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Control of suspended solids and nitrogenous compounds through bioremediation and artificial substrates in an emerging BFT System for Nile Tilapia

Controle de sólidos suspensos e compostos nitrogenados por meio de biorremediação e substratos artificiais em um sistema BFT emergente para Tilápia-do-Nilo

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Abstract: The effects of bioremediation with *Bacillus* spp. and an artificial substrate were compared on water quality, zootechnical and hematological parameters of Nile tilapia (*Oreochromis niloticus*) in a BFT system (biofloc technology). Twelve experimental units were divided into three groups: bioremediator (1.0g of bacilli per m3 of water daily); insertion of artificial substrate (AS); and control; in quadruplicate. Both treatments reduced NH_3 (bioremediador 0.73 mg.L^{-1} and AS 0.69 mg.L^{-1}) than the control group (1.33 mg.L^{-1}), meanwhile the bioremediator increased nitrite (31.71 mg.L^{-1}) compared to the control (12.76 mg. L^{-1}) and the AS did not diverge between treatments. AS (1.75 mL), bioremediator (3.10 mL) and control (3.87 mL) presented lower floc volume, respectively. The treatments reduced the food conversion ratio (bioremeaditor 0.84 and AS 0.86) than control group (1.07), meanwhile the AS promoted higher specific growth rate (3.05 %.day^{-1}) compared to the bioremediator (2.97 %.day^{-1}) and control (2.94 %.day^{-1}) which did not diverge between treatments. The percentage of circulating thrombocytes varied significantly among treatments, being highest in the bioremediator group (46.4 %), followed by the artificial substrate group (34.0 %) and the control (18.5 %). The bioremediator and AS led to improvements in water quality by removal of NH_3 and floc volume maintenance; promoted better zootechnical performance and changed the hematological profile for Nile tilapia.

Keywords: *Bacillus*; biofloc; bioremediation; *Oreochromis niloticus*.

Resumo: Os efeitos da biorremediação com *Bacillus* spp. e de um substrato artificial foram comparados quanto à qualidade da água, aos parâmetros zootécnicos e hematológicos de tilápia-do-nilo (*Oreochromis niloticus*) em um sistema BFT (biofloc technology). Doze unidades experimentais foram divididas em três grupos: biorremediador (1,0 g de bacilos por m^3 de água diariamente), inserção de substrato artificial (SA) e controle, em quadruplicata. Ambos os tratamentos reduziram a concentração de NH_3 (biorremediador: 0,73 mg·L^{-1}; SA: 0,69 mg·L^{-1}) em relação ao grupo controle (1,33 mg·L^{-1}). Entretanto, o tratamento com biorremediador aumentou o nitrito (31,71 mg·L^{-1}) em comparação ao controle (12,76 mg·L^{-1}), enquanto o SA não apresentou diferença significativa entre os tratamentos. Os volumes de flocos foram menores nos tratamentos com SA (1,75 mL), biorremediador (3,10 mL) e controle (3,87 mL), respectivamente. Os tratamentos reduziram a conversão alimentar aparente (CAA) (biorremediador: 0,84 e SA: 0,86) em relação ao grupo controle (1,07). Por outro lado, o SA promoveu uma taxa de crescimento específico (TCE) superior

(3,05 %·dia^{-1}) em comparação ao biorremediador (2,97 %·dia^{-1}) e ao controle (2,94 %·dia^{-1}), que não diferiram entre si. A porcentagem de trombócitos circulantes variou significativamente entre os tratamentos, sendo maior no grupo biorremediador (46,4 %), seguido pelo grupo com substrato artificial (34,0 %) e pelo controle (18,5 %). O biorremediador e o substrato artificial promoveram melhorias na qualidade da água, por meio da redução de NH_3 e da manutenção do volume de floco; proporcionaram melhor desempenho zootécnico e alteraram o perfil hematológico da tilápia-do-nilo.

Palavras-chave: *Bacillus*; biofloco; biorremediação; *Oreochromis niloticus*.

1. Introduction

Global consumption of aquatic foods has grown significantly, and the world's populations consume five times more fish compared to 60 years ago. This increase was mainly driven by aquaculture production [(1)]. Fish farming is the main activity of Brazilian aquaculture, and production in 2023 amounted to 887,029 tons, an increase of 3.1 % compared to the previous year [(2)].

Like most human activities, aquaculture causes environmental pollution because it promotes the degradation of water quality (culture environment and effluents) by the intensification of production systems. The loss in water quality compromises the growth, reproduction and quality of fish. Therefore, continuous monitoring of the farming environment is necessary in order to maintain animal well-being and reduce susceptibility to disease [(3,4)].

In recent years, biofloc technology (BFT) has gained prominence due to growing interest in closed aquaculture systems that ensure higher biosecurity and reduce water use [(5,6)]. The principle of BFT is to recycle nutrient by maintaining a high carbon-to-nitrogen ratio, stimulating the growth of heterotrophic bacteria that convert ammonia into microbial biomass, which supplements the diet of cultured organisms [(7)]. In this context, Nile tilapia has been widely adopted in BFT systems to improve feed conversion and enhance productivity [(5)].

Moreover, bioremediation is a beneficial process that uses biological agents to treat contaminated water. Among these contaminants, nitrogenous compounds, which are formed from the accumulation of ammonia from fish excretion, stand out. Bacterias, when used as bioremediators, promote the removal or conversion of these compounds [(8)]. Bioremediators can promote other benefits, such as strengthening the immune system and growth [(4,9)]. Bacteria from the *Bacillus* genus are commonly used in aquaculture as biological agents that improve the culture environment and the health of organisms. The benefits offered by these bacteria include removal of nitrogenous chemical compounds [(10)] and organic matter [(4)], secretion of enzymes that modulate fish intestinal physiology and food digestibility [(11)], increased humoral immune response in fish.

In contrast, artificial substrates are structures that increase the contact surface within the culture environment, promoting the attachment of bioflocs and microorganisms as biofilm [(12)]. The biofilm is formed by a group of microorganisms, mainly heterotrophic bacteria, which secrete proteins and carbohydrates to create an extracellular matrix. However, the biofilm also contains, albeit in smaller quantities, nitrifying bacteria, which are responsible for reducing the total ammoniacal nitrogen (TAN) concentration by converting it into nitrite, and subsequently into nitrate [(13,14)]. The availability of contact area is related to microbial activity, enhancing its diversity, including the development of microbial communities capable of performing the nitrification process [12,15,16]. Thus, substrates contribute to the metabolism of nitrogenous compounds and, consequently, to maintaining water quality, resulting in ammonia and nitrite concentrations at levels compatible with fish farming [17].

This study aimed to compare the effects of bioremediation, using *Bacillus* spp., and artificial substrate on control of settleable solids and nitrogen compounds, and their impact on the zootechnical performance and hematological parameters of Nile tilapia (*Oreochromis niloticus*) reared in an emerging BFT system.

2. Material and methods

The experiment lasted six weeks and was conducted at the Aquaculture Laboratory (LAQ), Instituto Federal Catarinense (IFC), campus Araquari, Santa Catarina, Brazil. This research was approved by the Ethics Committee on Animal Use (ECAU) under protocol number 533/2021.

2.1 Biological material

Strains of *B. amyloliquefaciens* and *B. subtilis*, provided by BioHall, were used for bioremediation. A total of 240 Nile tilapia fingerlings with an average weight of 0,8 g were provided by the LAQ, Instituto Federal Catarinense (IFC), campus Araquari.

2.2 Biofloc preparation

Occurred ten days before fish stocking when water in the tanks was fertilized with a source of carbon (refined sugar) and a nitrogen source (powdered feed) to maintain a C:N ratio of 10:1 [18,19]. After fish settlement, it continued at the same rate with the aim of neutralizing 40 % of the nitrogen in the feed and maintaining NH_3 below 1.0 mg.L^{-1}.

2.3 Experimental design and experimental management

Twelve experimental provided with 20 fingerlings each, the units were divided into three treatments in quadruplicate. Four units received 1.0 g for each m^3 of bioremediator (*B. amyloliquefaciens*, 1.86 x 10^{12} CFU and *B. subtilis*, 1.52 x 10^{13} CFU) daily. Artificial substrates were added to another four units, adapted from Schveitzer *et al.* [20], the material was bidim blanket (polyester) and the dimension consisted of 0.464 m^2 considering both sides. In addition, they were placed vertically and oriented perpendicularly to the aeration flow in the middle of the rectangular tanks. The rest units formed the control group. Therefore, twelve polyethylene boxes with a capacity of 250 L (useful) were used, each equipped with a constant aeration system and heater to maintain the temperature. The aeration system consisted of a diffused air system with microperforated hose (Aero-tube™) arragend on the bottom of each tank.

Biometries were carried out weekly to monitor fish growth, as well as to adjust the amount of feed provided. This ranged from 2.5 % to 6.0 % of the total biomass in each tank. Fish were fed three times a day (8h 11 and 16h) with commercial 3 mm pellet food containing 32 % crude protein, 5 % ether extract and 13 % moisture. Water lost to evaporation was replaced daily.

2.4 Water quality parameters

The culture environment was evaluated daily for the variables of dissolved oxygen, temperature (YSI PRO20 Oximeter) and floc volume (Imhoff cone) with sedimentation time of 30 min. And weekly by checking TAN, $N\text{-}NO_2$, $N\text{-}NO_3$ using a photocolorimeter (Alfakit®) and applying the Nessler, alpha-naphthylamine and brucine sulfate methods, respectively. Alkalinity, electrical conductivity, pH (pHep®) and total suspended solids (TSS) were also checking weekly (21).

2.5 Zootechnical performance

At the end of the experiment, biometry was performed on all fish in the experiment to evaluate the average final mean weight, total biomass, survival, weekly weight gain, food conversion rate (FCR), specific growth rate (SGR) and yield according to the formulas below:

$$Survival\ (\%) = \left[\frac{(Initial\ population\ -\ Final\ population)}{Initial\ population}\right] \times 100$$

$$Weekly\ weight\ gain\ (g.week^{-1}) = \left[\frac{(Average\ final\ weight\ -\ Average\ initial\ weight)}{Cultivation\ weeks}\right]$$

$$FCR = \left[\frac{(Consumed\ feed)}{(Final\ biomass\ -\ Initial\ biomass)}\right]$$

$$SGR\ (\%.day^{-1}) = \left[\frac{(Ln(Final\ weight) - (Ln(Initial\ weight))}{Cultivation\ days}\right] \times 100$$

$$Yield\ (kg.m^{-3}) = \left[\frac{(Final\ biomass\ -\ Initial\ biomass)}{Experimental\ unit\ volume}\right]$$

2.6 Hematological parameters

Five fish from each experimental unit were anesthetized with Eugenol (50 $mg.L^{-1}$). Blood was collected from the caudal vein using syringes with 10 % EDTA anticoagulant. Duplicates of blood smears were performed and stained with MayGrunwald/Giemsa/Wright (19) for differential leukocyte counts.

2.7 Statistical analysis

The data obtained were submitted to the Kolmogorov-Smirnov and Levene test to verify the normality and homoscedasticity, respectively. After that, data were subjected to analysis of variance (ANOVA) to observe significant differences and to the SNK test (Student-Newman-Keuls) to separate means. All analyses were performed with a significance level of 5 % (23).

3. Results and discussion

3.1 Water quality parameters

Temperature, dissolved oxygen, alkalinity, pH, nitrate, total suspended solids and conductivity did not differ among groups (Table 1). Meanwhile, the values for floc volume (FV) differed among the groups in that these values were higher in the control, bioremediator and substrate groups, respectively. Ammonia was both lower in the bioremediator and substrate group than the control, while nitrite in treatments was higher than control.

Table 1. Water quality parameters (mean ± standard deviation) of a BFT system integrated with either a bioremediator based on *Bacillus* spp. or artificial substrate.

	Control	Substrate	Bioremediator
Temperature (°C)	28.67 ± 0.43	28.62 ± 0.13	28.46 ± 0.27
Dissolved oxygen (mg.L^{-1})	8.13 ± 0.11	7.82 ± 0.11	7.95 ± 0.12
Floc volume (mg.L^{-1})	3.87 ± 0.37^{a}	1.75 ± 0.38^{c}	3.10 ± 0.27^{b}
Alkalinity (mg.L^{-1})	44.00 ± 8.00	49.50 ± 4.50	48.25 ± 13.25
pH	6.74 ± 0.22	6.91 ± 0.31	6.77 ± 0.28
TAN (mg.L^{-1})	1.33 ± 0.39^{a}	0.69 ± 0.35^{b}	0.73 ± 0.22^{b}
Nitrite (mg.L^{-1})	12.76 ± 12.25^{a}	45.91 ± 35.85^{ab}	23.71 ± 3.42^{b}
Nitrate (mg.L^{-1})	670.00 ± 35.00	678.13 ± 39.38	670.63 ± 34.38
TSS (mg.L^{-1})	76.77 ± 11.39	59.97 ± 16.24	51.23 ± 24.81
Conductivity (S.m^{-1})	670.00 ± 35.00	678.13 ± 39.38	670.63 ± 34.38

*Different letters in the same row indicate significant differences ($p < 0.05$) as determined by ANOVA and SNK's test.

The average temperature, oxygen and pH of the tanks remained within a range suitable for the cultivation of *Oreochromis niloticus* [24]. Thus, it appears that the presence of *Bacillus* spp. did not increase the biochemical oxygen demand of the system, or if it did, the system provided the necessary oxygen without reducing availability to the fish.

Nitrogenous compounds are the main pollutants in the culture environment, which can be toxic to aquatic ecosystems [4]. The addition of *B. amyloliquefaciens* and *B. subtilis* decreased ammonia levels in the bioremediator treatment since these bacteria, upon addition to water, can assist in establishing nitrifying bacterial colonies that promote the conversion of ammonia in nitrite. Moreover, similar results were observed when using *B. subtilis* and *B. licheniformis* in Nile tilapia raised in concrete ponds[25] and in Nile tilapia raised in a BFT system [26]. In the study made by Roveda *et al*. 2024 [26], two treatments were evaluated: an acidifying remediator and a *Bacillus* spp.-based bioremediator. Positive results were obtained, indicating that both strategies were effective in removing nitrogenous compounds [26]. This suggests that the inoculation of *Bacillus* spp. into the water of a BFT system can reduce ammonia levels as a form of bioremediation.

Meanwhile, nitrite levels were higher in the bioremediator treatment compared to the control, which can be explained by the fact that the biofloco system was still emerging. The conversion of nitrite to nitrate relies on the establishment of nitrite-oxidizing bacterial (NOA) populations, which had not yet been fully established. As a result, incomplete nitrification led to accumulation of nitrite [27].

Meanwhile, no significant differences were observed in ammonia and nitrite concentrations between the artificial substrate and bioremediator treatments. Although external microorganisms were not added in the substrate treatment, as was done with the bioremediator, there was a larger contact area for biofilm attachment and the development of the microbiota responsible for the nitrification process in the artificial substrate treatment [(15)]. Thus, the substrate is an effective strategy in promoting the nitrogen compound cycle, as reported by Da Paz Serra *et al.* [(17)]. When testing the use of substrate in BFT, they were able to maintain ammonia and nitrite levels safe for the cultivation of *L. vannamei*.

Floc volume among treatments was considered not enough for the cultivation of *Oreochromis niloticus* because the appropriate range is 25 to 50 mL [(27)]. However, this low value is justified given that floc formation was in its initial stage, so, the culture would have progressed along with the concentration of flocs. The lowest FV was observed in bioremediator and the artificial substrate treatments, respectively (Figure 1).

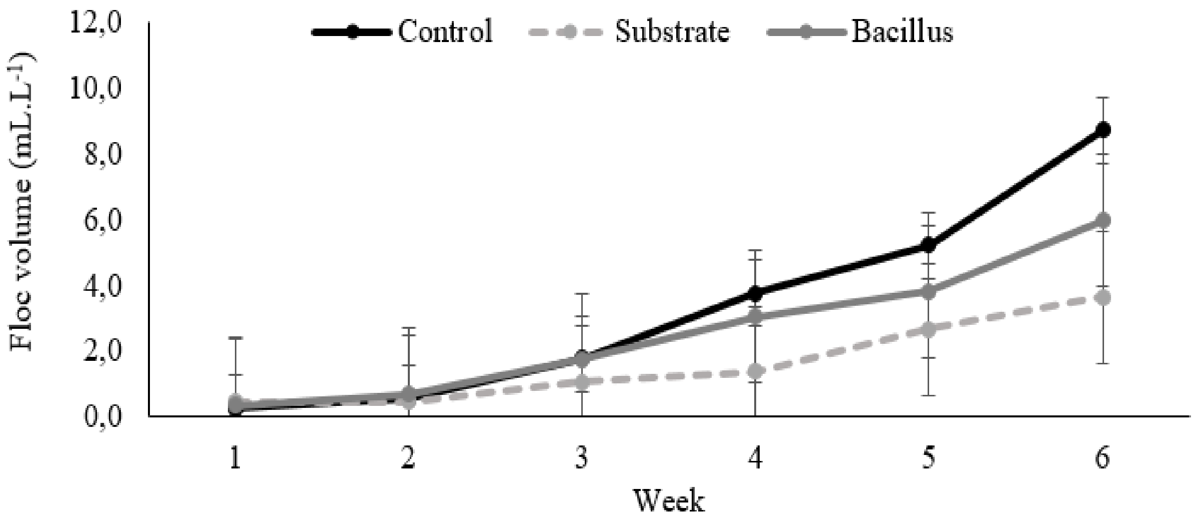

Figure 1. Floc volume of a BFT system integrated with either a bioremediator based on *Bacillus* spp. or artificial substrate.

By increasing the contact surface within the culture environment, where the artificial substrate was placed vertically and oriented perpendicularly to the aeration flow, promoted aggregation of the biofloc as biofilm, favoring the adherence of flakes to their structure. Accordingly, the floc had less presence within the water column, which explains the decrease in FV. In addition, no floc sedimentation was observed at the bottom of the tanks, which can be attributed to the effectiveness of the aeration system. Although the TSS were not significantly different among treatments, the changes in SST values among treatments could be observed from the fifth week of the experiment onwards (Figure 2).

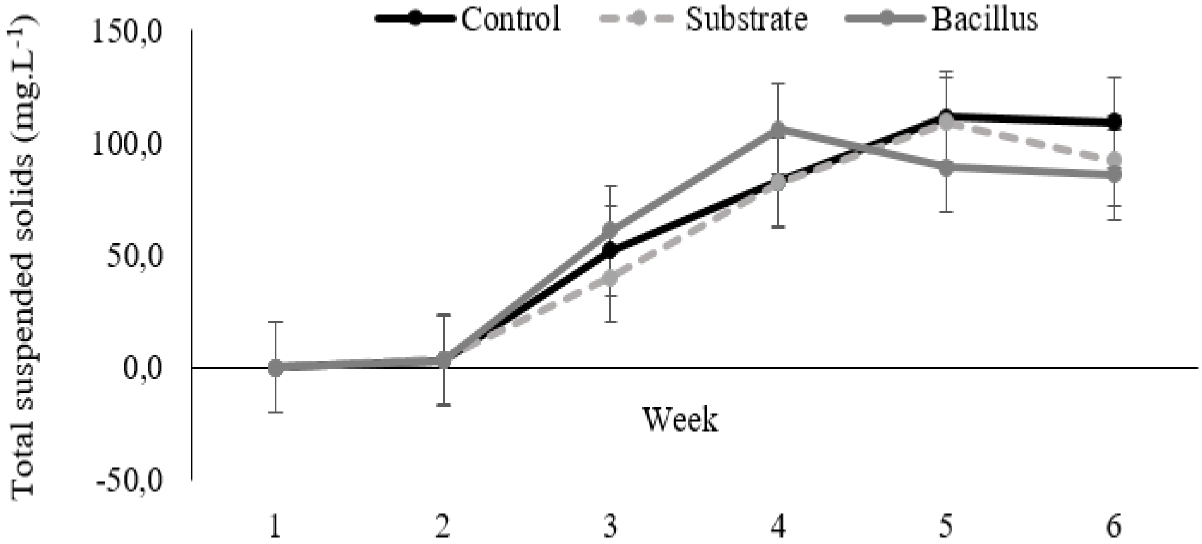

Figure 2. Total suspended solids of a BFT system integrated with a bioremediator based on *Bacillus* spp. or artificial substrate.

The lowest TSS values were obtained from the artificial substrate group and bioremediator group, respectively. TSS are formed by various particles, including unconsumed food, feces, debris and the various microorganisms that make up the biofloc [(28)]. It is proposed that the bioremediator decreased TSS since bacteria of the *Bacillus* genus have the ability to remove organic matter, as also observed by Roveda *et al.* [(26)]. Therefore, the addition of *Bacillus* spp. favors the decomposition of organic matter by promoting the recycling of suspended solids that accumulate in a BFT system. Whereas in the artificial substrate group, the TSS values decreased due to the control of suspended in the water column, in a similar way to what occurred with the FV, according to Ferreira *et al.* [(29)].

3.2 Zootechnical parameters

The average final mean weight, total biomass, survival, weekly weight gain and yield did not differ among the groups (Table 2). Meanwhile, the FCR was higher in the control relative to the substrate and the bioremediator groups, the latter two differing from each other. The SGR was lower in the bioremediator group relative to the artificial substrate group, while the control group did not differ from the others.

Table 2. Zootechnical parameters (mean ± standard deviation) of Nile tilapia reared in a BFT system integrated with a bioremediator derived from *Bacillus* spp. or artificial substrate.

	Control	Substrate	Bioremediator
Final mean weight (g)	24.83 + 4.61	26.38 ± 1.65	24.98 ± 1.41
Total biomass (g)	466.6 ± 72.94	495.0 ± 39.38	473.7 ± 16.24
Survival (%)	95.00 ± 5.00	93.75 ± 3.75	95.00 ± 2.50
Weekly weight gain (g.week^{-1})	23.95 ± 4.61	25.23 ± 1.6	24.10 ± 1.36
Food conversion rate	1.07 ± 0.07^{b}	0.86 ± 0.10^{a}	0.84 ± 0.03^{a}
Specific growth rate (%.day^{-1})	2.94 ± 0.16^{ab}	3.05 ± 0.04^{a}	2.97 ± 0.03^{b}
Yield (kg.m^{-3})	2.33 ± 0.36	2.48 ± 0.2	2.37 ± 0.08

*Different letters in the same row indicate significant differences ($p < 0.05$) as determined by ANOVA and SNK´s test.

Positive results were obtained from the both bioremediator and substrate treatments, as reflected in lower FCR when compared to the control. In the case of the bioremediator, this probably occurred because these bacteria colonized the gastrointestinal tract of the fish. *B. subtilis* can promotes this by the secretion of exogenous enzymes, modulating the intestinal physiology [11]. Thus, these bacteria facilitate the digestion of nutrients. Similar results were observed when using *B. subtilis* [35] and when using several *Bacillus* species [25].

In the artificial substrate treatment, besides achieving a better FCR, it was also possible to obtain a better SGR compared to the bioremediator treatment. In this case, the microorganisms adhering to the substrate in the form of biofilm form a well-established microbial community, which can act as an alternative food source for the cultivated organisms, contributing to the fish performance. This is similar to what has been observed in nile tilapia cultivation, where the use of substrates shows promising results for zootechnical variables [36].

3.3 Hematological parameters

The percentages of thrombocytes were higher in the bioremediator group, followed by the artificial substrate and the control. In the differential leukocyte count, the percentage of lymphocytes, monocytes, neutrophils, eosinophils and basophils did not differ among the groups (Table 3).

Table 3. Differential leukocyte and thrombocytes count (mean ± standard deviation) of Nile tilapia reared in a BFT system integrated with either bioremediator based on *Bacillus* spp. or artificial substrate.

Cells (%)	Control	Substrate	Bioremediator
Thrombocytes	18.5 ± 5.34^{c}	34.0 ± 6.98^{b}	46.4 ± 12.1^{a}
Lymphocytes	86.7 ± 9.90	97.9 ± 8.52	97.6 ± 13.3
Monocytes	11.6 ± 7.41	18.3 ± 14.5	16.9 ± 13.7
Neutrophils	6.61 ± 5.08	8.22 ± 5.77	9.37 ± 7.38
Eosinophils	0.33 ± 0.48	0.33 ± 0.59	0.5 ± 0.2
Basophils	0.22 ± 0.55	0.33 ± 0.77	0.32 ± 0.95

*Different letters in the same row indicate significant differences ($p < 0.05$) as determined by ANOVA and SNK's test.

An assessment of hematological parameters can reveal the health status of fish in response to changes related to water quality and diseases [32]. Some *Bacillus* species promote immunomodulatory activity by interacting with cells of the immune system, however, there is no exact clarifications on how probiotics stimulate these blood parameters [37]. Similar results were obtained by Rodrigues *et al.* [33] in *Pseudoplatystoma reticulatum* and TAchibana *et al.* [8] in *Oreochromis niloticus*.. Thrombocytes carry out phagocytosis and hemostasis, and their increased levels indicate an enhancement of the nonspecific immune response [38].

4. Conclusion

This study demonstrated that the application of a bioremediator (*B. amyloliquefaciens*, 1.86 × 10^{12} CFU and *B. subtilis*, 1.52 × 10^{13} CFU) and the use of artificial substrates enhanced water quality, zootechnical performance, and hematological profile of Nile tilapia in an emerging BFT system. Both strategies contributed to nitrogen compounds control through the establishment of microbial communities, while suspended solids were reduced either by biofilm formation and floc adherence to the substrate or by organic matter degradation mediated by *Bacillus* spp. Additionally, both treatments increased the proportion of thrombocytes, indicating a positive modulation of the hematological profile. Therefore, both strategies can be used as tools for maintenance of water quality and, hence better zootechnical performance.

Conflicts of interest statement

The authors declare no conflicts of interest.

Data availability statement

The data will be provided upon request.

Author contributions

Conceptualization: Lasala, M.; Dartora, A.; Andrade, J.; Jatobá, A.; Data Curation: Lasala, A.; Dartora, A.; Formal Analysis: Jatobá, A.; Funding acquisition: Jatobá, A.; Investigation: Lasala, M.; Dartora, A.; Morais, K. F.; Machado, A. O.; Ferreira, G. B.; Andrade, J.; Jatobá, A. Methodology: Andrade, J.; Jatobá, A.; Project administration: Lasala, M.; Resources: Jatobá, A.; Andrade, J.; Software: Lasala, A.; Dartora, A.; Supervision: Jatobá, A.; Andrade, J.; Validation: Lasala, M.; Jatobá, A.; Visualization: Lasala, M.; Stockhausen, L.; Jatobá, A.; Writing – First version: Lasala, M.; Stockhausen, L.; Jatobá, A.; Writing – Review & Editing: Lasala, M.; Stockhausen, L.; Jatobá, A.

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