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MICROBIOLOGY

In vitro manipulation of the bacterial community to improve the performance of bioflocs in aquaculture systems

JÉSSICA L.S. DA SILVA, ANA P.C. CARNEIRO, ANNA L.C. BRITO, ANA V.S. OLIVEIRA, JHONES L. VIEIRA, RAQUEL C. SOARES, ROBÉRIO M. DE FREITAS & OSCARINA V. DE SOUSA

Abstract: Although biofloc technology is already recognized as advantageous and practical for aquaculture for the effects of maintaining water quality and improving the health status and resistance of cultivated animals against pathogens, little is known about the way of action involved. This study aimed to evaluate the performance of bacterial groups as inducers in the formation of flocs compared to a system with spontaneous formation. Therefore, three microsystems were built in 3L tanks with constant aeration to induce the biofloc aggregation with addition of bacterial consortiuns with differentiated functions. It was used a control, without addition of bacterial consortium; B1 with addition of probiotic bacteria consortium; and B2, with adding nitrifying bacteria consortium. During the experimental period were evaluated physicochemical variables and quantifications of bacterial cultivable groups: Heterotrophic Bacteria and Vibrio. Also was the microscopic characterization of the flakes and tests of antimicrobial activity against pathogenic bacteria. Systems B1 and B2 showed promising results in relation to control (spontaneous bioflocs), showing more homogeneous flake formation, antimicrobial activity against the tested pathogens and greater biological diversity in the systems. The bacteria used in these tests were able to optimize the formation of microbial aggregates, showing potential for application in cultivation systems, in order to obtain improvements in productivity.

Key words: Microbial aggregates, community, functional bacteria, aquaculture.

INTRODUCTION

Biofloc technology (BFT) is characterized by the formation of microbial flocs through the manipulation of the carbon: nitrogen ratio (C: N) in the water, with high density of organisms, and with zero or minimal water exchange, in highly oxygenated tanks and fertilized with an external carbon source. The systems based on this technology have demonstrated a good productive efficiency, because in addition to reducing the use of water, it also reduces the emission of effluents into the environment (Ahmad et al. 2017, Panigrahi et al. 2018, Avnimelech 2007).

The basis of the BFT system are bioflocs, which are suspended microbial conglomerates or aggregates consisting of microalgae, ciliate, flagellate, protozoa, rotifers, bacteria, remains of dead organisms and debris (uneaten feed and feces) (Avnimelech 2009). All the components interact in the system and have a specific function (Manan et al. 2017). The aggregates cover a wide range of particles of different sizes, presenting an irregular and very fragile shape, being held together by bacterial secretions,

a tangle of filamentous microorganisms and electrostatic interactions (Samocha 2019, De Schryver et al. 2008). Among the microorganisms that compose these aggregates, those of greatest interest and most abundant and diverse are bacteria, (up to 100 million bacteria/mL), since they are responsible for producing adherent mucus (exopolysaccharides) that allows the aggregation of other particles, leading to an increase in the size of the flocs (Lara et al. 2017, Samocha 2019).

Among the bacterial groups present in the flocs, cultivable heterotrophic bacteria (CHB) are dominant because of its accelerated metabolism, since the biomass production per nitrogen unit in heterotrophic is about 40 times greater than autotrophic (nitrifying) (Furtado et al. 2014). Thus, CHB can recycle organic matter through the absorption of toxic nitrogen compounds (ammonia and inorganic nitrite-nitrogen), incorporating into their biomass, as long as organic carbon is available (Samocha 2019, Souza et al. 2014). In addition to the production of microbial protein, an extra source of food for cultivated organisms, which makes it possible to offer a low-protein diet, toxic ammonia is removed from the system, which reduces water renewal during cultivation (Ballester et al. 2010).

Despite all the benefits of using the biofloc system, it is necessary to improve studies that involve the formation and maintenance of aggregates for the management of their effects on cultivation systems, whether in the remediation of nitrogen compounds or aiming an improving on the animal diet (Ray et al. 2010). One way to optimize these systems is the manipulation of the microbiota, through the addition of bacterial strains with probiotic or nitrifying characteristics (Cienfuegos et al. 2017, Ferreira et al. 2017, Silva et al. 2020) and microalgae (Jiménez-Ordaz et al. 2021).

Thus, the present study aimed to verify the capacity of formation and development of induced bioflocs, from the addition of a consortium of probiotic bacteria and a consortium of nitrifying bacteria, compared to a system with spontaneous formation of bioflocs.

MATERIALS AND METHODS

Experimental system

Three experimental groups were evaluated in laboratory conditions, being: control (C), formed by spontaneous development of bioflocs; treatment (B1), formed by the addition of a bacterial consortium with probiotic potential; and treatment (B2), formed by the addition of a consortium of nitrifying bacteria. In all experimental groups the adjustment of the C: N ratio (10: 1) was performed using liquid molasses, with carbon content of approximately 30%, as a carbon source, and shrimp feed, with 35% crude protein content, as a nitrogen source (Lima et al. 2018). This ratio was calculated based on the necessary amount of carbon for the initial formation of bioflocs, according to the methodology described by Avnimelech (1999), based on the percentage of protein in the feed and the feed rates used.

The experimental systems were prepared in tanks each with a volume of 3 L of water from a shrimp nursery *Litopenaeus vannamei*, from the Center of Studies in Coastal Aquaculture - CEAC from the Federal University of Ceará - UFC. The tanks received constant aeration without water renewal during 12 days of experiment.

Formation of bacterial consortia

For the formation of bacterial consortia, two groups of strains were used (Table I). Consortium B1: composed of probiotic bacteria isolated from the intestinal tract of cultivated marine shrimp (*Litopenaeus vannamei*) (Abreu

Table I. Characterization of bacterial strains used in the assembly of consortia.

Consortia	Strain	Enzymatic expression	Aggregation	EPS
B1	*Bacillus* sp.	C-F	+	+
	Bacillus sp.	C-F	+	+
	Isoptericola jiangsuensis	F	+	+
B2	*Rhizobium rosettiformans*	-	+	-
	Pseudomonas sp.	C-F-L	+	+
	Burkholderia sp.	C-F-L-G	+	+

Source: Authors. C - Caseinase; F - Phospholipase; G - Gelatinase; L - Lipase. EPS - Ability to produce exopolysaccharides.

2019); Consortium B2: composed of bacteria with nitrifying capacity, originally isolated from a Nile tilapia cultivation system (*Oreochromis niloticus*) (Silva 2018).

The strains used in this study are part of the microbiological collection Profª Regine Vieira, from the Laboratory of Environmental and Fish Microbiology (LAMAP), at the Institute of Marine Sciences (Labomar), Federal University of Ceará.

For the introduction of bacterial consortia in the systems, 2 mL aliquots of each strain, adjusted to the turbidity equivalent to 0.5 on the McFarland scale (concentration corresponding to 1×10^8 CFU mL^{-1}), were added for each 1000 mL of culture water.

Physicochemical analysis of water quality

The physicochemical indicators of water quality during the period of in vitro cultivation were evaluated according to the methods presented in Table II.

Microscopic characterization of flocs

The development of the biofloc was monitored by optical microscopy during the cultivation period, at times of 24 hours, T5, T7 and T12, using an Olympus CX31 equipment with a focus on the 100x objective, connected to an imaging program. Aliquots of each treatment were removed and placed on a glass slide and taken to the microscope to characterize the forms, aggregation and constitution of microorganisms present (Silva et al. 2020). The observations were made in triplicate.

Microbiological analysis

The evaluation of microbiological quality of the cultivation systems was performed using the Standard Plate Counting technique (Apha 2000), for the quantification of cultivable heterotrophic bacteria (CHB) and *Vibrio* in water, before the beginning of the experiment, and in water and sedimentable solids after 12 days of cultivation. The samples were serially diluted to 10^{-5} using 1% NaCl saline as diluent. Aliquots of 1 mL of each dilution were inoculated, using the pour plate technique, in duplicate, in Plate Counting Agar (PCA) culture medium (Difco®), for the quantification of cultivable heterotrophic bacteria (CHB). To quantify *Vibrio* species, aliquot of 100 µL of each dilution were inoculated, using the spread plate technique, in duplicate, in differential medium Biostrate Bile Citrate and Sucrose (TCBS). The inoculated plates were incubated at 35°C for 24h and 48h, respectively, for growth of *Vibrio* and CHB. The results were expressed in Colony Forming Units per mL (UFC mL^{-1}).

Table II. Physical-chemical analyzes of water quality and the methodologies employed.

Analysis	Method	Unit	References	Observations
Alkalinity	Titration	mg $CaCO_3$ L^{-1}	Boyd e Tucker (1998)	T0, T5, T9 e T12
pH	Portable	-	-	T0, T9 e T12
Salinity	Portable	-	-	T0
NAT	Indophenol	mg L^{-1}	APHA (2000)	T0 e T12
Nitrite	Griess-Islova	mg L^{-1}	APHA (2000)	T0 e T12
Sedimentable Solids	Cone *Imhoff*	mL L^{-1}	Gaona *et al.* (2017)	T12

Antimicrobian activity

At the end of the cultivation period, the flocs formed were decanted in Cones Imhoff and evaluated for antimicrobial activity against Gram negative strains *Vibrio harvey* and *V. parahaemolyticus* (isolated from marine shrimp farming environment) and a Gram positive strain *Staphylococcus aureus* (ATCC 25923). It was used the Agar well diffusion method (Balouiri et al. 2016). The surface of agar plate Mueller-Hinton was inoculated by spreading of the microbial inoculum (*V. harvey, V. parahaemolyticus and Staphylococcus aureus* (ATCC 25923)) over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm was punched, and a volume (100 µL) of the flocs, for each treatment employed, in the concentration of 100 µL / mL, was introduced into the well. Sterile distilled water was used as a negative control and the commercial antibiotic Kanamycin at a concentration of 30 µg mL^{-1} (BD®) was used as a positive control. The result was observed after 48 h of incubation at 35 ºC.

RESULTS

Physico-chemical analysis of water from experimental systems

In all treatments there were formation of bioflocs, however, B1 produced, numerically, more volume of bioflocs than B2 and Control. It was formed, respectively, 100 ml x L^{-1} and, 83 ml x L^{-1} and 45 ml x L^{-1} of sedimentable solids (Figure 1).

The water quality variables observed during the experimental period are shown in Figure 2. The alkalinity values varied over the days of experimentation, and after 12 days, the lowest levels were detected in the Control and in the treatment B2, 59 and 62 mg x L^{-1}, respectively (Figure 2). In the B1 treatment, the value was around 80 mg x L^{-1}. The experimental group B1 presented a greater alkaline reserve. The pH showed a considerable decrease in treatments,

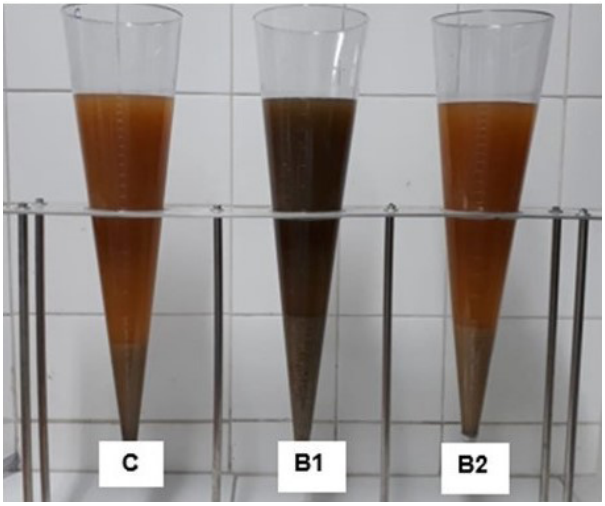

Figure 1. Imhoff cone with water samples from Control C, Treatment B1 and B2 to determine the sedimentable suspended solids at the end of the experimental period. Source: Authors.

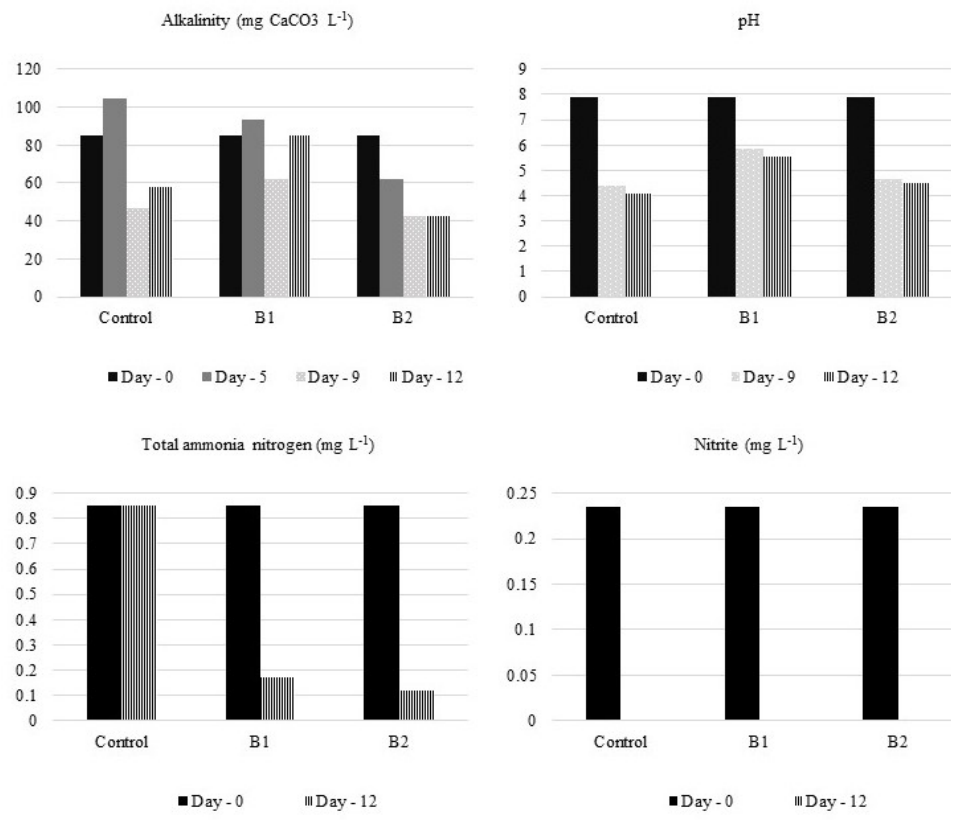

Figure 2. Total alkalinity, pH, total ammonia nitrogen and nitrite values of the three experimental groups tested. Source: Analysis Data.

reaching values below 6. Treatment B1 showed a pH equal to 5.8, a value slightly higher than the other experimental tanks, at the end of the 12 days of cultivation.

In the tanks with groups of probiotic (B1) and nitrifying (B2) bacteria, the NAT concentration decreased after 12 days, with values below 0.2 mg L^{-1} being detected. The Control group had a NAT level equal to 0.85 mg L^{-1}, a value 4.25 times higher than the treatments added to biofloc-producing bacteria (Figure 2). The Control group had a NAT level equal to 0.85 mg L^{-1}, a value 4.25 times higher than the treatments added to biofloc-producing bacteria (Figure 2).

Microscopic characterization of flocs

The development of the flocs was monitored microscopically throughout the cultivation period. It was observed both in treatments (B1 and B2) and in the Control an increase in the density of aggregates throughout the cultivation, reaching a more mature stage and with a greater number of microbial aggregates at 12 days.

The physical characteristics (size and three-dimensional shape) of the flocs formed during the experimental period were also monitored microscopically. In the Control treatment, the formation of large but irregular aggregates was observed, less structured and more dispersed in relation to treatments B1 and B2, which presented flakes of homogeneous sizes and regular shapes. (Figure 3).

Several microscopic organisms from different phyto and zooplankton classes were registered (Figure 4) in induced bioflocs. This diversity was not observed on the spontaneously formed bioflocs (Control).

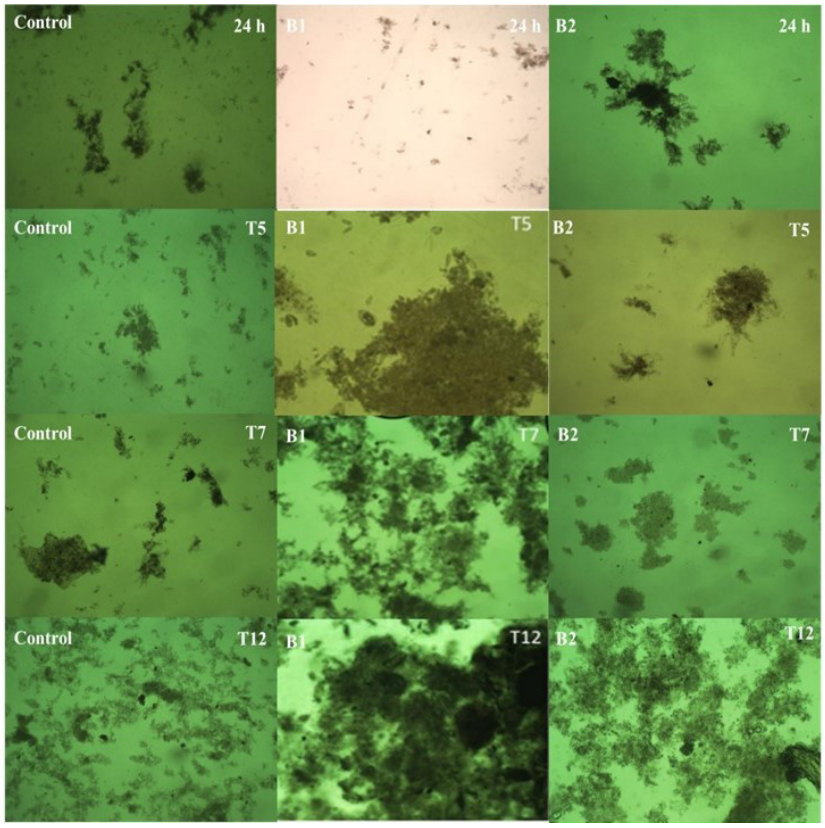

Figure 3. Optical microscopy images of BFT formation during the 12 days of *in vitro* culture.

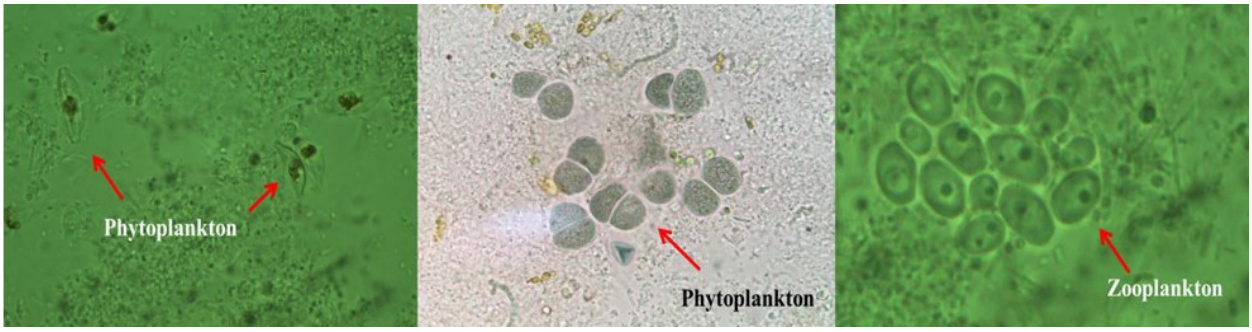

Figure 4. Phytoplankton and zooplankton microorganisms observed in treatments B1 and B2.

Microbiological analysis

The amount of cultivable heterotrophic bacteria (CHB) increased about four logarithmic cycles after the 12-day culture period in the water in all experimental systems. The number of CHB present in the nursery water used in the systems at the beginning of the experiment (Day 0) was 1.56 x 10^4 UFC mL^{-1}, which was increased after 12 days of cultivation to 10^8 UFC mL^{-1} (Control and B1) and 10^7 UFC mL^{-1} (B2) in the systems (Table III).

For the Vibrio spp counts, the values of 1.0 x 10^2 UFC mL^{-1} were initially detected in the nursery water used in the cultivation. However, at the end of the experiment, no bacteria of the genus *Vibrio* were detected in Control and treatment B2, either in water samples or in bioflocs. Already in the B1 system there was an increase in both water and bioflocs, in the number of bacteria of the genus *Vibrio,* after 12 days of cultivation (Table III).

Table III. CHB and Vibrio count (UFC mL^{-1}) at the beginning and after 12 days of cultivation in water and flocs in treatments B1 (consortium of probiotic bacteria), B2 (consortium of nitrifying bacteria) and Control (spontaneous biofloc).

Cultivation days			CHB	*Vibrio*
0	Nursery water		1,56 x 10^4	1,0 x 10^2
12	Control	Water	1,01 x 10^8	ND
		BFT	5,08 x 10^7	ND
	B1	Water	1,15 x 10^8	1,5 x 10^3
		BFT	1,34 x 10^8	9,69 x 10^4
	B2	Water	2,68 x 10^7	ND
		BFT	4,36 x 10^7	ND

ND: not determined.

Antimicrobian activity

Bioflocs from the B1 system showed antibiotic activity against the three pathogens tested, visualized by the formation of an inhibition halo around the inoculum. Control and B2 systems did not show antibiotic activity.

DISCUSSION

Physicochemical quality of water from cultivation

The production of bioflocs is favored by carbon and nitrogen sources and the aggregates are formed by organic matter from various sources in its composition: bacteria, microalgae, protozoa, small metazoans, larval forms of invertebrates, exoskeletons and remains of dead organisms (Monroy Dosta et al. 2013).

In all treatments there was a daily addition of molasses (carbon source) and feed (nitrogen source), aiming to stimulate the natural productivity of the water, thus increasing the amount of solids suspended in the water column. The greater volume of sedimentable solids obtained in treatments B1 and B2, in relation to the Control, resulted from the occurrence of the multiplication of bacteria in the formation of bioflocs. The addition of a microbial consortium accelerated the process of floc formation in these treatments when compared to the biofloc spontaneously developed (Control). According to Harun et al. (2019), the introduction of bacterial consortia with flocculant capacity accelerates the process of formation of microbial aggregates. The same interference in the formation of aggregates was showed by Soares et al. (2021), using functional bacteria isolated from the intestinal microbiota of cultivated shrimp as bio-augmenters, verified a greater volume and lower sedimentation rate compared to the spontaneously formed biofloc.

Analyzing factors related to water quality, all cultivation tanks the water acidification process took place. In the early stages of biofloc formation, variations in the chemical parameters of water are common, mainly in pH and alkalinity, which reduce with the incresing of suspended solids (Furtado et al. 2014). Through stoichiometric engineering analysis, it was possible to correlate the acidification of water with the increase in bacterial biomass in the system and the consumption of ammonia (Ebeling et al. 2006).

For the cultivation of aquatic organisms, the correction of water quality parameters with the use of chemical products is a routine practice in

order to maintain the physiological processes of organisms cultivated in a biofloc system at desirable levels (Furtado et al. 2011). This interference was not used in our experiment, so acidification of the water was expected.

Other patterns like reduction in NAT concentration at B1 and B2 treatments, after 12 days, may be related to the nitrifying activity of two distinct bacterial groups used to induce the formation of flocs. The growth of these bacterial groups, including some potentially probiotic strains, as species of the genus Bacillus, can promote a reduction of ammonia which is converted into bacterial protein, thereby enabling constant removal of ammonia and nitrite from cultivation environments (Cao et al. 2019, Khanichaidecha et al. 2018).

The absence of nitrite in all treatments may be associated, in addition to the presence of biofloc-inducing bacteria, to the volume of sedimentable solids in suspension (Souza et al. 2019). It is enough to observe that the Control treatment presented the smallest volume, and this is indirectly related to a better efficiency of removal of nitrogen compounds, thus being able to induce the nitrifying capacity of the flakes.

Microscopic characterization of flocs

Through microscopic monitoring of the formation of flocs throughout the growing period, it was possible to observe differences in the formation of spontaneous aggregates of the Control treatment, compared to the formation of aggregates from treatments B1 and B2 (Figure 3). Likewise, Hapsari (2016) observed microscopically that the flocs accumulated in the BFT system added with probiotic bacteria were denser and larger, when compared to the control without adding bacteria. Harun et al. (2019) found that the introduction of biofloculant-producing bacteria like inoculum proved to accelerate the formation of bioflocs during the production of *L. vannamei* white shrimp.

Phyto and zooplankton microorganisms, observed in biofloc systems with addition of bacterial groups B1 and B2, are considered responsible for maintaining the aquatic balance, recycling nutrients (Santos 2018). According to Lara et al. (2017) the presence of ciliate and protozoa in the biofloc stimulates the growth of the cultivated animal, as it presents a rich nutritional composition in unsaturated fatty acids. Furthermore, favors the control of pathogenic microorganisms in the system (Thompson et al. 1999). Loureiro et al. (2012), observed through the analysis of shrimp intestine content, the presence of protozoa and rotifers, mostly in the initial and intermediate stages of culture, indicating that the shrimp preferentially grazed these microorganisms, probably due to the size adjustment of prey and predators, which allows microorganisms to be preyed upon directly by the shrimp.

Failure to observe these microorganisms in bioflocs formed spontaneously (Control) may be because the elements that produce the flocs, such as the carbon source, the balanced feed and the bacteria in the system, can directly influence the groups of organisms that develop. Since the heterotrophic growth of protozoa, rotifers, cyanobacteria and diatoms in the BFT system contributes to improving growth, survival and reduced feed demand (Loureiro et al. 2012, Panigrahi et al. 2020).

Microbiological water quality of cultivations.

Microorganisms, especially bacteria, are known for their ability to degrade synthetic and natural substances, reducing toxic compounds and can sometimes be used as bioremediation agents, therefore, important components in maintaining water quality (Manan et al. 2017).

The number of cultivable heterotrophic bacteria (CHB) found is considered high when compared to that found by Arias-Moscoso et al. (2018), that in experiments using bioflocs with the addition of probiotic strains, obtained maximum CHB counts in the order of 10^6 UFC mL^{-1}, indicating a great ability of the microorganisms tested, due to the high bacterial multiplication.

Comparing the CHB counts in water and bioflocs, after 12 days of cultivation, it is noticed that there is a greater amount in the Control and B1 groups compared to the B2 group (Table III). The lowest amount of bacteria in the B2 system happens, because it was composed with the addition of nitrifying bacteria, generally autotrophic, which compete with CHB and may cause a slight reduction in their quantities. However, CHB are extremely important for the nitrification process and for the efficiency of the biofloc system in maintaining water quality, promoting significant and constant removal of ammonia and nitrite in aquaculture systems, through the consumption of inorganic carbon and nitrogen compounds used in their growth (Souza et al. 2019).

Silva et al. (2020), studying the floc formation process from the addition of a consortium of nitrifying bacteria, found, at the end of 18 days of in vitro culture formation, count values equal to 1.43 x 10^7 CFU mL^{-1} of CHB in the bioflocs. According to the authors, in the biofloc system with the addition of a nitrifying bacterial consortium, there may be a competition between the added exogenous bacterial groups and the autochthonous ones, causing a reduction in the quantification of bacterial groups, indicating a possible stability of the environment.

The absence of bacteria of the genus *Víbrio*, after 12 days of cultivation, in the Control and B2 systems, suggests that both bioflocs induced by the addition of nitrifying bacteria (B2 system) and spontaneous (Control), may have a population-reducing effect of *Vibrio* spp. This absence of *Víbrio* spp. in Control and B2 systems shows the high protective potential of this technology. Monroy-Dosta et al. (2013) only observed a decline in vibrio counts after 5 weeks of cultivation with spontaneous biofloc.

The reduction of *Vibrio* spp. in water from systems C and B2, and its increase in system B1 may be associated with competition for nutrients caused by the addition of a consortium of nitrifying bacteria and the dominance of heterotrophic bacteria that are prevalent in biofloc technology due to their high growth rate and substrate utilization (Manan et al. 2017). In an experiment carried out by these same authors, with biofloc test systems formed spontaneously in shrimp farming, *Vibrio* spp. were also detected, although in small quantities. Arias-Moscoso et al. (2018) identified a significant increase in balance in BFT systems, both added with commercial probiotics and control without probiotics during the cultivation days, reaching values in the order of 10^3 UFC mL^{-1}.

Interactions (biochemical inhibition, substrate and/or nutrient competition) that occur between the biofloc microbiota (heterotrophic and autotrophic bacteria, microalgae, yeast, etc.) and potentially pathogenic bacteria (such as vibrio) can reduce or cancel the incidence of pathological events in cultivated organisms, with biocontrol action of the flocs on the pathogens installed in the systems (Manan et al. 2017, Arias-Moscoso et al. 2018, Monroy-Dosta et al. 2013, Emerenciano et al. 2017). The study conducted by Kumar et al. (2020) demonstrated that the biofloc system increased the survival of *L. vannamei* challenged against a strain of *V. parahaemolyticus* AHPND. The results highlight that in the biofloc system, AHPND-causing *V. parahaemolyticus* possibly changes from a free-living virulent planktonic phenotype to a non-virulent biofilm phenotype. This transition can

be registered by the decreased transcription of motility genes related to flagella, Pir toxin and AHPND plasmid genes and increased expression of the AlkPhoX gene which is a marker of phenotype change *in vitro* and *in vivo* conditions.

The consortium used in the B1 treatment consisted of two strains of *Bacillus* sp., group recognized for presenting probiotic properties, which aid in growth, metabolism, digestion and resistance to diseases (Daniel & Nageswari 2017, Aguilera-Rivera et al. 2014, Hapsari 2016). Thus, the presence of vibrios in the water and in the flocs of treatment B1 will not harm the animals grown, since they will have an immune system strengthened by having fed on the flocs rich in *Bacillus sp*., which will help to fight infectious conditions, in case environmental imbalances occur causing exacerbated proliferation of the vibrio. Corroborating with the present study, Aguilera-Rivera et al. (2014) found a group of strains of exclusive Vibrio in the floc treatment in which probiotics were added during the cultivation of the shrimp *L. vannamei*. However, no level of lesions in the tissues of the animals was observed, suggesting that the probiotic contributed to homeostasis and prevented the outbreak of opportunistic pathogenic species.

Antimicrobial activity of microbial aggregates

The bacteria that compose the B1 system are characterized as probiotic bacteria and can produce inhibitory compounds against pathogenic microorganisms (Mohapatra et al. 2013). The group of lactic acid bacteria that was used in the B1 system has already been reported to be able to inhibit the growth of *Vibrio harveyi* and *V. parahaemolyticus* in antagonism tests and to strengthen the immune system of shrimp infected with these pathogens (Panigrahi et al. 2018, Sha et al. 2016).

In a previous study, the strain *Bacillus sp*., used in the formation of the B1 consortium of the present study, showed positive antagonism against the pathogen *Vibrio harveyi* (Abreu 2019) and this capacity was probably transferred to the B1 system bioflocs, after the establishment and colonization of the consortium bacteria at the end of the 12 days of cultivation.

The reduction in the population of *Vibrio* spp. during cultivation with bioflocs from Control and B2 system was not the result of an antimicrobial ability attributed to microbial flocs, but rather to a capacity for competitive exclusion that certain heterotrophic bacterial populations have on pathogenic bacteria (Wu et al. 2012). Monroy-Dosta et al. (2013), stated that the increase in heterotrophic microorganisms prevents the development of bacteria of the genus *Vibrio*.

CONCLUSIONS

The biofloc technology was more efficient with the addition of bacterial consortia, since the use of microorganisms, with pre-established functions, accelerates the formation and structure processes of the aggregates, reduction of ammonia and nitrite in the evaluated treatments. In addition to attributing benefits to flocs, as antagonistic activity against pathogens.

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JÉSSICA L.S. DA SILVA[1]
https://orcid.org/0000-0001-5449-0816

ANA P.C. CARNEIRO[2]
https://orcid.org/0000-0001-5404-6205

ANNA L.C. BRITO[1]
https://orcid.org/0000-0001-5267-3782

ANA V.S. OLIVEIRA[2]
https://orcid.org/0000-0002-0854-7188

JHONES L. VIEIRA[3]
https://orcid.org/0000-0002-7321-3571

RAQUEL C. SOARES[2]
https://orcid.org/0000-0003-2964-9893

ROBÉRIO M. DE FREITAS[2]
https://orcid.org/0000-0002-7871-9889

OSCARINA V. DE SOUSA[4]
https://orcid.org/0000-0003-3907-7964

[1]Universidade Federal do Ceará, Laboratório de Microbiologia Ambiental e de Pesca, Instituto de Ciências do Mar, Avenida da Abolição, 3207, Meireles, 60165-081 Fortaleza, CE, Brazil

[2]Universidade Federal do Ceará, Programa de Pós-Graduação em Engenharia de Pesca, Bloco 825, Avenida Mister Hull, Pici, 60455-760 Fortaleza, CE, Brazil

[3]Universidade Federal do Ceará, Programa de Pós-Graduação em Ciências Marinhas Tropicais, Instituto de Ciências do Mar, Avenida da Abolição, 3207, Meireles, 60165-081 Fortaleza, CE, Brazil

[4]Universidade Federal do Ceará, Instituto de Ciências do Mar, Avenida da Abolição, 3207, Meireles, 60165-081 Fortaleza, CE, Brazil

Correspondence to: **Jéssica Lucinda Saldanha da Silva**
E-mail: jessicalucinda@hotmail.com

Author contributions

Jéssica Lucinda Saldanha da Silva: responsible for writing and general organization of the text of the article.
Ana Patrícia Cavalcante Carneiro: responsible for tabulating and interpreting the results and contributed to the discussion. Anna Luisa de Carvalho Brito: contributed to the construction of the introduction. Ana Vládila da Silva Oliveira: contributed to the discussion. Jhones de Lima Vieira: contributed to the interpretation of the results. Raquel Cavalcante Soares: contributed to the introduction. Robério Mires de Freitas: contributed to the construction of the introduction and methodology. Oscarina Viana de Sousa: was responsible for the general correction of the article.