarty, 1998).

Aside from bacteria, fungi such as yeasts and molds are also present in biofloc systems. Yeasts in particular have been reported to occur naturally in sea and estuarine water (Walker, 1998) and, as chemoorganotrophic microorganisms, obtain energy from organic carbon through hexose sugars, such as glucose and fructose (Emerenciano et al., 2017), thereby benefiting from the high carbon addition often used to ensure optimum heterotrophic bacteria growth in intensive, minimal-exchange production systems (Avnimelech, 2007). The lower yeast counts when the rotifer was present can be attributed to its grazing habits (Ray et al., 2010) and the composition of its diet (Ben-Amotz et al., 1987) as well as the influence of the zooplankton in the system.

Hemocytes are the most important cells of the immunological system in crustaceans (Vazquez et al., 2009), playing important roles in the immune response when combating the invasion of pathogens (Soderhall, 2016; Tassanakajon et al., 2013). In the present study, the total hemocyte count remained similar among the treatments. In contrast, Abreu et al. (2019) found that the mean hemocyte count was higher in shrimp in tanks with the addition of different densities of the microalga *Navicula*

sp. after a saline stress test compared to shrimp treated with biofloc alone. In the present investigation, the addition of *B. plicatilis* did not exert an influence on higher THC values at the end of the nursery phase, but the shrimp were not submitted to any stress after the cultivation cycle.

5. Conclusion

The addition of the rotifer *B. plicatilis* four times at 10-day intervals as supplemental feed during the 42-day nursery phase of shrimp contributed significantly to the improved performance, especially yield and final weight, with clear benefits for system through increase in *Bacillus* quantity, decrease of fungi concentration in animals and reduction in sucrose-negative colonies in the water and animals during the nursery phase.

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8. Appendices

Table 1. Water quality parameters in the culture of *Litopenaeus vannamei* under nursery biofloc system with the different frequencies addition of *Brachionus plicatilis* during a 42-day experimental period.

Parameters	Treatments			
	BFT	**BFT-1F**	**BFT-2F**	**BFT-4F**
DO (mg L^{-1})	5.04 ± 0.13	5.17 ± 0.08	4.99 ± 0.14	5.14 ± 0.16
DO (%)	78.44 ± 1.92	79.98 ± 1.20	77.90 ± 1.98	79.37 ± 1.86
Temperature (°C)	32.74 ± 2.81	31.42 ± 0.23	31.85 ± 0.24	31.23 ± 0.40
Salanity (g L^{-1})	31.39 ± 0.35	31.40 ± 0.32	32.63 ± 2.95	31.49 ± 0.38
TAN (mg L^{-1})	0.21 ± 0.26	0.17 ± 0.18	0.20 ± 0.17	0.17 ± 0.20
N-NO_2 (mg L^{-1})	0.75 ± 0.30	0.71 ± 0.29	0.80 ± 0.33	0.72 ± 0.32
N-NO_3 (mg L^{-1})	7.90 ± 8.11	5.90 ± 5.67	7.69 ± 8.03	7.52 ± 7.88
pH	7.89 ± 0.18	7.90 ± 0.19	7.89 ± 0.20	7.91 ± 0.20
Alkalinity (mg L^{-1} $CaCO_3$)	131.76 ± 16.28	136.79 ± 17.95	138.10 ± 22.84	136.19 ± 19.70
SS (mL L^{-1})	2.77 ± 3.02	2.42 ± 2.53	2.65 ± 2.03	2.48 ± 2.14
Ortophosphate (mg L^{-1})	40.77 ± 40.36	42.67 ± 31.01	33.36 ± 23.07	44.23 ± 48.39

The data correspond to the mean ± SD. Results were analyzed by friedman test. BFT (biofloc); BFT-1F (one addition of 30 org ml^{-1} of *Brachionus plicatilis* in the 1st day); BFT-2F (two addition of 30 org ml^{-1} of *Brachionus plicatilis* in the 1st and 10th day) and BFT-3F (three addition of 30 org ml^{-1} of *Brachionus plicatilis* in 1st, 10th, 20th and 30th). DO -dissolved oxygen; TAN - total ammonia nitrogen; N-NO_2 - nitrite-nitrogen; N-NO_3 - nitrate-nitrogen; SS - Settleable solids.

Table 2. Nursery shrimp zootechnical parameters with different frequencies addition of *Brachionus plicatilis* during a 42-day experimental period.

Parameters	Treatments			
	BFT	**BFT-1F**	**BFT-2F**	**BFT-4F**
Survival (%)	88 ± 0.10^{a}	92 ± 0.02^{a}	86 ± 0.01^{a}	88 ± 0.02^{a}
Final weight (g)	0.61 ± 0.02^{d}	0.65 ± 0.01^{c}	0.69 ± 0.03^{b}	0.73 ± 0.01^{a}
SGR (% day^{-1})	13.92 ± 0.06^{c}	13.98 ± 0.15^{bc}	14.22 ± 0.10^{ab}	14.31 ± 0.08^{a}
Yield (Kgm^{-3})	1.60 ± 0.14^{b}	1.72 ± 0.13^{ab}	1.78 ± 0.06^{ab}	1.88 ± 0.10^{a}
FCR	1.50 ± 0.20^{a}	1.33 ± 0.07^{a}	1.30 ± 0.04^{a}	1.25 ± 0.09^{a}
PER (%)	1.50 ± 0.20^{a}	1.68 ± 0.08^{a}	1.71 ± 0.05^{a}	1.78 ± 0.14^{a}

The data correspond to the mean ± SD. Results were analyzed by performing ANOVA one way and the Duncan test. Mean values in the same row with different superscripts differ significantly ($p < 0.05$). BFT (biofloc); BFT-1F (one addition of 30 org ml^{-1} of *Brachionus plicatilis* in the 1st day); BFT-2F (two addition of 30 org ml^{-1} of *Brachionus plicatilis* in the 1st and 10th day) and BFT-4F (three addition of 30 org ml^{-1} of *Brachionus plicatilis* in 1st, 10th, 20th and 30th). SGR - specific growth rate; FCR - feed conversion rate; PER- protein efficiency rate.

Table 3. Values concerning to statistic R (p-value) found in analysis of similarity (ANOSIM) for phytoplankton community among the treatments (BFT, BFT-1F, BFT-2F, BFT-4F)) and days (0, 7, 14, 21, 28, 35 and 42). Side by side comparison in the groups by 999 permutations were carried out. R values equal to 1 indicate completely dissimilarity and equal to -1 show completely similarity.

Phytoplankton community among treatments						
Global R = -0.106	BFT		BFT-1F		BFT-2F	
BFT	-					
BFT-1F	-0.078 (0.84)		-			
BFT-2F	-0.075 (0.82)		-0.131 (0.97)		-	
BFT-4F	-0.093 (0,92)		-0.138 (0.99)		-0.113 (0.99)	
Phytoplankton community among days						
Global R = 0.818	0	7	14	21	28	35
0	-					
7	1(0.029)	-				
14	1(0.029)	0.917 (0.029)	-	-		
21	1(0.029)	0.854 (0.029)	0.958 (0.029)	-	-	
28	1(0.029)	0.781 (0.029)	0.74 (0.029)	0.969 (0.029)	-	
35	1(0.029)	0.646 (0.029)	0.76 (0.029)	0.917 (0.029)	0.656 (0.029)	
42	1(0.029)	0.99 (0.029)	0.969 (0.029)	1 (0.029)	1 (0.029)	1 (0.029)

Table 4. Values concerning to statistic R (p-value) found in analysis of similarity (ANOSIM) for zooplankton community among the treatments (BFT, BFT-1F, BFT-2F, BFT-4F) and days (0, 7, 14, 21, 28, 35 and 42). Side by side comparison in the groups by 999 permutations were carried out. R values equal to 1 indicate completely dissimilarity and equal to -1 show completely similarity.

Zooplankton community among treatments						
Global R = -0.114	BFT		BFT-1F		BFT-2F	
BFT	-					
BFT-1F	-0.146 (0.98)		-			
BFT-2F	-0.126 (0.95)		-0.118 (0.90)		-	
BFT-4F	-0.073 (0.74)		-0.082 (0.76)		-0.117 (0.89)	
Zooplankton community among days						
Global R = 0.875	0	7	14	21	28	35
0	-					
7	1(0.029)	-				
14	0.958(0.029)	0.698 (0.057)	-			
21	1(0.029)	1 (0.029)	0.417 (0.057)	-	-	
28	1(0.029)	1 (0.029)	0.646 (0.057)	0.656 (0.029)	-	
35	1(0.029)	1 (0.029)	0.896 (0.029)	1 (0.029)	0.979 (0.029)	-
42	1(0.029)	1 (0.029)	0.823 (0.029)	0,979 (0.029)	0.823 (0.029)	0.729(0.029)

Table 5 - Colony-forming units (CFU) for water and shrimp samples from the TCBS medium of *Litopenaeus vannamei* under nursery biofloc system with *Brachionus plicatilis* addition in different frequencies during 42-day experimental period.

Colony-forming units (CFU)						
	Water			Shrimp		
	CFU (10^4 mL^{-1})	% Sucrose-fermenting (Yellow)	% Non-sucrose fermenting (Green)	CFU (10^4 g^{-1})	% Sucrose-fermenting (Yellow)	% Non-sucrose fermenting (Green)
Initial sample	366.7±171.0	85.04	14.96	288.9± 125.9	28.49	71.51
Treatments						
BFT	60.9±18.1^{a}	100	0	13.3±4.5^{a}	100	0
BFT 1F	76.6±12.7^{a}	100	0	14.9±4.6^{a}	100	0
BFT 2F	237.7±167.7^{a}	100	0	40.7±20.0^{ab}	100	0
BFT 4F	571.6±124.2^{b}	100	0	63.2±10.4^{b}	100	0

Mean values were analyzed by Welch's ANOVA (allowing for unequal variance) and Games-Howell Post-Hoc test to determine differences ($p< 0.05$) between the treatments. BFT (biofloc); BFT-1F (one addition of 30 org ml^{-1} of *Brachionus plicatilis* in the 1st day); BFT-2F (two addition of 30 org ml^{-1} of *Brachionus plicatilis* in the 1st and 10th day) and BFT-4F (three addition of 30 org ml^{-1} of *Brachionus plicatilis* in 1st, 10th, 20th and 30th).

Table 6. Colony-forming units (CFU) for water and shrimp samples from the MYP medium of *Litopenaeus vannamei* under nursery biofloc system with *Brachionus plicatilis* addition in different frequencies during 42-day experimental period.

	Colony-forming units (CFU)	
	Water (10^4 mL^{-1})	**Shrimp** (10^4 g^{-1})
	MYP	**MYP**
Initial sample	192.2±45.10	1.39±0.534
Treatments		
BFT	450.0±144.7[a]	2.07±0.365[a]
BFT 1F	416.7±135.7[a]	5.91±0.946[b]
BFT 2F	456.7±113.7[a]	4.87±1.69[ab]
BFT 4F	425.0±136.6[a]	2.71±1.67[a]

Mean values were analyzed by Welch's ANOVA (allowing for unequal variance) and Games-Howell Post-Hoc test to determine differences ($p< 0.05$) between the treatments.

Table 7. Colony-forming units (CFU) for water and shrimp samples from the Sabouraud medium of *Litopenaeus vannamei* under nursery biofloc system with *Brachionus plicatilis* addition in different frequencies during42-day experimental period.

	Colony-forming units (CFU)					
	Water			**Shrimp**		
	CFU (10^4 mL^{-1})	**% Yeast**	**% filamentous**	**CFU (10^4 g^{-1})**	**% Yeast**	**% filamentous**
Initial sample	5.17±1.63	75.67	24.33	0.24±0.34	53.09	45.91
Treatments						
BFT	6.66±1.56 [a]	79.98 [a]	20.02 [b]	2.02±0.033[b]	70.30 [a]	29.70 [a]
BFT 1F	5.63±1.14 [a]	100.00 [a]	0 [a]	0.073±0.009[a]	94.14 [a]	5.86 [a]
BFT 2F	6.80±1.37 [a]	100.00 [a]	0 [a]	0.051±0.007[a]	100.00 [a]	0.00 [a]
BFT 4F	5.20±1.40 [a]	91.58 [a]	8.42 [a]	0.04±0.020[a]	83.16 [a]	16.84 [a]

Mean values were analyzed by Welch's ANOVA (allowing for unequal variance) and Games-Howell Post-Hoc test to determine differences ($p< 0.05$) between the treatments. BFT (biofloc); BFT-1F (one addition of 30 org ml^{-1} of *Brachionus plicatilis* in the 1st day); BFT-2F (two addition of 30 org ml^{-1} of *Brachionus plicatilis* in the 1st and 10th day) and BFT-4F (three addition of 30 org ml^{-1} of *Brachionus plicatilis* in 1st, 10th, 20th and 30th).

Table 8. Total haemocyte count (THC) in *Litopenaeus vannamei* under nursery biofloc system with *Brachionus plicatilis* in different frequencies addition.

Treatments	THC (x10^4 cél mL^{-1})		
BFT	603.77	±	311.51[a]
BFT-1F	598.58	±	95.59[a]
BFT-2F	778.14	±	162.49[a]
BFT-4F	773.48	±	134.43[a]

The data correspond to the mean ± SD. Results were analysed by performing Duncan test. Mean values in the same row with different superscripts differ significantly ($p < 0.05$). BFT (biofloc); BFT-1F (one addition of 30 org ml^{-1} of *Brachionus plicatilis* in the 1st day); BFT-2F (two addition of 30 org ml^{-1} of *Brachionus plicatilis* in the 1st and 10th day) and BFT-4F (three addition of 30 org ml^{-1} of *Brachionus plicatilis* in 1st, 10th, 20th and 30th).

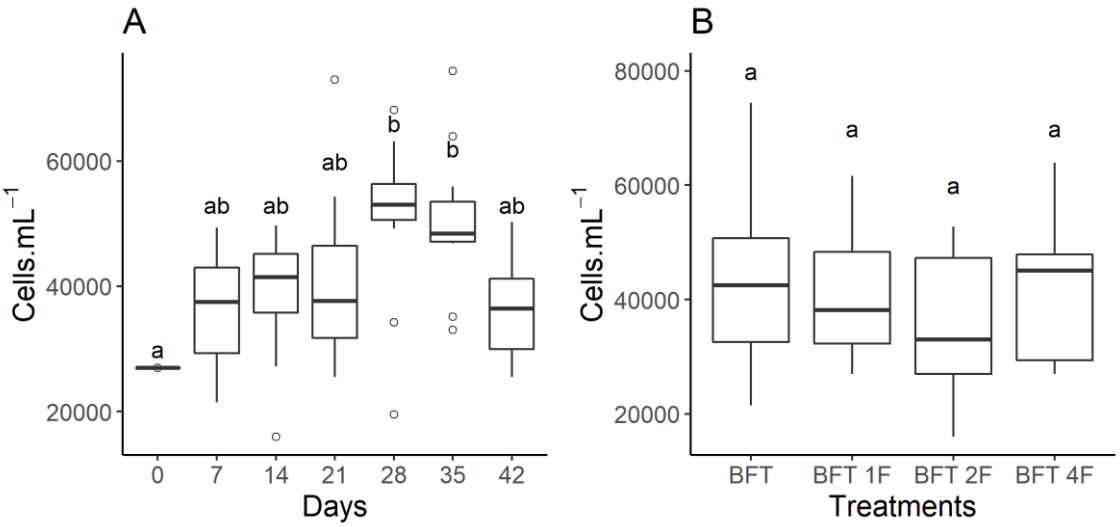

Figure 1. Variation of phytoplankton density (cells mL^{-1}) during cultivation days (A) and between treatments (B). Different letters showed significant differences ($p<0.05$) on statistical Kruskal-Wallis test followed by Dunn test post hoc.

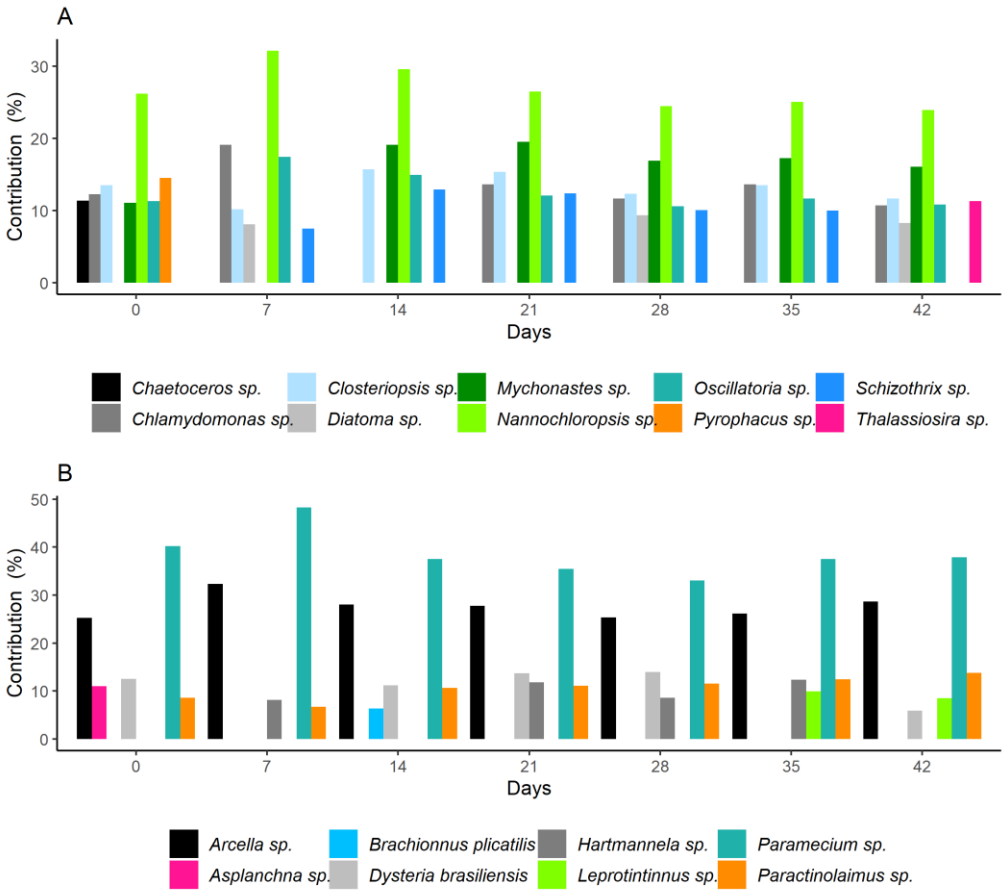

Figure 2. Contribution of genera and / or species during cultivation days for the phytoplankton (A) and zooplankton (B) community through the analysis of SIMPER.

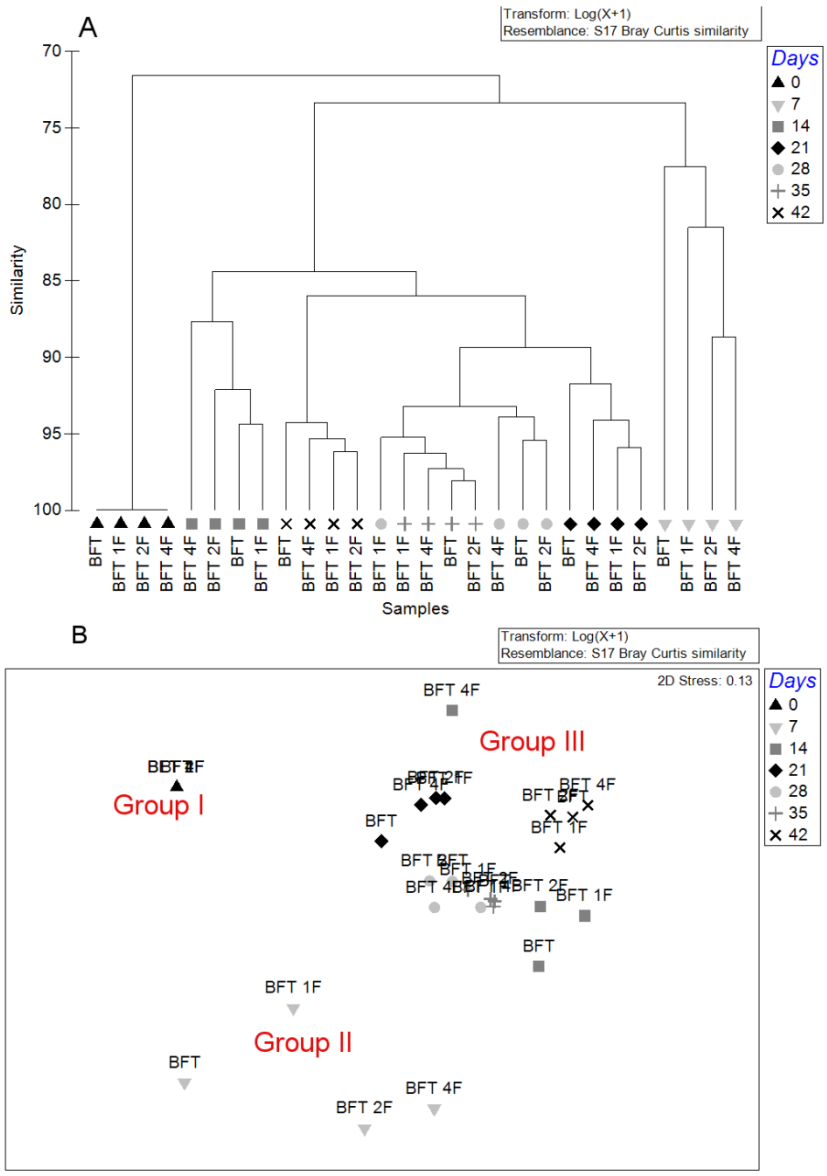

Figure 3. Cluster (A) and MDS (B) analyzes of the phytoplankton community during the cultivation of *L. vannamei* shrimp in BFT system with different inoculation frequencies of the rotifer *Brachionus plicatilis*.

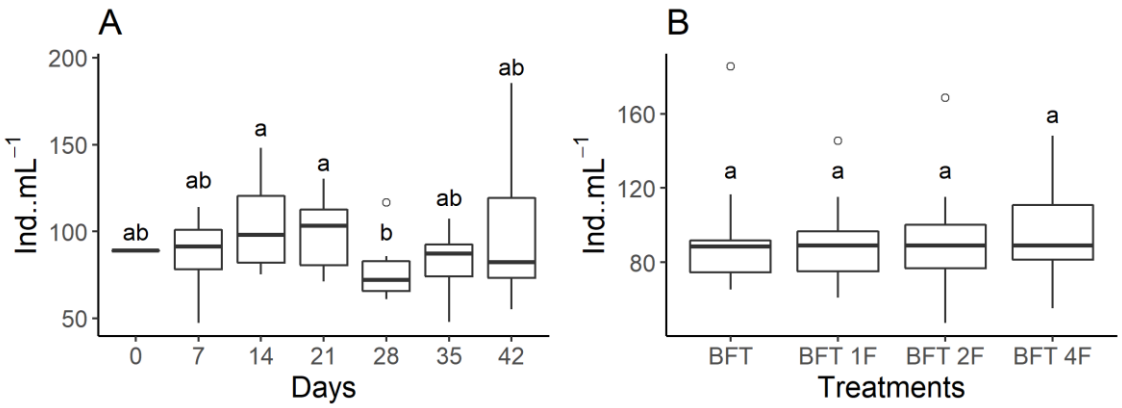

Figure 4. Variation in the density of zooplankton during cultivation days (A) and between treatments (B). Different letters showed significant differences ($p<0.05$) on statistical Kruskal-Wallis test followed by Dunn test post hoc.

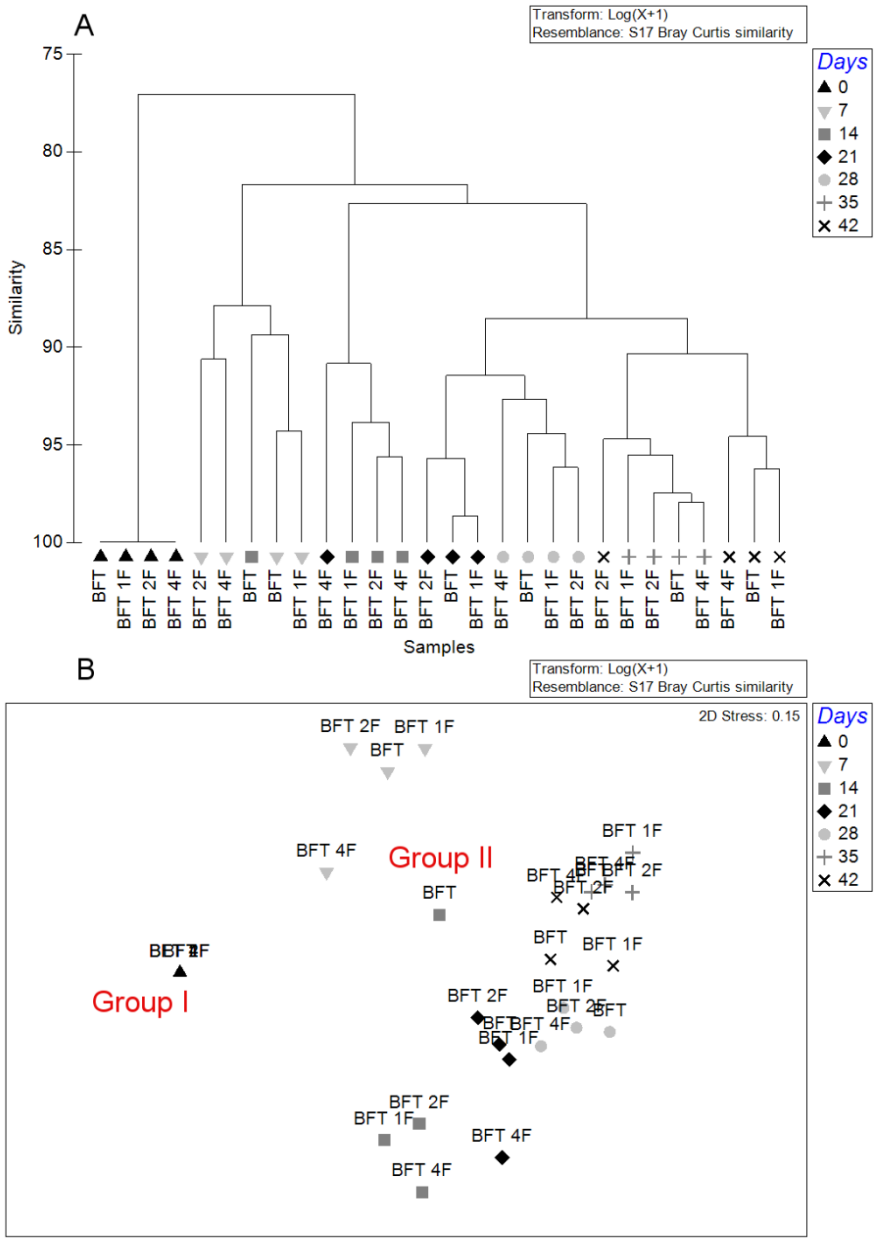

Figure 5. Cluster (A) and MDS (B) analyzes of the zooplankton community during the cultivation of *L. vannamei* shrimp in BFT system with different inoculation frequencies of the rotifer *Brachionus plicatilis*.

3. Considerações finais

No presente estudo foi constatado que a utilização de sistemas intensivos com bioflocos e adição do zooplâncton *B. plicatilis*, mostra-se como uma boa opção para o cultivo em berçário de *L. vannamei*. A adição de quatro vezes de rotífero (*B. plicatilis*) em densidade de 30 org mL^{-1} a cada 10 dias, durante o berçário de camarões marinhos com duração de 42 dias, proveu uma fonte de suplementação alimentar que contribuiu significativamente na melhora do desempenho, em particular, na produtividade e peso final. A fertilização orgânica, a partir de processos anaeróbico e aeróbico juntamente com o mix de bactérias adicionados aos tanques de cultivo, proporcionou a redução da presença de *víbrios* sacarose negativa, além de um aumento de *Bacillus*, crucial ao controle dos compostos nitrogenados.

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