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CASE REPORT

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Effects of a commercial multi-strain bioremediator on the growth and microbial community of Nile tilapia (*Oreochromis niloticus*) under biofloc conditions: a field study

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Abstract

The present field study evaluated the effects of a commercial multi-strain bioremediator (*Thiobacillus denitrificans*, *Bacillus subtilis*, *Bacillus licheniformis*, and *Paracoccus* sp.) on the frying phase of Nile tilapia (*Oreochromis niloticus*) under biofloc conditions. The bioremediator was applied directly to the biofloc water at a dosage of 3 g m^3 (150 g per tank) every three days, following the manufacturer's recommendations. Significant differences were observed in the aquatic and intestinal microbial communities, as well as in tilapia growth performance. The greatest diversities of phyla, orders, and genera were observed in the bioremediator group. The control group presented a greater number of potentially pathogenic species in the BFT water, with an emphasis on *Aeromonas hydrophila, Aeromonas veronii*, *Edwardsiella* spp., *Flavobacterium* spp., and *Mycobacterium* spp.. In the intestinal microbiome, the bioremediator group showed a decrease in bacterial diversity; however, the number of pathogenic bacteria, such as *A. hydrophila* and *A. veronii* decreased in the intestine of the fish in this group.

Keywords Aquaculture, Tilapia culture, Microbiome, Probiotic, Bioremediation

Introduction

In recent years, tilapia production has gained prominence in systems with biofloc technology (BFT) [1]. BFT cultures, which involve the production and management of microbial communities, stands out as a sustainable technology with low environmental impact and high fish densities [2, 3], and its fundamental principle is the establishment of a high carbon/nitrogen ratio (C:N) in water to stimulate the growth of heterotrophic bacteria [4]. These bacteria metabolize nitrogenous compounds and transform them into microbial biomass that can eventually be used as a dietary supplement for cultivated organisms [5]. Furthermore, BFT can provide a food source composed of fiber and essential fatty acids, further improving tilapia nutrition [6, 7]. Several studies have investigated the importance of BFTs for tilapia farming, with promising results [2, 3, 8]. For example, the diversity of bacterial groups present in biofloc can contribute to the digestive metabolism and performance of *O. niloticus* [9], reduce its dependence on commercial feed and improve its economic viability in production [10].

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Recent research has shown that systems with BFT technology exert a protective effect against pathogens such as *Vibrio harveyi*, *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Streptococcus iniae*, which cause major economic losses in fish farms [11]. This protective effect occurs due to the presence of several bioactive substances in the system, including chlorophyll, polyphenols, carotene, taurine, polysaccharides, phytosterols and vitamins, which have antagonistic effects on pathogens, suppress disease outbreaks and improve immunity in farmed fish [12].

The water microbiome in BFT systems is composed of a wide variety of microorganisms, such as fungi, algae, protozoa and bacteria [13, 14]. According to In-Kwon [15], more than 2000 bacterial species can grow properly in the BFT system, among which nitrifying bacteria and heterotrophic bacteria stand out as the most important groups [4]. Such microbial communities in aquatic habitats respond to changes in their environment immediately. These changes can be subtle, manifesting as activation or inactivation of specific metabolic pathways in the bacterial community or through changes in the composition and functionality of fish endogenous processes [16]. Thus, one possible cause of changes in biofloc microbial communities is the direct application of probiotics to culture water.

In recent decades, laboratory-scale research has investigated the bioremediation potential of probiotics based on bacteria from the *Bacillus* genus, and promising results have been reported [2, 17]. It is already known that some strains of the *Bacillus* genus are capable of heterotrophic nitrification [18]. At the same time, *Thiobacillus denitrificans* and *Paracoccus* sp. are bacteria that participate in the sulfur oxidation and nitrogen demineralization cycle [19, 20]. Furthermore, several authors have reported that the flakes formed in BFTs are also formed by probiotic bacteria, which can improve the digestion and absorption of nutrients, thus positively affecting the immune system of the fish [4, 7, 18, 21]. However, evaluations under real cultivation conditions, i.e., on a commercial scale, are scarce in aquaculture and require technical proof, as the predictability and repeatability of microbiome manipulations in BFTs on a commercial scale are currently limited.

The advantages of using probiotics as feed additives in aquaculture have been proven, but little is known about the influence of these additives on the dynamics of the microbiome in commercial BFT systems. This knowledge is essential for developing effective management strategies to control diseases and maximize the growth of aquatic organisms. Therefore, the present field study, based on evidence from previous research under laboratory conditions, aimed to evaluate the possible benefits of bioremediators in a commercial Nile tilapia fish farm under biofloc conditions, considering the water quality, changes in the intestinal and aquatic microbial community, and the growth performance of the fish.

Materials and methods

This study was carried out during the production cycle of Nile tilapia fingerlings under biofloc conditions on a commercial fish farm located in the municipality of Santa Fé do Sul, São Paulo, Brazil. The fish farm used circular high-density polyethylene (HDPE) tanks with a capacity of 50 m^3, which were individually equipped with constant aeration systems. The biometry and water quality data were obtained during the company's daily management routine, while the biological samples for intestinal analysis were collected from the company's own meatpacking plant. The ethics committee for the use of animals in research at the Federal University of Santa Catarina allowed the research to be carried out in accordance with its standards.

Experimental design

The juvenile Nile tilapia *Oreochromis niloticus* strain GIFT (genetically improved farmed tilapia) was supplied through a partnership with a company. For the field study, 780 thousand fingerlings of Nile tilapia with an average initial weight of 1.36 ± 0.26 g were distributed in six 50 m^3 circular tanks under biofloc conditions (BFT) and subjected to two different treatments: a control group with no bioremediator and a group with a bioremediator. The fish were fed four times daily with commercial feed suitable for tilapia containing 38% crude protein at a feeding rate equivalent to 3% of the fish biomass. A bioremediator (*T. denitrificans, B. subtilis, B. licheniformis, and Paracoccus* sp. at a concentration of 1×10^9 CFU g^{-1}) was applied directly to the biofloc water at a dosage of 3 g m^3 (150 g per tank) every three days, following the manufacturer's recommendations. This evaluation phase lasted 24 days (Fig. 1).

Initially, the six experimental units were inoculated with a 5% aliquot from a biofloc matrix tank that presented the following water quality parameters: pH 6.83, temperature 26.5 °C, dissolved oxygen 6.35 mg L^{-1}, alkalinity 128 mg L^{-1} $CaCO_3$, total ammonia (TAN) 0.12 mg L^{-1}, toxic ammonia (N-NH_3) 0.00 mg L^{-1} (below detectable levels), nitrite (N-NO_2) 0.05 mg L^{-1}, nitrate (N-NO_3) 9.25 mg L^{-1}, total suspended solids (TSS) 174 mg L^{-1}, and electrical conductivity 1.45 μS^{-1}.

After settling the fish in the system, water quality variables were measured twice daily, and the C:N ratio was maintained at 15:1 throughout the experimental period by the daily addition of molasses (30%) as a carbon source. The C:N ratio was calculated based on the

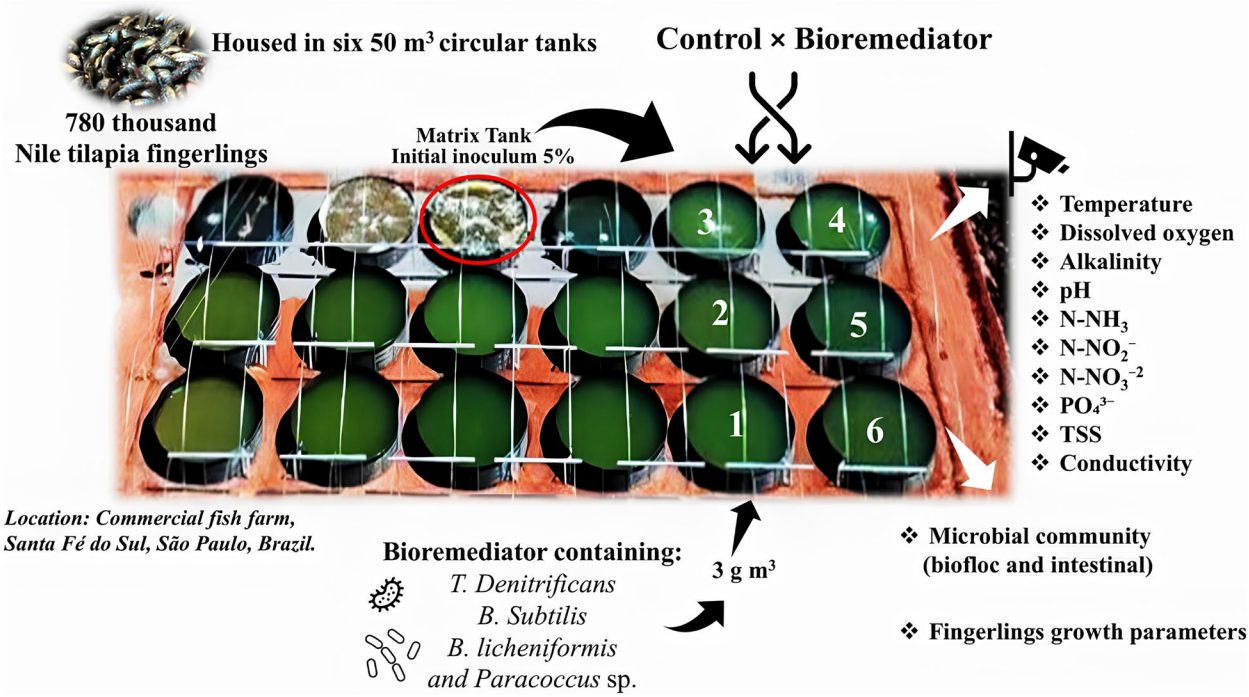

Fig. 1 Schematic representation of the experimental design. Nile tilapia fingerlings (1.36 ± 0.26 g) were randomly distributed in six 50 m^3 circular tanks (1 to 6) under biofloc conditions. Matrix tank (red circle). Santa Fé do Sul is a municipality in the state of São Paulo, Brazil. It is situated at a latitude of 20º12'40" south and a longitude of 50º55'33" west

alkalinity and total ammoniacal nitrogen (TAN) concentration in the tanks. The cultivation environment was assessed based on dissolved oxygen, temperature, and floc volume, using an Imhoff cone. Additionally, weekly monitoring included testing for TAN, N-NH_3, N-NO_2, N-NO_3, $CaCO_3$, orthophosphate (PO_43^-), electrical conductivity (µS–1), pH (Alfakit®), and TSS according to Jatobá et al. [2].

Biofloc bacterial community

To investigate the biofloc bacterial community, water samples were collected aseptically from each production unit and kept in sterile containers under refrigeration, according to Mello Júnior et al. [22]. Total DNA extraction was performed using the phenol/chloroform method. The DNA concentration of each pool was estimated using Picogreen dsDNA. To identify the microbial population, the bank of DNA libraries was adjusted to a final concentration of 11 pM, and amplification of the V3-V4 region of the 16S ribosomal RNA gene (rRNA) was conducted through polymerase chain reaction (PCR) using primers 341F (5' CCTACGGGRSGCA GCAG 3') and 805R (5' GGACTACCAGGGTATCTAAT 3'). Sequences were grouped into taxonomic operational units (OTUs) using USEARCH (version 10.0.240) at 97% similarity to the UPARSE algorithm. The SILVA database (version 132), with 91% identity, was used for taxonomic attribution. Richness and diversity indices were calculated using the vegan R package, and Good's coverage was calculated using the QsRutils R package.

Intestinal bacterial community

At the end of the production cycle, the biological samples for intestinal analysis were collected from the company's own meatpacking plant. The samples were composed of pools from the fish's intestinal tract. Samples from the intestine of 30 fish from each experimental unit were collected aseptically, without the digestive content, which was considered one sample; that is, six samples were collected (three from each group). The samples were stored at −80 °C until processing. Subsequently, deoxyribonucleic acid (DNA) was extracted with the commercial QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany, DE), as recommended by the manufacturer. The extracted material was quantified using a NanoDropTM 1000 spectrophotometer (Thermo Scientific DE, USA). Samples were maintained above 60 µg $µL^{-1}$.

For polymerase chain reaction (PCR), amplified DNA samples were sent to the company Macrogen® for high-throughput sequencing (HTS). PCR amplification of the microbial population was first performed by amplifying the V3-V4 region of the 16S ribosomal ribonucleic acid

(rRNA) gene. PCR was performed using primers for bacteria 341F (5'-CCT ACG GGN GGC WGC AG-3') and 805R (5'-GAC TAC HVG GGT ATC TAA TCC-3') and gel purified.

High-throughput sequencing was performed using Illumina SBS technology, and the nucleotides bound in each cycle were marked by fluorescence. Taxonomic analyses of the sequential reads were performed after filtering the reads and removing extra cuttings. Noise sequences were removed from the cluster, and the remaining representative reads from the clusters were clustered using a greedy algorithm into operational taxonomy units (OTUs) through fast short read length adjustment (FLASH), and the reads were clustered with 100% identity (ID) using CD-HIT-DUP in a single file. Sequences were then analyzed using Quantitative Insights in Microbial Ecology (QIIME). OTUs were collected using a quality filter to ensure 97% species-level identification. For sequencing, a minimum alignment of 300 base pairs (bp) was used with 20 k reads per sample.

Growth parameters

The biometric data were obtained during the company's daily management routine over a period of 24 days. The fish were counted and weighed on a precision scale (±0.01 g) to evaluate weight gain, final biomass, daily weight gain, productivity, specific growth rate, feed conversion and survival according to the following equations:

- Weight gain (g) = (final average weight − initial average weight)
- Biomass gain (kg) = (final biomass − initial biomass)
- Daily weight gain (%) = {(final weight − initial weight) / (cultivation days)}
- Productivity (kg $m.^{-3}$) = {(final biomass − initial biomass) / (experimental unit volume)}
- Specific growth rate (% $day.^{-1}$) = {(Ln (final weight) − Ln (initial weight) / (cultivation days)} × 100
- Feed conversion = (total feed intake/weight gain)
- Survival (%) = {(final number of animals/initial number of animals)} * 100.

Statistical analysis

The data obtained for growth performance and water quality were subjected to the Kolmogorov–Smirnov test to assess whether the data distribution was within the normality curve and Levene's test to verify homoscedasticity. The data obtained that met the prerequisites of normality and homoscedasticity were subjected to Student's t test. All analyses were significant at the 5% level. Metagenomics data were performed using R [23]. The main packages employed were nVennR [24], vegan [17], phyloseq [25] and iNEXT [8].

Table 1 Water quality parameters during cultivation of Nile tilapia *Oreochromis niloticus* under biofloc conditions (BFT) on a commercial scale. The data represents the control group without bioremediator and a group with bioremediator. The data were submitted to Student's -t test. All analyses have 5% significance

Water quality indicators	*Treatments*		
	Control	*Bioremediator*	*p-value*
Temperature (°C)	27.5 ± 0.26	27.33 ± 0.15	0.193
pH	7.80 ± 0.01	7.75 ± 0.05	0.115
Salinity (‰)	0.5 ± 0.0	0.5 ± 0.0	-
Toxic ammonia (mg L^{-1})	0.44 ± 0.31	0.14 ± 0.07	0.110
Nitrite-N (mg L^{-1})	0.06 ± 0.05	0.02 ± 0.01	0.153
Nitrate–N (mg L^{-1})	0.02 ± 0.01	0.01 ± 0.01	0.245
Dissolved oxygen (mg L^{-1})	4.67 ± 0.29	4.83 ± 0.58	0.253
Total suspended solids (mg L^{-1})	203.2 ± 40.41	213.33 ± 56.86	0.387
Alkalinity (mg L^{-1} $CaCO_3$)	152.22 ± 14.24	132.18 ± 54.23	0.290
Electrical conductivity (μS^{-1})	258.36 ± 193.23	196.17 ± 31.78	0.316

Results and discussion

The application of the bioremediator did not significantly change ($p > 0.05$) the water quality variables between treatments (Table 1). Using bioremediators directly in aquaculture pond water is beneficial for treating aquaculture sludge and enhancing fish health by improving water conditions and suppressing pathogens [15, 19, 26–28]. In the current field study, although no significant changes in water quality variables were observed between the groups, it is worth noting that the bioremediation group achieved a significantly higher final biomass. This likely led to increased waste generation in the water. Therefore, the statistical similarity in water quality between the groups is likely attributed to the bioremediator, which helped maintain similar levels of nitrogen compounds and total suspended solids in both groups.

The presence of 13 phyla, 41 orders, 114 genera and 142 bacterial species in the biofloc water were identified (Fig. 2 and Supplementary file 1). However, a greater diversity of phyla, orders, and genera was observed in the tanks to which the bioremediator was added. The presence of bacteria from the genera *Pseudomonas* and *Nitrobacter* highlights the importance of these species in the bioremediation process. The fact that *Nitrobacter* contributes to the conversion of nitrite to nitrate is relevant, as it helps to maintain acceptable levels of these substances in tanks, preventing possible negative impacts on water quality.

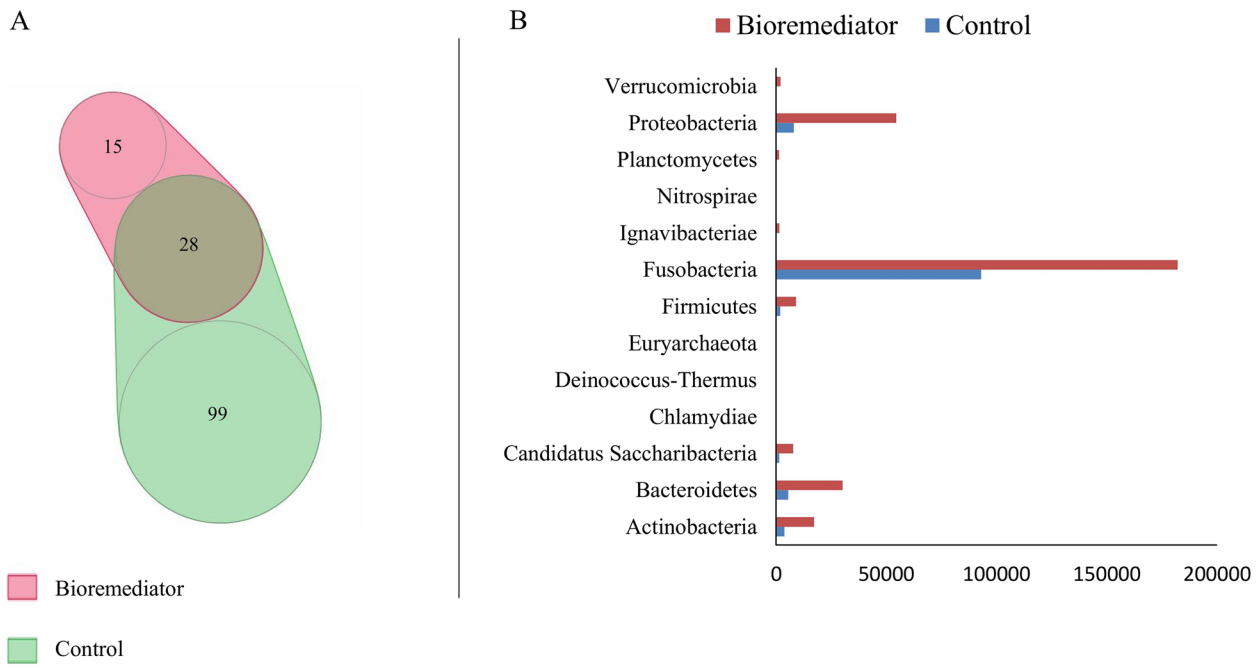

Fig. 2 Bacterial community in water during cultivation of Nile tilapia (*Oreochromis niloticus*) under biofloc (BFT) conditions. The data represents the control group without a bioremediator and the group with a bioremediator. **A** Diagram showing the bacterial community shared between treatments in BFT water. **B** The bacterial phyla found in the respective treatment groups

Regarding phyla, we highlight the Proteobacteria, which play a role in nutrient cycling, degradation of organic residues, nitrogen fixation, protection against pathogens and biodegradation of toxic compounds [29]. In contrast, the order Fusobacteriales, belonging to the genus *Cetobacterium*, plays an important role in aquaculture due to several essential functions, such as degradation of organic compounds, nutrient cycling, and competition with pathogens, so this order helps prevent diseases and promote fish health [30]. On the other hand, bacteria of the genus *Cetobacterium*, which play important roles in aquaculture as probiotics for fish, improve the digestion of nutrients [31]. In this context, the use of a bioremediator favored the presence of potentially beneficial phyla, promoting greater bacterial diversity for BFTs and desirable improvements in aquaculture.

In addition to the beneficial effects on increasing the diversity of bacterial phyla, orders and genera, the present field study highlighted the presence of 20 potential pathogenic bacteria in BFT water, with an emphasis on *Vibrio cholerae*, *Aeromonas* spp., *Edwardsella* spp., *Flavobacterium* spp., *Mycobacterium* spp., and *Streptococcus agalactiae*, which are known in aquaculture for their economic impact on commercial production [21]. Due to the action of the bioremediator, which was applied directly to the water, potentially pathogenic bacteria were less frequent in the BFT water, indicating that the bioremediator was an important ally in the health of the fish and was reflected in growth indicators. Such benefits strengthen the use of bioremediators in commercial BFTs.

In the present field study, 186 bacterial species were also identified in the tilapia intestine (Supplementary file 2). A total of 174 bacterial genera were identified in the control group, while 52 species were identified in the bioremediation group (Fig. 3). The control and bioremediation groups shared 40 species of bacteria. The bioremediation group showed a decrease in bacterial diversity; however, it demonstrated the potential to reduce the number of pathogenic bacteria in the intestine.

Early colonization of the intestine of fish has been studied in recent years to alter and direct the growth of beneficial intestinal bacteria during early life and determine how such changes affect the intestinal microbiota in later stages of life [6]. Although water and feed are the two primary sources of microorganisms available to fish, the factors underlying the successful colonization of ingested microorganisms and the gut microbial community are not yet fully understood. According to Giatsis et al. [6], studies that focus on the long-term effects of legacy probiotics during the early stages of animal development are desirable in aquaculture. Despite this, the present study reported that a *Bacillus*-based bioremediator caused significant changes in the intestinal microbial community of Nile tilapia under BFT conditions, and such changes in

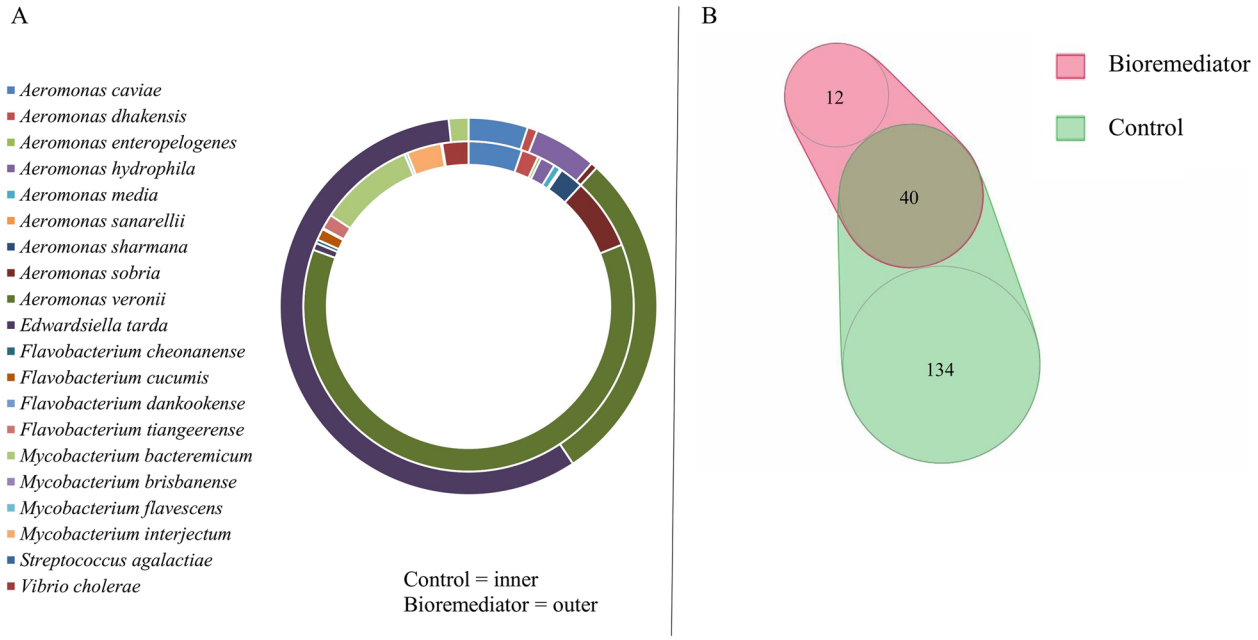

Fig. 3 Potentially pathogenic bacterial species found in the intestine of Nile tilapia (*Oreochromis niloticus*) cultured in biofloc conditions (BFTs). The data represents the control group without a bioremediator and the group with a bioremediator. In (**A**), the graph shows the main species identified in the intestine of fish that are pathogens of aquacultural importance. **B** Diagram showing 186 species of bacteria in the intestine of tilapia and the bacterial community shared between treatments

the microbiome were verified after just 24 days of cultivation, indicating that these fish obtained some advantages for the next stages of production when transferred to the final fattening phase.

Regarding growth performance indicators, significant differences ($p < 0.05$) were observed for final weight, weight gain, daily weight gain, biomass gain, specific growth rate, productivity, and feed conversion rate (Table 2). Certain probiotic bacteria are capable of improving water quality in intensive production, decomposing organic matter, and reducing the presence of pathogenic bacteria. Furthermore, they can lead to greater liver integrity and improvements in intestinal morphometry, important benefits that are related to better growth of fish [28, 32–34]. Indeed, the improvement in growth indicators in the present study may also have been caused by benefits in intestinal morphometry, resulting in greater absorption and use of food nutrients. However, these indicators were not evaluated in this field research.

Laice et al. [35] observed beneficial effects on the productive performance of Nile tilapia after using a combination of prebiotics and probiotics in biofloc water without causing significant changes in intestinal morphometry. However, the intestinal microbiota plays an essential role in the development and maturation of the gastrointestinal tract and, consequently, in the metabolism of nutrients [7]. This means that greater absorption and use of nutrients are not only related to improvements in intestinal morphometry but also

Table 2 Growth performance indexes of Nile tilapia *Oreochromis niloticus* under biofloc conditions (BFT) on a commercial scale. The data represent the control group without bioremediator and a group with bioremediator. The data was submitted to Student's -t test. All analyses have 5% significance. (*) Significant

Productive indexes	***Treatments***		
	Control	***Bioremediator***	***p-value***
Culture days	24	24	-
Initial fish (n)	390,000	390,000	-
Initial weight (g)	1.38 ± 0.26	1.35 ± 0.26	-
Initial biomass (kg)	530.4 ± 11.40	530.4 ± 11.40	-
Final total biomass (kg)	1,382.59 ± 17.74	1,454.16 ± 81.60	0.061
*Final weight (g)**	3.74 ± 0.51	4.20 ± 1.46	0.002
*Weight gain (g)**	2.38 ± 0.36	2.84 ± 0.85	< 0.001
*Daily weight gain (g day^{-1})**	0.09 ± 0.01	0.12 ± 0.03	0.040
*Biomass gain (kg)**	852.19 ± 6.34	923.76 ± 72.2	0.016
*Specific growth rate (% day^{-1})**	4.26 ± 0.04	4.61 ± 0.12	< 0.001
*Productivity (kg m^{-3})**	17.04 ± 0.12	18.67 ± 1.40	< 0.001
*Feed conversion rate**	1.10 ± 0.02	1.29 ± 0.11	0.019
Survival (%)	94.8 ± 4.82	88.68 ± 4.14	0.085

related to microorganisms that permanently establish themselves within the gastrointestinal tract without harming the host under normal conditions, which would justify the best results for growth performance in the bioremediation group.

The associations of Proteobacteria, Bacteroidetes, Fusobacteria and Actinobacteria with digestive processes and the production of fish-relevant enzymes indicate a possible route of action of the bioremediator to improve tilapia performance indicators. The decrease in the distribution of pathogenic bacteria in the intestine of fish after the application of the bioremediator was confirmed on a commercial scale. This research contributes to the development of more effective and ecologically sustainable management strategies for farms under BFT conditions, with the potential to reduce the incidence of diseases and improve fish production efficiently.

Conclusion

The bioremediator promoted the maintenance of water quality and improved the growth indicators of Nile tilapia under biofloc conditions. Furthermore, the bioremediator reduced the presence of gram-negative and gram-positive aquaculture pathogens both in the BFT water and in the intestine of the fish on a commercial scale.

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Animal welfare statement
All animal procedures carried out in this research were approved by the Ethics Committee on the Use of Animals of the Federal University of Santa Catarina.

Authors' contributions
W.P.M. conceptualization, experimental execution, and writing—original draft. M.S.O. writing—review and editing, visualization, supervision, and project administration. N.I.F. experimental execution, investigation, analysis. L.O.M.D.G. experimental execution, investigation, analysis. T.S. experimental execution, investigation. J.L.P.M. Supervision, project administration, experimental execution, investigation, resources, data curation. M.L.M. conceptualization, methodology, investigation, writing—review and editing, visualization, supervision, and project administration.

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Data availability
The original data measured in this study are available from the corresponding author and may be made available upon prior request.

Declarations

Compting interests
The authors declare no competing interests.

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