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Research Article

Impact of *Bacillus* spp. and benzoic acid supplementation on *Oreochromis niloticus* intestinal health

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ABSTRACT. This study aimed to evaluate the effects of a probiotic mix composed of *Bacillus* spp. and benzoic acid, all together and separately, in intestinal microbiology, liver, and intestinal histology, as well as in the composition of the intestinal microbiota by high-throughput sequencing analysis of juvenile Nile tilapias *Oreochromis niloticus*. Three hundred and twenty fish were divided into four experimental groups with four replicates. They were fed for 54 days: control (no supplementation), 0.1% benzoic acid ($BA_{0.1\%}$), mix of *Bacillus* spp. (*Bacillus*), and feed supplemented with *Bacillus* spp. and 0.1% benzoic acid ($B+BA_{0.1\%}$). Classical microbiological analysis showed that $B+BA_{0.1\%}$ increased the concentration of total heterotrophic bacteria (4.94 ± 0.77) compared to the control group (4.43 ± 0.90). The results showed significant liver changes such as ballooning, congestion of large vessels, and pancreatic congestion between treatments $B+BA_{0.1\%}$ and $BA_{0.1\%}$, the latter resulting in less (15.91 ± 16.61) pancreatic congestion than the other treatments. Vacuolation in the intestine was greater in supplemented animals with $BA_{0.1\%}$, $B+BA_{0.1\%}$, and *Bacillus* spp. than in the control group. The width of the villi was greater in the group supplemented with $B+BA_{0.1\%}$ (22.19 ± 10.91) than in the *Bacillus* group. The abundance of Firmicutes and Bacteroidetes was higher in the $B+BA_{0.1\%}$ group. *Cetobacterium somerae* was more abundant in the *Bacillus* and $B+BA_{0.1\%}$ groups in the diversity index. Notably, joint supplementation with the probiotic mixture *Bacillus* spp. and 0.1% benzoic acid had a synergistic effect on the intestines of tilapia, positively modulating the intestinal microbiota.

Keywords: *Bacillus*; aquaculture; tilapia; microbiology; additive; histology

INTRODUCTION

Feed additives increase production and resistance to diseases to minimize the problems related to fish health in aquaculture (Pereira et al. 2020). Probiotics, organic acids, and their salts have shown positive results by supporting fish immune systems, promoting enzyme production, regulating gut flora, and improving both performance and well-being (Albuquerque et al. 2013, Rodrigues et al. 2015, Pereira et al. 2020, Libanori et al. 2021).

Among the food additives used in aquaculture, probiotics can control pathogenic bacteria through competition for nutrients and/or adhesion sites, produce

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digestive enzymes by contributing to the better use of the nutrients present in the diet offered, and improve the immune system (Jesus et al. 2016).

Regarding probiotic bacterial species, those of the genus *Bacillus* have advantages over other strains because they can produce antimicrobial substances active against many microbes, are non-pathogenic and/or non-toxic to the host, and form spores, which make them heat-resistant and therefore suitable for the extrusion process (Kuebutornye et al. 2019). In addition, species of the genus *Bacillus* exhibit mechanisms already scientifically well described, such as the modulation of digestive enzymes, antioxidant enzymes, immune gene expression, stress, liver indices, disease resistance, and growth (Kuebutornye et al. 2019).

In addition to probiotics, organic acids and their salts are feed additives with beneficial effects on diet, metabolism, and the ability to fight microorganisms in the intestinal tract (antimicrobial activity) (Silva et al. 2012, Lim et al. 2015, Pereira et al. 2019, Libanori et al. 2021). Among the organic acids, benzoic acid has dietary benefits and potential antimicrobial function and is considered the most important aromatic carboxylic acid for animal dietary supplementation (Gheler et al. 2009, Santos et al. 2023). Libanori et al. (2021) demonstrated that feeding 0.1% benzoic acid to fish improved the growth performance and survival of Nile tilapia *Oreochromis niloticus* after a challenge with *Streptococcus agalactiae*. Furthermore, in a complementary work to the previous one, Libanori et al. (2023) identified that benzoic acid supplementation positively influenced and modulated the gut health of *O. niloticus*.

Thus, it is believed that the combined use of probiotics, organic acids, and/or their salts may have beneficial effects on the animals' digestive tracts, such as promoting the growth of beneficial bacteria and inhibiting the growth of pathogenic bacteria, thereby protecting the host from bacterial infections and disease transmission (Bolivar et al. 2018, Ebeid et al. 2021, Santos et al. 2023). Although there is a limited amount of research on the combined use of probiotics and organic acids for fish, Santos et al. (2023) evaluated the positive effects of combining the probiotic *Bacillus* spp. and benzoic acid in tilapia, observing improvements in zootechnical parameters, hematological and immunological profiles, and increased resistance after challenge with *S. agalactiae*. These results encouraged further investigation into the effects of these additives on intestinal microbiology, microbial community, and hepatic and intestinal changes.

Therefore, this study aimed to evaluate the influence of dietary supplementation of probiotic *Bacillus* spp. mixed with benzoic organic acid and separately for juvenile Nile tilapia on classical gut microbiology, liver, and gut histology, as well as on the composition of the gut microbiota, using high-throughput sequencing (HTS).

MATERIALS AND METHODS

This study continues the study by Santos et al. (2023), which evaluated the influence of 54-day dietary supplementation with a probiotic mixture of *Bacillus* spp. and 0.1% benzoic acid for Nile tilapia juveniles, focusing on zootechnical performance, hematological and immunological parameters, and resistance to experimental infection via gavage with *S. agalactiae*.

Biological material

Nile tilapia of the GIFT lineage were obtained from a monosexual male population donated by Empresa de Pesquisa Agropecuária and Extensão Rural de Santa Catarina (EPAGRI). A commercial probiotic mix (PureGro™) composed of *B. subtilis* and *B. licheniformis* at a concentration of 10^{12} colony-forming units per gram (CFU g^{-1}) (product used in aquaculture) and benzoic acid (VevoVitall™) was donated by DSM®. The animal handling procedures were approved by the Ethics Committee on Animal Use of the Federal University of Santa Catarina (CEUA/UFSC, by its Portuguese acronym, 2793230320).

Preparation of experimental diets

The experimental diets were formulated to meet the nutritional requirements of the species, according to nutrient requirements of fish and shrimp (NRC 2011). The proximate composition of the diet was determined at the Nutrition Laboratory of UFSC (LabNutri/UFSC), following the standard procedures detailed by the Association of Official Analytical Chemists (AOAC 1997). A detailed description of the diet can be found in Santos et al. (2023), which prepared four different diets: supplementation with no inclusion of benzoic acid or *Bacillus* spp. (control), supplementation with 0.1% benzoic acid ($BA_{0.1\%}$), with *Bacillus* spp. (*Bacillus*), and combined supplementation of *Bacillus* spp. and 0.1% benzoic acid ($B+BA_{0.1\%}$).

The food inoculum of *Bacillus* spp. probiotic mix was first prepared and activated in a sterile solution of NaCl prepared at 0.65% with distilled water. In the group supplemented with only 0.1% benzoic acid, the preparation followed the preparation of the mother

solution (8% benzoic acid, diluted in 50% cereal alcohol and 50% in 0.65% saline solution). From this, a dilution was made in saline solution to obtain concentrations of 0.1% of benzoic acid. The group was supplemented with *Bacillus* spp. and 0.1% benzoic acid; the inoculum was composed of 50% *Bacillus* spp. probiotic mix, and 50% of 0.1% benzoic acid. Subsequently, the feed was sprayed with their respective inoculum in the proportion of 100 mL kg^{-1} of feed. The mixture was incubated at 35ºC for 30 min in a hermetically closed container. Afterward, this container was opened and dried in a bacteriological oven with air recirculation at 30°C for 12 h. The diet of the non-supplemented group (control) was sprayed only with sterile saline solution (SSE) in the same proportions and conditions. This process was carried out weekly.

Microbiological count in the diet

To assess the permanence of the probiotic mix *Bacillus* spp. in the diet of the supplemented groups, the count of total heterotrophic bacteria was performed at the beginning and the end of each week. For this purpose, 1 g of the diet of the supplemented and control groups was macerated, added of 9 mL of 0.65% saline solution and serially diluted eight times in a factor of 1:10. The present concentrations of total heterotrophic bacteria were quantified by seeding the dilutions 10^{-4} to 10^{-8} in Tryptone Soy Agar (TSA, Himedia®) culture medium and incubated at 30°C for 24 h. The present concentrations of microorganisms were measured in CFU g of $feed^{-1}$.

The initial bacterial concentrations in the supplemented groups (*Bacillus* spp. together with benzoic acid 0.1%) were maintained after 7 days of spraying in the concentration of 1.32×10^9 and 4.35×10^9, respectively, indicating the method of inoculation of the probiotic mix was efficient. Thus, there was no significant difference between the initial concentrations and after 7 days of inoculation. However, there was a significant difference between the groups receiving the probiotic mix and those without bacterial supplementation. Santos et al. (2023) provide the microbiological feed count information.

Experimental design

The experimental design was similar to that described by Santos et al. (2023). Three hundred twenty fish with an initial mean weight of 5.76 ± 0.24 g were divided into 16 polyethylene tanks with a capacity of 100 L and a usable volume of 80 L, with 20 fish per tank and four replicates in each experimental group. The fish were acclimated for 15 days under experimental conditions. Thus, the experimental groups for evaluating the effects of the probiotic mixture of *Bacillus* spp. and benzoic acid were as follows: non-supplemented fish (control 0%), fish fed a diet supplemented with 0.1% benzoic acid ($BA_{0.1\%}$), fish fed a diet supplemented with a probiotic mix of *Bacillus* spp., and fish fed a diet supplemented with a probiotic mix of *Bacillus* spp. and 0.1% benzoic acid ($B+BA_{0.1\%}$).

Biweekly biometric measurements were used to monitor the fish's growth. When excess food was observed, the amount was reduced by 10% the following day; conversely, if no food was noticed, 10% more food was added the next day. Excess food and excreta were removed from the tanks twice daily via siphoning. The experiment lasted for 54 days.

During the trial, the experimental units (EUs) were connected to a semi-open water recirculation system with a flow rate of 0.022 L s^{-1}, composed of a decanter and mechanical and biological filters (anaerobic and aerobic, respectively), including ultraviolet sterilization. A controlled photoperiod of 12 h was used (Owatari et al. 2018b). The water quality was measured daily. The oxygen, alkalinity, pH, total ammonia, toxic ammonia, nitrite, and nitrate levels were measured using the colorimetric method (Labcon® test kit, Brazil). The water quality parameters were kept within the following ranges: water temperature 28.90 ± 2.36°C, dissolved oxygen 7.00 ± 1.07 mg L^{-1}, alkalinity 49.08 ± 28.48 mg L^{-1} of $CaCO_3$, pH 6.70 ± 0.34, total ammonia 4.25 ± 1.56 mg L^{-1}, toxic ammonia 0.02 ± 0.01 mg L^{-1}, nitrite 1.93 ± 0.72 mg L^{-1}, and nitrate 3.5 ± 1.5 mg L^{-1}.

Microbiological analysis of the intestine

At the end of the fattening experiments, the intestines of 6 fish from each tank were excised (one pool per tank), weighed, macerated and serially diluted in the proportion of 1:10 and then the dilutions of 10^{-4} to 10^{-9} were seeded. in: Man, Rogosa and Sharpe (MRS) agar medium Himedia® for cultureable total lactic acid bacteria) with aniline blue, and TSA (Himedia® for cultureable total heterotrophic bacteria) and dilutions 10^{-1} to 10^{-4} were seeded on: Thiosulfate Citrate Bile Sucrose agar (TCBS, Himedia® for cultivable vibrionases) and Cetrimid agar (Himedia® for cultivable *Pseudomonas* sp.). All culture media were incubated at 30ºC for 24 h, except for MRS, which was incubated at 35ºC for 48 h (Jatobá et al. 2008).

Histological analyses

After 54 days of testing, the liver and intestinal portions (anterior and posterior) of four fish per EU were collected for histological analysis. The organs were fixed in 10% buffered formalin. After 24 h of fixation, the samples were prepared for routine histological techniques, dehydrated serially in ethyl alcohol, and embedded in paraffin at 60°C for posterior cross sections of 3 µm thickness (microtome PAT-MR10) and stained with Harris Haematoxylin and Eosin (HHE). After staining, the slides were mounted with Entellan® and analyzed under Differential Interference Contrast Microscope (DIC) Axio Imager A.2 (Zeiss) (Addam et al. 2019).

Concerning intestinal morphology, the length, width, perimeter, and area of villi were measured, as well as the counts of goblet cells per villi, with the aid of Zen Pro software (Zeiss, Germany). All organs were assigned to histological changes according to the degree of intensity: 0 (absence of change), 1 (mild alteration, corresponding to less than 25% of the organ area), 2 (moderate alteration, 25 to 50% of the organ area) and 3 (severe alteration, more than 50% of the organ area), according to the method described by Schwaiger et al. (1997) adapted by Brum et al. (2018).

Metagenomic analysis

Sampling procedure

After 54 days of feeding, the animals were subjected to a 24-h fast. Subsequently, only the intestinal mucosa (without the presence of content) from each experimental unit (n = 9 for treatment) was aseptically collected in a laminar flow hood, pooled, fixed in absolute ethanol, and stored in a freezer at -80°C for later analysis.

DNA extraction

For deoxyribonucleic acid (DNA) extraction, 200 mg of the collected intestine was weighed. After incubation with 50 mg mL^{-1} of lysozyme at 37°C, DNA was extracted by the QIAamp® DNA Stool mini kit (QIAGEN, Hilden, Germany, DE) following supplier specifications. In the end, the amount of DNA was quantified by NanoDrop™ 1000 spectrophotometer (Thermo Scientific DE, US). Samples were maintained above 60 µg $µL^{-1}$.

PCR amplification

After the DNA extraction, these samples were sent to the Macrogen Company® for high-throughput sequencing (HTS). Briefly, the microbial population's polymerase chain reaction (PCR) amplification was first performed by amplifying the ribosomal ribonucleic acid (rRNA) 16S gene V3-V4 region at 55°C in 35 cycles. PCR was performed using bacterial primers described by Edwards et al. (1989): 341F (5′ CCT ACG GGN GGC WGC AG 3′) and by Herlemann et al. (2011): 805R (5′ GAC TAC HVG GGT ATC TAA TCC 3′).

High-throughput sequence (HTS) data processing

Taxonomic analyses of the sequential readings were performed after filtering the readings and removing the extra gravel. Noise sequences were removed from the cluster using statistical calculations, and the remaining representative reads from the clusters were grouped into operational taxonomic units (OTUs) using a greedy algorithm through Fast Length Adjustment of SHort reads (FLASH). The reads were generated using the HiSeq 2500 (Illumina) with Paired-End genetic sequencing. The readings were grouped with 100% identity (ID) using the CD-HIT-DUP in a single file. The sequences were analyzed using Quantitative Insights into Microbial Ecology (QIIME 2, Bolyen et al. 2019). The OTUs were collected using a quality filter to guarantee a 97% ID at the species level. For sequencing, a minimum alignment of 300 bp and 100 k of readings per sample was used, and the results were compared to the public sequence database of Silva (Quast et al. 2012, Pereira et al. 2020). Chimera removal was performed using the methodology described by Smyth et al. (2010).

A Venn diagram was generated using the InteractiVenn Platform (Heberle et al. 2015) to identify bacterial species that were unique and common to the treatments. The abundance of the bacterial phyla was estimated for each treatment group. PAST data analysis software was used for principal coordinate analysis (PCoA) and species diversity index (Hammer et al. 2001). PCoA interprets the spatial distribution of bacterial communities with similar and distinct compositions and abundances through an ordering diagram. A diversity index was used to explore microbial richness (Pereira et al. 2020).

Statistical analysis

The histology and classical microbiology data were subjected to Shapiro-Wilk and Levene tests to assess normality and variance homogeneity. Data not meeting at least one of these criteria were transformed using $\log_{10}(x+1)$. Subsequently, the data were analyzed using one-way ANOVA, and means were compared using the Tukey test with the assistance of Statistica 10.0

software. A significance level of ≤ 5% was considered for all tests. Quantitative insights into microbial ecology were used to identify the core microbiota, defined in this study as OTUs related to their corresponding taxa. The results of microbiome characterization are presented at the taxonomic level.

Heat map plots for phylum, class, and genus were produced using Heatmapper (Babicki et al. 2016). The OTUs were clustered using the average linkage method (average linkage) and Euclidean distance (Yang & Xu 2020).

Interactive Venn software was used for the Venn diagram (Heberle et al. 2015). Furthermore, diversity profiles and PCoA were performed using the PAST 4.03 program (Hammer et al. 2001). For PCoA, the similarity index was calculated using Euclidean distance.

RESULTS

The combined supplementation of the probiotic mix *Bacillus* spp. with benzoic acid resulted in an increase in the concentration of total heterotrophic bacteria compared to the control group and a reduction in the potentially pathogenic bacteria, *Pseudomonas* sp., compared to animals that received only *Bacillus* spp. In contrast, the animals that received $BA_{0.1\%}$ alone showed a lower concentration of *Pseudomonas* sp. than the other experimental groups. In addition, *Bacillus* spp. supplementation alone resulted in an increase in potentially beneficial lactic acid bacteria compared to animals that received only the $BA_{0.1\%}$ supplementation (Fig. 1).

Histological analysis

Morphological changes in the liver showed an increase in a ballooned appearance (Fig. 2a) in the group supplemented with $B+BA_{0.1\%}$ (P = 0.025) compared with the groups supplemented with $BA_{0.1\%}$ and *Bacillus* spp. alone; however, the control group showed intermediate data. Congestion in the great vessels was greater (P = 0.007) in animals supplemented with the $B+BA_{0.1\%}$ diet (Fig. 2b) and *Bacillus* spp. than in the control group; however, the $BA_{0.1\%}$ group showed intermediate value. The pancreatic congestion was lower (P = 0.032) in fish supplemented with $BA_{0.1\%}$ (Fig. 2c) compared to the other groups (Table 1).

In terms of intestinal morphological changes, animals supplemented only with *Bacillus* spp. showed significant differences in necrosis compared to the control group (Fig. 3a) (P = 0.019). Vacuolization was higher in animals supplemented with $BA_{0.1\%}$, $B+BA_{0.1\%}$, and *Bacillus* spp. than those in the control group (Fig. 3b) (P = 0.004) (Table 2).

Significant differences in the midgut morphometry in animals supplemented with $BA_{0.1\%}$ and the control group (P = 0.000) compared to the groups $B+BA_{0.1\%}$ and *Bacillus* spp. in terms of the villus length. The width of the villi of the animals was greater in the group supplemented with $B+BA_{0.1\%}$ compared to the *Bacillus* spp. group (P = 0.003) (Table 2).

Metagenomic analysis

The Shannon and Simpson diversity indices showed that the control group has higher species diversity (0.083 and 0.223, respectively) compared to the $BA_{0.1\%}$ group (0.050 and 0.134, respectively), the $B+BA_{0.1\%}$ group (0.015 and 0.055), and the *Bacillus* spp. group (0.003 and 0.015, respectively). Regarding species richness, the Chao-1 index revealed greater richness in the BA+B and *Bacillus* spp. groups (21 and 21, respectively) compared to the control group and the $BA_{0.1\%}$ group (6 and 4, respectively).

The Venn diagram (Fig. 4) demonstrates the number of OTUs at the species level that were unique or shared between the experimental groups. A total of 50 OTUs were identified. The *Bacillus* spp. group showed the highest number of gut bacterial species (20 OTUs), of which 10 strains were unique and were influenced by this supplementation. The second group with the highest number of bacterial species was $B+BA_{0.1\%}$, which presented 20 OTUs, nine of which were exclusive. The $BA_{0.1\%}$ and control groups contained four and six OTUs, respectively, and zero exclusive bacterial species. The $B+BA_{0.1\%}$ group shared four species with the *Bacillus* spp. group: *Bosea thiooxidans*, *Escherichia coli*, *Ralstonia pickettii*, and *Tabrizicola aquatica*. In contrast, the *Bacillus* spp., $B+BA_{0.1\%}$, and $BA_{0.1\%}$ groups only had one species in common, *Plesiomonas shigelloides*. The core bacterial groups had three species in common: *Cetobacterium somerae*, *Clavibacter michiganensis*, and *Cryobacterium psychrotolerans* (Fig. 4).

The heat map analysis showed a higher abundance of Firmicutes and Bacteroidetes in the $B+BA_{0.1\%}$ group and Actinobacteria in the control group (Fig. 5).

The principal coordinate analysis revealed similar compositions of microbial communities in the control and $BA_{01\%}$ groups (Fig. 6).

Among the OTUs, the most abundant species was *C. somerae*, which was most abundant in *Bacillus* spp. and groups $B+BA_{0.1\%}$ (Fig. 7).

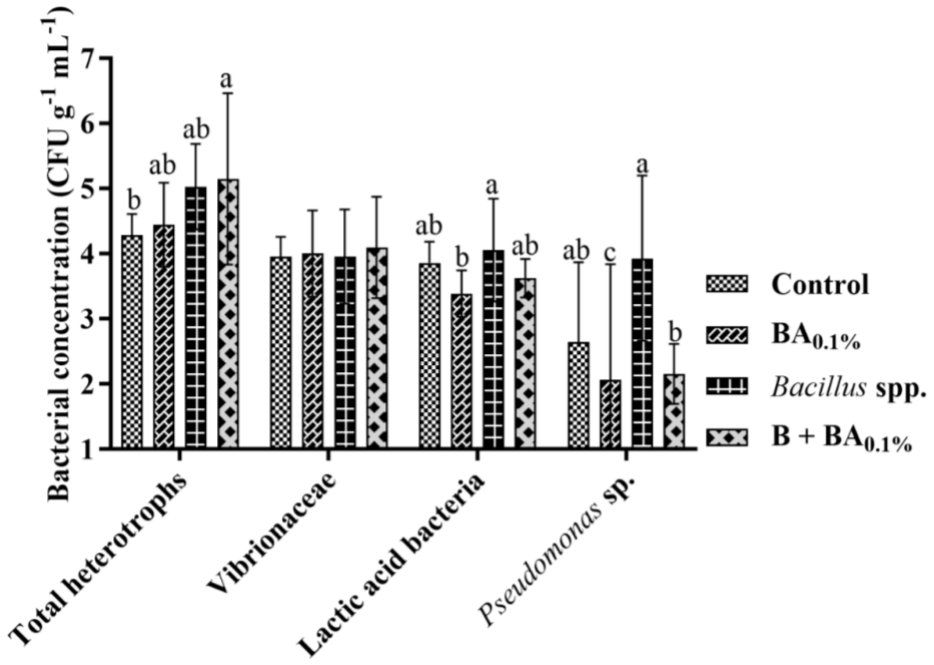

Figure 1. The concentration of bacteria per gram of intestine, at $\log_{10}$ (x+1) of colony forming units (CFU g^{-1}), of Nile tilapia *O. niloticus* fed a diet supplemented with 0.1% benzoic acid ($BA_{0.1\%}$), probiotic mix, *Bacillus* spp. (*Bacillus*), probiotic mix, and 0.1% benzoic acid ($B+BA_{0.1\%}$) and not supplemented (0% control) for 54 days. The bacterial concentrations are total heterotrophs, Vibrionaceae, total lactic acid bacteria, and *Pseudomonas* sp. Data are presented as mean ± standard deviation. *Different letters indicate a significant difference by Tukey's test ($P < 0.05$).

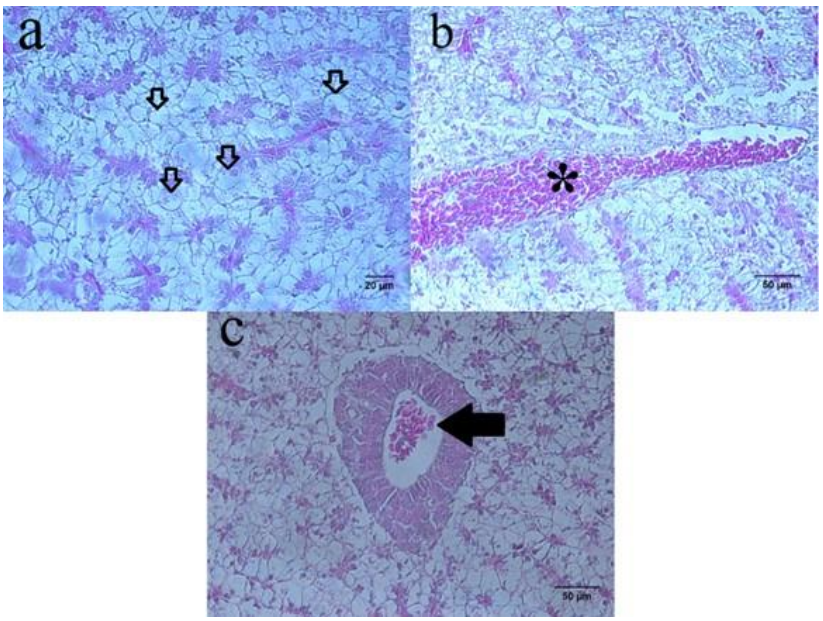

Figure 2. Histopathological changes in the liver of Nile tilapia (*O. niloticus*) were observed when fed a diet supplemented with 0.1% benzoic acid ($BA_{0.1\%}$), *Bacillus* spp. probiotic mixture (B), 0.1% benzoic acid ($B+BA_{0.1\%}$) probiotic mixture, and a non-supplemented control (0%) for 54 days. a) Open arrows indicate ballooning hepatocytes in the $B+BA_{0.1\%}$ group; b) asterisks (*) denote a large congested and dilated vessel in the $B+BA_{0.1\%}$ group; and c) filled arrows indicate pancreas congestion in the $BA_{0.1\%}$ group.

Table 1. Intensity of histological changes in Nile tilapia *O. niloticus* liver fed an unsupplemented diet (control 0%), supplemented with 0.1% benzoic acid ($BA_{0.1\%}$), with *Bacillus* spp. probiotic mix with 0.1% benzoic acid ($B+BA_{0.1\%}$) and *Bacillus* spp. alone (*Bacillus*) for 54 days. Data are presented as mean ± standard deviation. USCN: uniformity in the size of cells and nucleus, PIAZ: pancreas with intact acini and zymogen granules, DHN: displacement of hepatocyte nucleus, LNPA: loss of nucleus of pancreatic acini.

Parameter (%)	$Control_{0\%}$	$BA_{0.1\%}$	$B+BA_{0.1\%}$	*Bacillus* spp.	*P*-value
Loss of cord aspect	47.92 ± 31.01	29.16 ± 9.73	39.58 ± 32.06	52.08 ± 31.21	0.137
USCN	58.33 ± 16.28	75.82 ± 14.57	63.54 ± 18.03	66.66 ± 15.92	0.083
PIAZ	66.66 ± 19.46	0.42 ± 12.87	59.37 ± 19.24	64.58 ± 19.38	0.626
Bile ducts	0.42 ± 0.51	0.75 ± 0.45	0.66 ± 0.48	0.63 ± 0.49	0.374
Ballooning aspect	56.25 ± 21.65^{ab}	41.66 ± 24.62^{b}	61.95 ± 19.76^{a}	46.87 ± 19.93^{b}	0.025
Great vessels congestion	16.66 ± 12.31^{b}	28.85 ± 27.86^{ab}	35.23 ± 21.35^{a}	43.75 ± 22.42^{a}	0.007
Pancreas congestion	33.33 ± 16.28^{a}	15.91 ± 16.61^{b}	31.25 ± 19.84^{a}	36.46 ± 20.83^{a}	0.032
Sinusoids congestion	58.33 ± 22.19	41.66 ± 22.19	53.12 ± 21.94	53.12 ± 18.52	0.227
DHN	62.50 ± 13.05	6.25 ± 21.65	63.54 ± 16.45	60.87 ± 19.69	0.712
Eosinophilic infiltrate	0.00 ± 0.00	6.25 ±11.31	6.25 ± 16.89	9.09 ± 16.45	0.382
Lymphocytic infiltrate	14.58 ± 12.87	2.50 ± 13.06	19.27 ± 17.52	25.00 ± 23.31	0.187
Hepatocytes hypertrophy	37.50 ± 13.06	9.17 ± 17.94	36.46 ± 12.72	34.37 ± 14.39	0.457
Necrosis	54.16 ± 20.87	45.83 ± 23.43	61.46 ± 18.03	54.35 ±16.26	0.413
Macrosteatosis	0.00 ± 0.00	6.25 ± 15.54	5.98 ± 12.98	4.17 ± 12.04	0.526
LNPA	2.08 ± 7.22	4.17 ± 9.73	11.46 ± 16.45	6.25 ± 13.29	0.182
Amyloidosis	1.042 ± 3.61	0.00 ± 0.00	1.56 ± 4.22	3.64 ±7.80	0.226

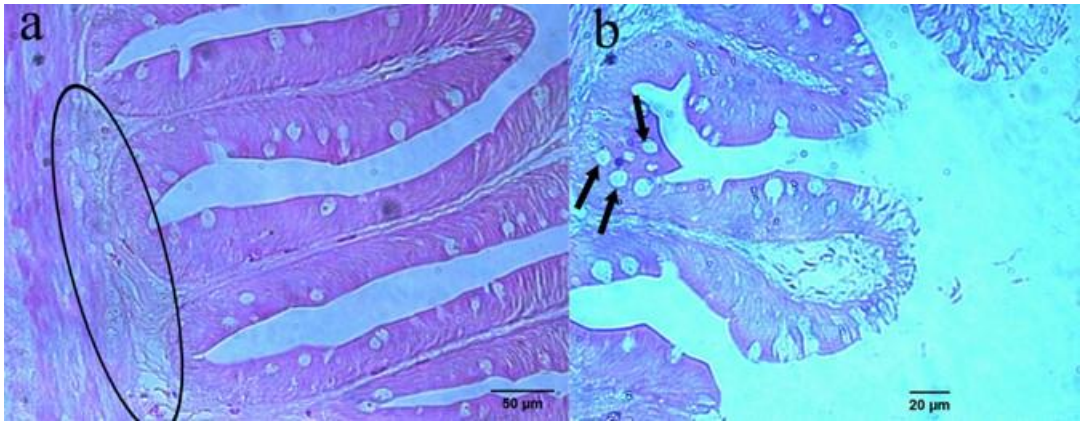

Figure 3. Histopathological changes in the intestine of Nile tilapia (*O. niloticus*) were observed when fed a diet supplemented with 0.1% benzoic acid ($BA_{0.1\%}$), *Bacillus* spp. probiotic mixture (B), probiotic mixture, and 0.1% benzoic acid ($B+BA_{0.1\%}$), and a non-supplemented control (0%) for 54 days. a) The black circle indicates necrosis in the *Bacillus* spp. group; and, b) the black arrows indicate vacuolization in the control group.

DISCUSSION

The combined use of the *Bacillus* spp. probiotic mix and benzoic acid 0.1% positively modulated the gut microbiota of *O. niloticus*, showing a synergistic effect of the additives in protecting against pathogenic bacteria and increasing the number of beneficial bacteria in the gut. In this study, fish supplemented with $B+BA_{0.1\%}$ showed an increase in the concentration of total heterotrophic bacteria compared to the control group and a reduction in the number of potentially pathogenic bacteria, specifically *Pseudomonas* sp., compared to the animals that received only *Bacillus* spp. The growth and reduction of total heterotrophic bacteria and *Pseudomonas* sp., respectively, may be associated with the possible synergistic effect of the probiotic and the organic acid mixture, as they might be contributing to the acidification of the gut, decreasing the pH in the gastrointestinal tract. This suppression of pathogenic bacteria proliferation and enhancement of

Table 2. Morphological and morphometric changes in the midgut of Nile tilapia *O. niloticus* fed an unsupplemented diet (control 0%), supplemented with benzoic acid 0.1% ($BA_{01\%}$), with *Bacillus* spp. probiotic mix with benzoic acid 0.1% ($B+BA_{0.1\%}$) and *Bacillus* spp. alone (*Bacillus*) for 54 days. Data are presented as mean ± standard deviation.

Parameter	$Control_{0\%}$	$BA_{0.1\%}$	$B+BA_{0.1\%}$	*Bacillus* spp.	*P*-value
Necrosis	35.42 ± 16.71^{b}	41.67 ± 19.17^{ab}	43.48 ± 22.88^{ab}	56.25 ± 19.85^{a}	0.019
Vacuolation	12.50 ± 13.18^{b}	40.28 ± 25.92^{a}	44.79 ± 23.29^{a}	41.67 ± 25.18^{a}	0.004
Nº villi	46.00 ± 2.00	40.00 ± 6.93	43.00 ± 2.31	45.00 ± 7.57	0.808
Length of villi	9.26 ± 18.43^{a}	55.90 ± 17.42^{a}	42.34 ± 14.26^{b}	34.87 ± 18.63^{b}	0.000
Width of villi	7.39 ± 3.25^{ab}	19.57 ± 3.67^{ab}	22.19 ± 10.91^{a}	16.34 ± 5.95^{b}	0.003
Total area of the villi	49837.9 ± 4714.18	45482.90 ± 11904.90	57744.36 ± 20552.94	40764.30 ± 23971.54	0.620
Total perimeter	6163.18 ± 277.10	4963.95 ± 1002.64	5903.96 ± 1816.26	4943.95 ± 2883.97	0.779
Globet cells	460.00 ± 296.00	287.33 ± 83.72	245.00 ± 53.60	314.00 ± 285.89	0.574

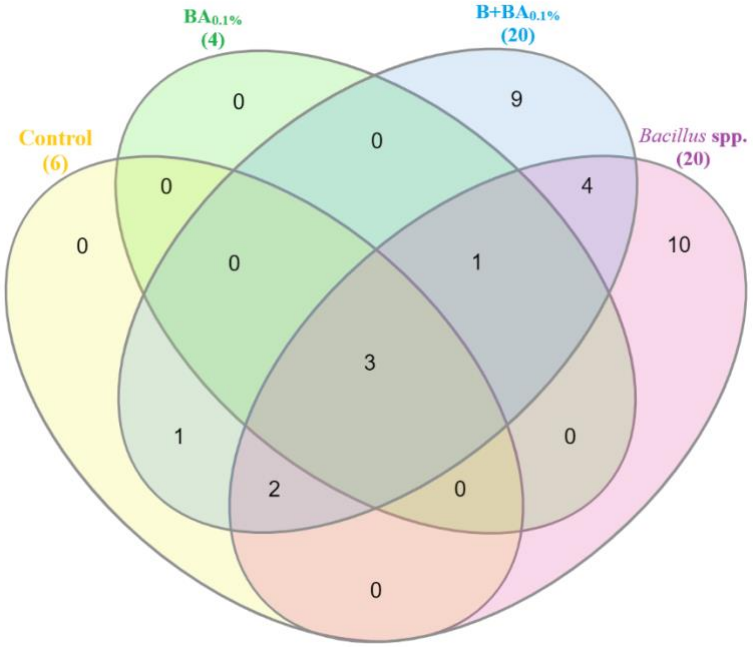

Figure 4. Operational taxonomic unit presentation (OTUs) of Nile tilapia (*O. niloticus*) fed an unsupplemented diet (control 0%) or supplemented with benzoic acid 0.1% ($BA_{0.1\%}$), a *Bacillus* spp. probiotic mix with benzoic acid 0.1% ($B+BA_{0.1\%}$), and only *Bacillus* spp. (*Bacillus*) for 54 days.

beneficial microorganisms could be responsible for the observed effects (Zulfqarul et al. 2017, Ebeid et al. 2021).

Another important factor in the action of organic acids, including benzoic acid, is the depletion of adenosine triphosphate (ATP), which allows organic acids to inhibit the growth and induce the death of pathogenic bacteria (Lim et al. 2015, Koh et al. 2016, Libanori et al. 2023), which could also explain the lower concentration of *Pseudomonas* sp. in animals supplemented with $BA_{0.1\%}$ in the present study, and the increase in total heterotrophic bacteria, as observed by Libanori et al. (2023) with 0.3% benzoic acid supplementation in tilapia, which positively modulated the concentration of these bacteria.

Additionally, there is an increase in lactic acid bacteria in animals supplemented with *Bacillus* spp. compared to the $BA_{0.1\%}$ group could be associated with the ability of probiotics to increase the synthesis of mucin genes (glycoproteins), which positively affect the bacterial interactions in the intestinal tract (Aliakbarpour et al. 2012). This process may contribute to greater adhesion of lactic acid bacteria, which is mainly facilitated by the action of peristalsis associated with the lubrication of mucins that protect intestinal epithelial cells (Ayivi et al. 2020). In addition, supple-

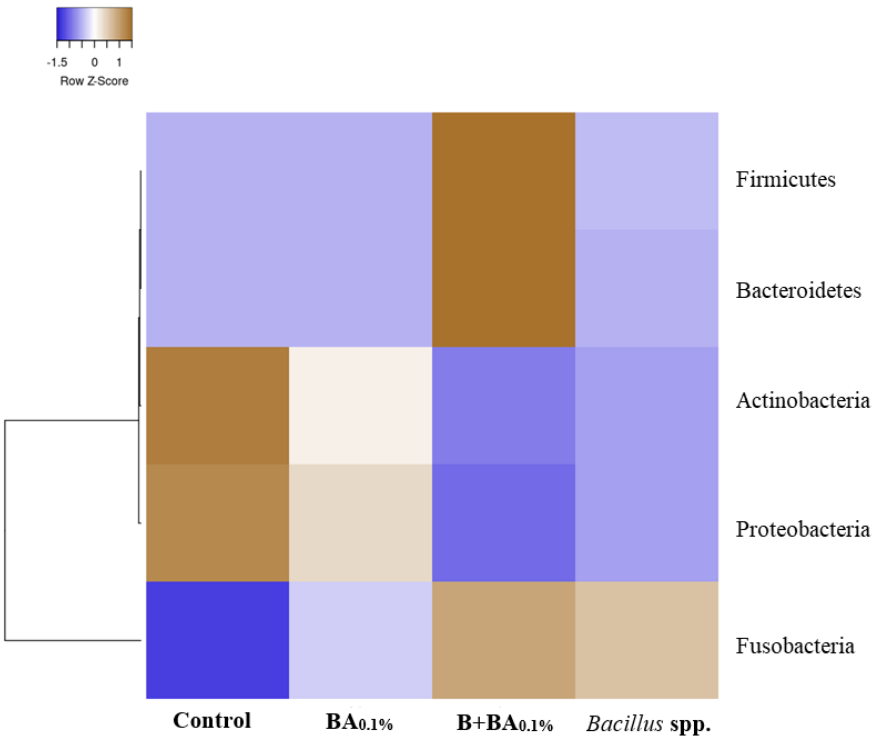

Figure 5. Presentation of the phylum-level heat map analysis of gut samples from Nile tilapia (*O. niloticus*) fed an unsupplemented diet (control 0%), supplemented with benzoic acid 0.1% ($BA_{01\%}$), with *Bacillus* spp. probiotic mix with benzoic acid 0.1% ($B+BA_{0.1\%}$) and *Bacillus* spp. alone (*Bacillus*) for 54 days.

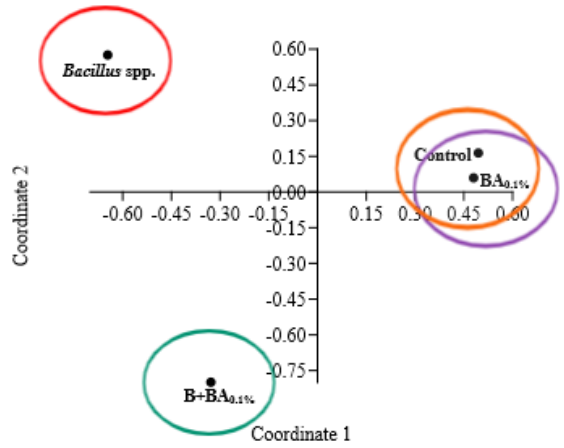

Figure 6. Principal coordinate analysis (PCoA) of gut samples from Nile tilapia (*O. niloticus*) fed an unsupplemented diet (control 0%), supplemented with 0.1% benzoic acid ($BA_{0.1\%}$), with *Bacillus* spp. probiotic mix with 0.1% benzoic acid ($B+BA_{0.1\%}$) and *Bacillus* spp. alone (*Bacillus*) for 54 days.

mentation with *Bacillus* spp. resulted in a higher number of OTUs in exclusive strains; that is, it promoted a favorable environment for the proliferation of specific bacterial species, most of which belonged to the lactic acid bacteria group.

In terms of the potential toxicity of supplemented substances, the liver is considered an organ of good pathological indication for nutrition due to the function of metabolizing substances from the gastrointestinal tract (Rašković et al. 2011). Thus, changes in the liver are related to interference in metabolic activity, which can be caused by toxic substances, food, and microorganisms occurring in diseases and even the death of animals (Honorato et al. 2014, Libanori et al. 2023). The histopathological findings of the liver in this study indicated significant changes in the ballooning aspect, congestion of the great vessels, and congestion of the pancreas.

The ballooning appearance is presumed to be caused by damage to the plasma membrane, allowing the influx of fluid into the cell, or damage to the cytoskeleton, resulting in the loss of cell shape (Romil 2018). This change may be due to the interference of metabolic activity caused by the additives, where the group supplemented with $B+B_{A0.1\%}$ compared to $BA_{0.1\%}$ and *Bacillus* spp. showed an increase in this aspect. According to Rašković et al. (2011), the liver is

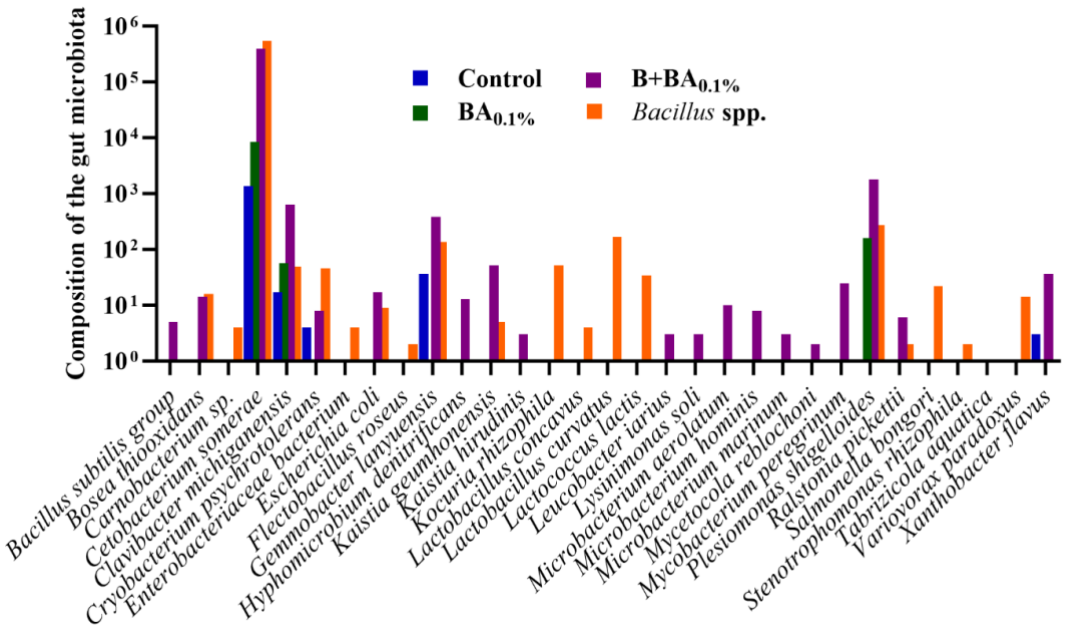

Figure 7. The most abundant species among the number of OTUs and diversity indices of the intestinal samples of Nile tilapia (*O. niloticus*) fed an unsupplemented diet (0% control), supplemented with 0.1% benzoic acid ($BA_{0.1\%}$), with a probiotic mixture of *Bacillus* spp. together with 0.1% benzoic acid ($B+BA_{0.1\%}$) and *Bacillus* spp. alone (*Bacillus*) for 54 days.

the organ of choice in histopathology. However, difficulties in interpreting results can occur because the liver is sensitive to any source of pollution that can be found under experimental conditions.

Blood vessel congestion may be associated with hepatocyte responses to toxic substances (Dyk et al. 2007). Owatari et al. (2018a) reported that passive congestion of blood vessels in the liver can occur in all animal species due to cardiac disorders; therefore, it is not potentially influenced by the supplemented substances.

As mentioned earlier, the liver can metabolize substances from the gastrointestinal tract, similar to the pancreas of fish, which secretes insulin and glucagon in response to nutrient absorption in the gut (Rotta 2003). In our study, the classical microbiological analysis and intestinal morphometry demonstrated that animals supplemented with $BA_{0.1\%}$ showed a reduction in possibly pathogenic bacteria, resulting in improved nutrient absorption and increased villus length. Thus, congestion in the observed pancreas may be associated with benzoic acid absorption in the gut, which was possibly secreted by the pancreas and showed less change in the $BA_{0.1\%}$ group than in the other groups. Addam et al. (2019), Pereira et al. (2019), and Libanori et al. (2023) also evaluated pancreatic congestion; however, they did not find significant differences between jundiá (*Rhamdia quelen*) and tilapia supplemented with different salts of organic acids.

In terms of intestinal morphological alterations, little is known about the effects of the combination of probiotics and organic acids on the biochemical mechanisms of action in the intestine, perhaps because these additives can modify some internal biological processes, resulting in increased necrosis in animals supplemented with $B+BA_{0.1\%}$ compared to the control group. The necrosis process is initiated by the accumulation of ATP depleted by the enzyme participating in DNA repair (PARP), during which cells and organelles swell and rupture with the leakage of cell contents into the microenvironment, causing an inflammatory response (Nunes et al. 2014). Therefore, the influence of food additives on this process is poorly understood. Thus, further studies addressing this issue are warranted.

The same explanation could be offered for fish that received diets supplemented with $BA_{0.1\%}$, $B+BA_{0.1\%}$, and *Bacillus* spp., which showed greater vacuolization compared to fish in the control group, demonstrating that these changes may be linked to biochemical functions or pathological issues. According to Cerezuela et al. (2012), the excessive accumulation of

vacuoles inside enterocytes and the dilated intercellular space between cells are related to pathological conditions such as gastroesophageal reflux disease in mammals. In contrast, Cerezuela et al. (2013) reported that vacuoles inside enterocytes are normal during lipid digestion, absorption, and transport; however, excessive accumulation can result in functional alterations in enterocytes. Thus, food additives (benzoic acid and/or *Bacillus* spp.) may have contributed to the better digestion, absorption, and transport of the lipids present in the diet, including the organic acid itself, as it is a short-chain lipid. Similar to the results of the present study, Cerezuela et al. (2013) verified intestinal histological changes in the vacuolization of the enterocytes of sea bream (*Sparus aurata* L.) supplemented with the probiotic *B. subtilis* (10^7 CFU g^{-1}). However, unlike previous studies, Libanori et al. (2023) found no histological changes in the intestines of Nile tilapia-fed diets supplemented with different concentrations of benzoic acid. Thus, owing to the divergence between studies regarding enterocyte vacuolization, further work should be conducted in this direction.

Regarding intestinal histomorphometry, Batista et al. (2016) reported that villus length and width were directly related to increased surface area, which may indicate improved intestinal nutrient absorption capacity. Studies have reported that the villus length in animals supplemented with organic acids is associated with antimicrobial effects in the gut (Addam et al. 2019, Pereira et al. 2019, Jesus et al. 2021, Libanori et al. 2023). According to Jesus et al. (2021), this effect is caused by the reduced shedding losses of villi, resulting in an increased area for nutrient absorption. In our study, the villus length of animals that received a diet containing $BA_{0.1\%}$ significantly differed from those that received $B+BA_{0.1\%}$ and *Bacillus* spp. Like this study, Libanori et al. (2023) also observed increased villus length in fish supplemented with benzoic acid 0.1% compared to the control and 0.2% benzoic acid groups.

The villus width was significantly greater in the $B+BA_{0.1\%}$ group than in the *Bacillus* spp. group. These results corroborate the classic microbiology results, in which the $B+BA_{0.1\%}$ group showed reduced pathogenic bacteria compared to the *Bacillus* spp. group. Pathogenic bacteria can affect intestinal mucosal integrity, and $B+BA_{0.1\%}$ supplementation contributed to the reduction of possibly pathogenic bacteria by improving intestinal mucosal integrity, thereby increasing villus width (Mello et al. 2013, Santos et al. 2023).

In terms of the analysis of bacterial metagenomics of the intestinal tract of the fish, the phyla Firmicutes and Bacteroidetes are part of the dominant phyla in the microbiota of fish and are associated with efficient energy absorption from the diet (Bereded et al. 2022) while Firmicutes are bacteria that can increase the activity of digestive enzymes and inhibit the growth of possibly pathogenic bacteria (Zhang et al. 2021); in our study, these phyla were the most abundant in the $B+BA_{0.1\%}$ group. These findings correspond to the histopathological data of the intestine, which showed a significant difference in the $B+BA_{0.1\%}$ group in terms of villus width, indicating better absorption and improved integrity of the mucosa, again confirming the hypothesis of a synergistic effect of the probiotics and benzoic acid. Actinobacteria are detected at varying levels in herbivorous fish species, including tilapia (Standen et al. 2015). According to Adeoye et al. (2016), the presence of Actinobacteria in the gut of tilapia aligns with previous research that utilized molecular techniques to investigate the microbiota. Adeoye et al. (2016) found an abundance of Actinobacteria in a tilapia control group supplemented with exogenous enzymes.

Despite the identification of bacteria from the *Pseudomonas* group using medium-dependent techniques (classical microbiology), these organisms were not observed in the metagenomic analyses (likely due to sampling) given that in microbiology, the intestines were macerated, diluted, and plated without distinguishing between intestinal content and the mucosa, while in metagenomics, the sample was obtained from the intestinal mucosa without the content. Therefore, the medium-dependent *Pseudomonas* spp. are not of autochthonous origin. Regarding bacteria from the *Bacillus* spp., the *Bacillus subtilis* group was found only in the $B+BA_{0.1\%}$ group, indicating that the incorporation of benzoic acid may have favored the colonization of this species in the intestinal tissue, effectively acting as a prebiotic. In this case, it is also important to note that the supply of *Bacillus* spp. by the *Bacillus* group likely resided in the lumen of the microbiota; therefore, in this group, this organism was allochthonous and did not adhere to the surface of the intestinal mucosa, as described by Soltani et al. (2019).

The diversity index showed a higher abundance of *C. somerae* in the *Bacillus* spp. and $B+BA_{0.1\%}$ groups. The genus *Cetobacterium* is predominant in the intestines of freshwater fish (Tsuchiya et al. 2008, Zhai et al. 2017). Moreover, this genus is considered beneficial to the host fish and capable of producing vitamin B12, which acts as a regulator of the gut

microbiota (Zhou et al. 2022). Studies have shown that probiotic *Bacillus* spp. increases the abundance of *Cetobacterium* in tilapia and carp (Wang et al. 2020, Zhang et al. 2021). According to Zang et al. (2021), the probiotic *B. licheniformis* can positively reduce the pathogenic gut microbiota and profoundly affect the diversity and composition of the gut microbiota in common carp *Cyprinus carpio*. In the B+$BA_{0.1\%}$ group, our study demonstrated that the combined effect of the additives provided a higher abundance of *C. somerae* species, clearly showing that the synergistic effect of the probiotic and organic benzoic acid was able to modulate beneficial bacteria in the gut of Nile tilapia positively.

PCoA showed greater similarity between fish supplemented with $BA_{0.1\%}$ and the control group. This similarity may be related to the intestinal morphometry data in this study. There was no difference in the villus lengths of either group, demonstrating that both groups had increased surface areas and maintained a similar bacterial community.

CONCLUSION

The probiotic mixture of *Bacillus* spp. and 0.1% benzoic acid promoted an increase in villus width, improved intestinal mucosal integrity, and potentially enhanced nutrient absorption capacity. Additionally, high-throughput sequencing (HTS) confirmed the possible synergistic effect of the probiotic mixture and 0.1% benzoic acid, showing a greater abundance of beneficial phyla and an increased species diversity index in tilapia microbiota. However, further study of the internal biochemical processes is needed to understand the synergistic effects better. More research is needed to evaluate the combined effects of probiotic and organic acid supplementation in tilapia and other fish species.

Credit author contribution

G.G. Santos: conceptualization, validation, methodology, formal analysis, writing-original draft; M.C.M. Libanori: conceptualization, validation, methodology, formal analysis; M.B. Ferreira: Conceptualization, validation, methodology, formal analysis; L. Cardoso: conceptualization, validation, methodology, formal analysis; D.S. Costa: conceptualization, validation, methodology, formal analysis; M.C. Fernandes: methodology, formal analysis; S.T. Fontes: methodology, formal analysis; K.G. Addam: methodology, formal analysis; T.A. Soligo: funding acquisition; M.L. Martins: methodology, validation, supervision, review, and editing; J.L.P. Mouriño: methodology, validation, supervision; S.A.P. Dutra: project administration, supervision, methodology, review, and editing. All authors have read and accepted the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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