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Preliminary evaluation of the use of bacteria isolated from the digestive tract of shrimp *Litopenaeus vannamei* as a source to accelerate the process of formation and development of bioflocs

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ABSTRACT. This study aims at investigating to follow the formation and development of biofloc aggregates in a system with the introduction of an *in vitro* selected bacterial consortium (*Bacillus thuringiensis, Bacillus sp., Staphylococcus cohnii*) in order to induce fast formation of biofloc and to compare it to the development of spontaneous formation biofloc. Two experimental groups were evaluated for biofloc formation, SFT and IFT. The first refers to spontaneous (conventional) formation of the flocs and the second to induced formation (IFT), achieved through the consortium of potentially inducing bacteria. Both treatments presented a constant increase of bioflocs, however, in the IFT treatment, the microbial aggregates were larger and more uniform. By the end of the experiment, we verified that the aggregates formed in the IFT showed higher volume and lower sedimentation rate in comparison to the spontaneously formed ones. The results show that domestication in microbial communities is efficient as related to bioflocs, reducing instability during its formation and development.

Keywords: aquaculture; probiotic; biofloc induction; microbial domestication.

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Introduction

The search for technologies that overcome the challenges faced by modern aquaculture promoting the increased productivity allied reduction of environmental impacts aroused interest in the cultivation in closed systems, mainly, due to the greater search for biosecurity and environmental advantages when compared to the cultivation conventional systems (extensive and semi-intensive; Azim & Little, 2008; Crab, Lambert, Defoirdt, Bossier, & Verstraete, 2010; Martínez-Córdova, Emerenciano, Miranda-Baeza, & Martínez-Porchas, 2015). In this context, Biofloc Technology System (BFT) is considered alternative technologies systems as the nutrients can be recycled continuously and reused in cultivation, aiming to intensify the activity in a sustainable (Azim & Little, 2008; Avnimelech, 2012). The sustainable approach this system based on the growth of microorganisms that maintain water quality and serve as high protein value feed, resulting in reduced feed conversion ratio and of feed costs (McIntosh et al., 2000; Burford, Thompson, McIntosh, Bauman, & Pearson, 2004; Avnimelech, 2007; 2009; Zokaeifar et al., 2013).

The basis of biofloc cultivation is to transform toxic nitrogenous compounds into bacterial biomass, stimulating the increase in the microbial biomass through optimization carbon-to-nitrogen ratio (C/N ratio), promote by fertilization of water with carbon-rich compounds (Emerenciano, Gaxiola, & Cuzon, 2013; Martínez-Córdova, Martínez-Porchas, Emerenciano, Miranda-Baeza, & Gollas-Galván, 2016). Bacterial communities growing in the system assist in the structuring and formation of suspended particles (bioflocs) which are composed of microalgae, bacteria, aggregates of particulate organic matter, protozoa and metazoa (Crab, Avnimelech, Defoirdt, Bossier, & Verstraete, 2007).

The biofloc process formation is relatively rapid; however, the microbial community can take up to six weeks to become stable in the system. Because of that, from the start of the culture until the stabilization of the microbial community, various problems might occur, e.g. high concentration of nitrogen compounds (especially in ammonia and nitrite form), which might affect the growth and even cause shrimp mortality when they are not removed from the system. In other hand, the faster the bioflocs develop and become large

enough to be consumed by the shrimp, more benefits they bring to the system (McIntosh et al., 2000; Krummenauer, Peixoto, Cavalli, Poersch, & Wasielesky Jr., 2011).

In order to accelerate the process and increase the production of suspended solids and ensure the establishment of nitrifying bacteria, some studies have used inoculum from mature biofloc removed from previous cultivation to start a new cultivation (Krummenauer et al., 2011; Gaona, Almeida, Viau, Poersch, & Wasielesky Jr., 2017). Thus, knowledge about proper management is necessary to accelerate the process of biofloc formation and the beneficial effects produced by them, thus optimizing their function in aquaculture systems, both in the removal of nitrogen compounds as well as in the feed of the cultured animal (Ray, Lewis, Browdy, & Leffler, 2010; Samocha, Wilkenfeld, Morris, Correia, & Hanson, 2010).

Bioremediation is a technique that consists of the inclusion of microorganisms in the aquatic environment, which can accelerate and promote the restoration of the ecological balance of the planned environment. The microbial community may be of spontaneous origin or genetically modified (Jiao et al*.,* 2011). Numerous microorganisms have been reported for their efficiency as biological agents in aquaculture. The bacteria of the genus *Bacillus sp*. the most common organisms used (Antony, Singh, Jose, Kumar, & Philip, 2011).

Considering the value of biofloc technology combined with bioremediation technique and the importance of ensuring the integrity and efficiency of the formed aggregates in short period of time an experiment *in vitro* was conducted to monitor the formation and development of microbial aggregates induced from the of the consortium introduction of potential inducing bacteria for rapid biofloc formation isolated from the digestive tract of the shrimp *Litopenaeus vannamei*.

Material and methods

The experiment was carried out at the Laboratory of Environmental Microbiology and Fish - LAMAP in Institute of Marine Sciences (Labomar), Federal University of Ceará (UFC), Fortaleza, state Ceará, Brazil.

Experimental conditions

In the present study were evaluated two experimental groups for biofloc formation, SFT and IFT. The first refers to the spontaneous (conventional) production of the flocs and the second the induced flocs through the introduction of the bacterial consortium. Since this is a preliminary study where the objective was to assess whether the presence of these bacteria would influence the formation of bioflocs, animals were not used in both treatments.

Selection the bacterial consortium

Were selected Among 191 strains isolated from the intestine and intestinal content from shrimp from two different cultivation systems, bioflocs and clear water, using techniques and culture media selective for groups Bacillus, Lactic Acid Bacteria (BAL), Proteolytics, Amylolytics, Lipolytics and Cellulolytics. All bacterial isolates were subjected to the tests of virulence factor expression, with the help of the following exoenzymes: gelatinase (GEL), elastase (Elas), caseinase (CAS), lipase (LIP), phospholipase (Phos), following the methodologies of Rust, Messing, and Iglewski (1994), Rodrigues, Ribeiro, Alves, and Hofer (1993) and Furniss, Lee, and Donovan (1978). They were also subjected to stress stability tests: Thermoresistance and pH variation tolerance test (Cai, Suyanandana, Saman, & Benno, 1999) as well as antimicrobial susceptibility tests (CLSI, 2010). Ability to form biofilms was determined through phenotypic test in plates containing Congo red agar (CRA) according to Freeman, Falkiner, and Keane (1989) and Abdallah, Chaieb, Zmantar, Kallel, and Bakhrouf (2009) with adaptations, and Adhesion microplate test (AMT), with modifications (Christensen, Simpson, Bisno, & Beachey, 1982). According to the results observed in the tests mentioned above, strains were selected for the development of the bacterial consortium for *in vitro* tests. The bacterial consortium used for floc formation in the IFT was comprised of four bacterial strains (Table 1).

Table 1. Identification of bacteria used as inoculum for the formation of bioflocs.

Functional group	virulence	Aggregation	pH 9.0	Temperature (40°C)	Taxonomic identification
Groups Bacillus	C-F	+	+	+	*Bacillus thuringiensis*
Lactic Acid Bacteria	C-F	+	+	+	*Bacillus sp.*
Proteolytics	G	+	+	+	*Bacillus sp.*
Cellulolytics	F	+	+	+	*Staphylococcus cohnii*

C: Caseinase, F: fosfolipase, G: Gelatinase.

Experimental system

The bacteria selected for the consortium were renewed on TSA agar at 35ºC for 24 hours. After this period, they were individually transferred to 0.85% sterile saline and adjusted to $1.5x10^8$ CFU mL^{-1} (0.5 McFarland standard). Subsequently, 1.0 mL aliquots of each strain were transferred to a falcon type tube containing TSB broth and incubated at 35°C for 48 hours.

To start the experiment, it was used the water sampled from a *Litopenaeus vannamei* shrimp pond with 30 days of cultivation. The water was added in 2-glass beakers containing either 1,000 mL water (3 for each treatment). The initialization sources of both treatments were used molasses powder as a source of carbon and Brain heart infusion broth (BHI) as a source of nitrogen maintained at a 10: 1 ratio (C: N) in the treatments. Ratio was calculated based on the necessary amount for initial biofloc formation, following the methodology suggested by Avnimelech (1999). Next, the bacterial consortium selected for biofloc formation was introduced to the test treatment (IFT). This system was kept under constant aeration for ten days where it was observed daily.

Figure 1 shows the scheme to start and monitor of the bioflocs during the experiment. Monitoring of the biofloc structure development was conducted through observation in a 40 × objective lens from the 1st to the 10th day of the experiment. In 10th day sedimentation rate was also observed by the volume of biofloc formed in *Imhoff* cones.

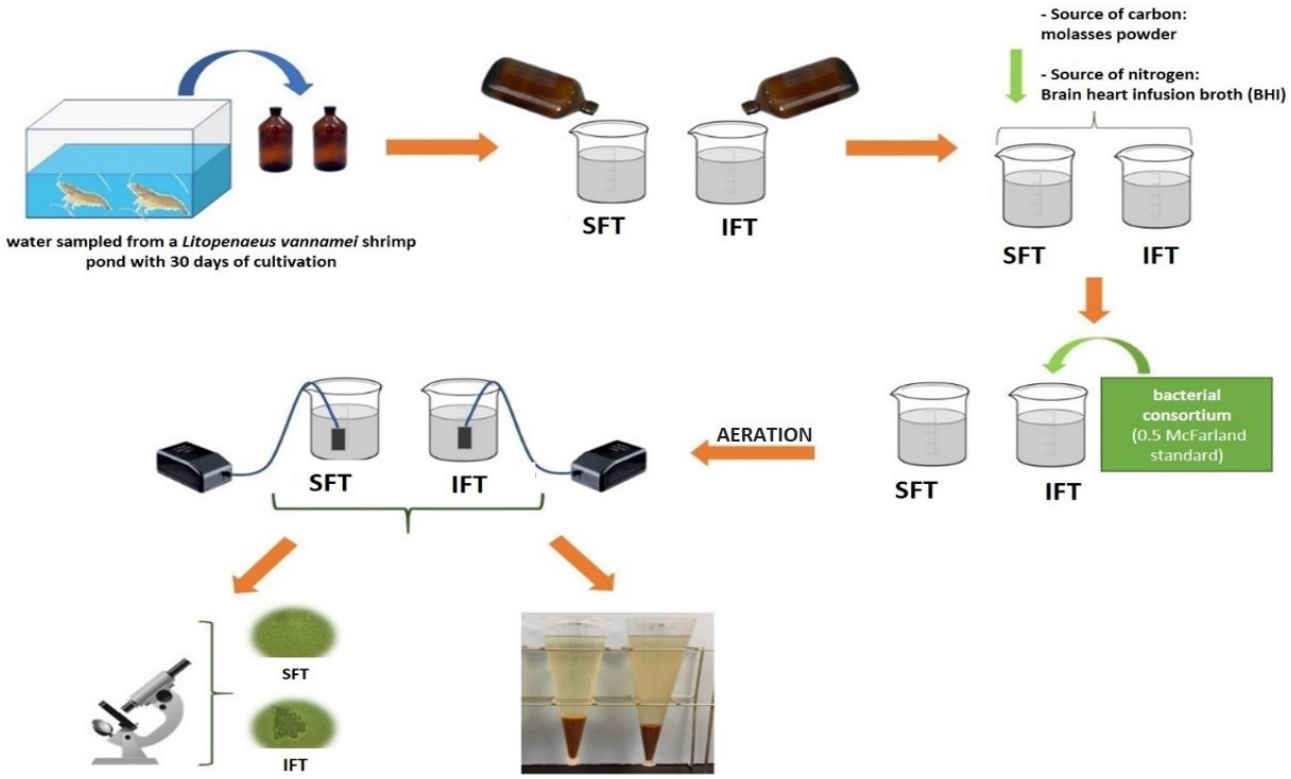

Figure 1. Scheme of installation of the experiment and monitoring of the bioflocs in the two treatments.

Results and discussion

From this study it was possible to verify that there was a higher volume of biofloc in the treatment with bacterial introduction. The treatment which started without the presence of the inoculate (SFT) showed lower average concentration of microbial aggregates and its structure presented a smaller size compared to the treatment with bacterial induction (IFT) (Figure 2).

During the bioflocs monitoring process, it was possible to verify the increase for bioflocs aggregates for both treatments throughout the experiment. However, microbial aggregates were more abundant in the treatment in which bacteria were introduced (IFT), presenting a higher amount of biofloc (8 mL L^{-1}) compared to the spontaneous formation treatment (SFT; 5.5mL L-1) (Figure 3). This is important, as the presence of these flocs results in reducing feeding costs, promoting improvement feed Conversion Ratio (FCR) (Avnimelech, 2009). Studies conducted by Fóes, Gaona, and Poersch (2012) in commercial shrimp farms using

the BFT system have shown that 29% of the feed consumed by *Litopenaeus vannamei* shrimp might be provided by microbial flocs present in cultivation water. This complementary food source enables the rise in shrimp stocking density of the white shrimp *L. vannamei*, increasing the productivity of the culture as it improves feed conversion and decreases the amount of requirement from in the feed.

The control treatment (SFT) was shown in flocculation, with irregular agglomerates, growing day by day, while the flocs in the IFT group were more abundant and uniform since the start of the experiment (Figure 4). Analysis by microscope revealed that from day 2 it was already possible to observe the microbial aggregates in both treatments (Figure 4A and B), but from day 5 on it was possible to distinguish a sharp difference between the amount and size of the aggregates observed in both treatments (Figure 4C and D). During the experiment it is observable that in both treatments the amount of aggregate increases in time and, in the last observation, note them in a more developed stage, with a higher number of microbial aggregates.

The flocs generally grow until they reach a size limit. Large flocs are broken in smaller fragments to be able to continue aggregating individually (Gregory, 1997; Tansel, 2018). Both the size and the shape of the flocs that form in different conditions vary depending of the interactions, type and concentration of the particles, as well as the chemical and physical characteristics of the water (Zouboulis & Avranas, 2000). Thus, the presence of culture aggregates in bioflocs was wider and more complex as time lapsed in the culture, possibly due to the increase in particulate material in the water.

On day 10, it was possible to verify that the volume of biofloc formed was larger in the treatment with the introduction of bacteria (IFT), and this same treatment showed a lower sedimentation rate, that is, after one hour without agitation, it showed more aggregates suspended in the water column than the treatment with spontaneous formation (SFT). This might have occurred because of the characteristics of microbial flocs (size shape, density and porosity) that might affect the speed of floc sedimentation. Some studies show that with the increase in floc size, porosity also increases, decreasing the density and sedimentation speed of the floc (Gregory, 1997; Tansel, 2018).

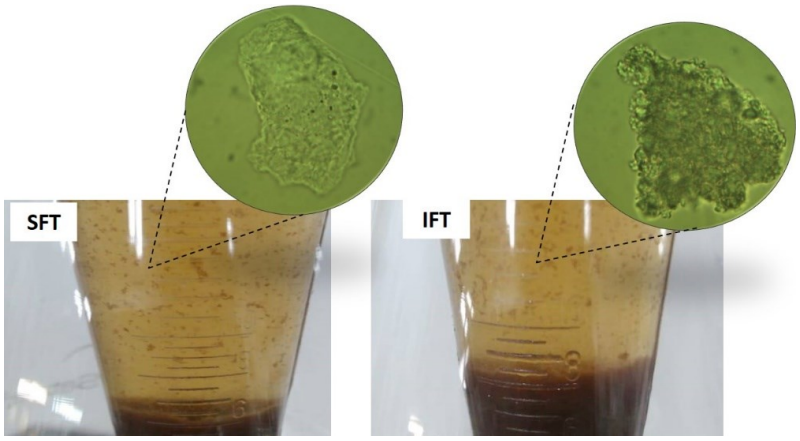

Figure 2. Representation of the structure of the aggregates formed in the two treatments.

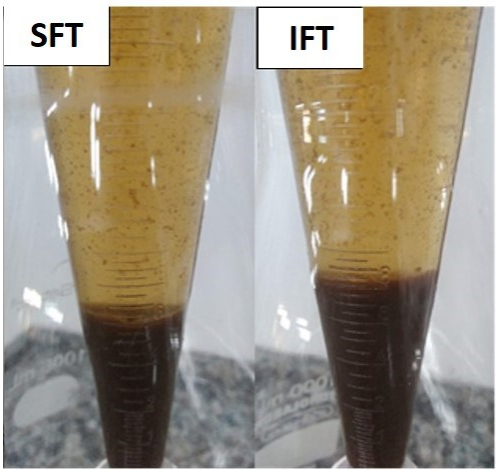

Figure 3. Analysis of the volume of bioflocs produced in the two treatments.

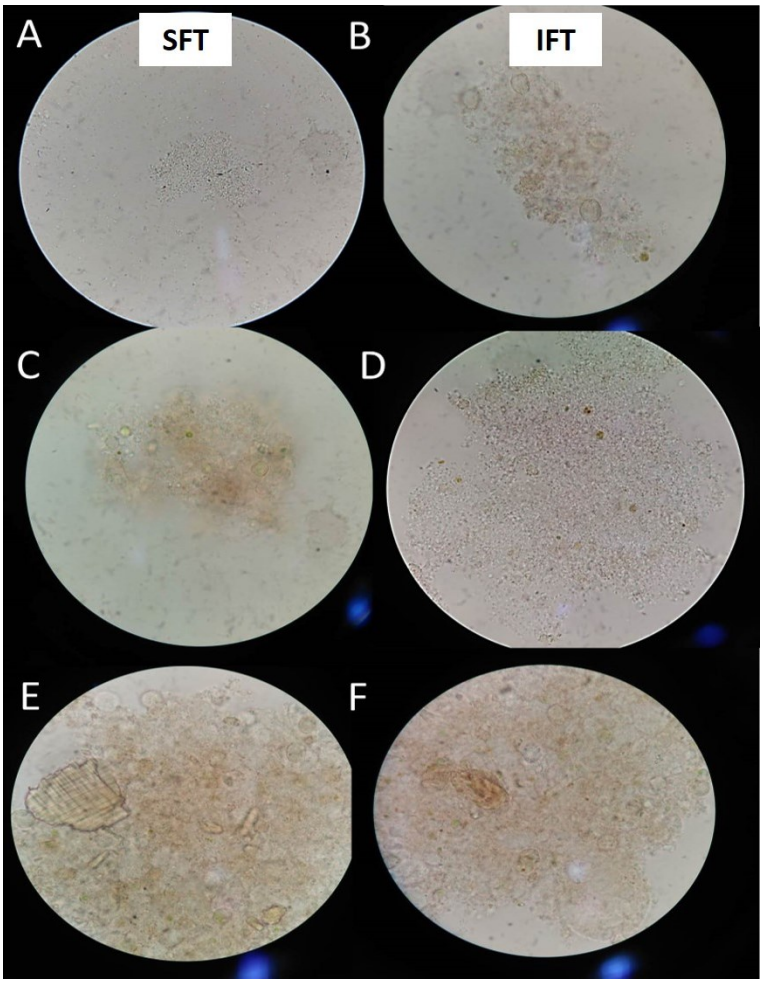

Figure 4. Monitoring of microbial aggregates observed under a microscope throughout the experiment in both treatments. SFT: treatment with spontaneous formation (conventional), IFT: treatment with the introduction of bacteria. A and B: 2th day, C and D: 5th day, E and F: 10th day.

The introduction of bacteria in order to induce biofloc formation was efficient, reaching better results in comparison to the spontaneous formation method, as the bacterial strains introduced colonized the treatment efficiently. They proved to be good candidates for the acceleration of biofloc formation and development process, not to mention their probable ability to act as specific probiotic and bioremediation bacteria. The domestication of microbial biofloc communities can promote a decrease in instability in the formation and development of microbial aggregates resulting in an expressive increase in survival and growth rates of animals cultured in these systems, besides the increase in water quality and protection against diseases.

Conclusion

The association of *Bacillus thuringiensis, Bacillus* sp. *e Staphylococcus cohnii* might be used as constituents of the microbiota in biofloc systems. These constituents will serve as a stimulant and bioregulator in the development of the bioflocs. Thus, allowing bacterial development with low variation over time using a single initial dose of bacterial inoculum.

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