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Prebiotic, probiotic and marine algae suplementation in juvenile tilapia diet

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ABSTRACT

This study aimed to evaluate the probiotic mannan oligosaccharide (MOS), and marine algae for Nile tilapia juveniles (*Oreochromis niloticus*) feeding. The parameters analyzed were fish-growth rate, hematological and immunological parameters, and intestinal microbiota. Also, the fish were submitted to experimental infection challenge with *Aeromonas hydrophila* to evaluate the immune response. Nile tilapia juveniles (mean weight 8.86 ± 3.22 g) were used in the six treatments with four replicates, for 63 days. The treatments were: control with basal diet; PAS-TR: basal diet plus 0.04 g·kg^{-1} of the probiotic (*Bacillus cereus* and *Bacillus subtilis*); MOS: basal diet plus 4 g·kg^{-1} of the prebiotic mannooligosaccharide, 4 g·kg^{-1} of the prebiotic *Kappaphycus alvarezii*. Two more diets were formulated by a combination of PAS-TR + MOS (4 +4 g·kg^{-1}), and PAS-TR + KAP (4 +4 g·kg^{-1}). For the challenge experiment, the fish were fed for 21 days, infected via intraperitoneal injection with *A. hydrophila* (1 x 10^{6} UFC·mL^{-1}), and the mortality rate was registered for 15 days post-infection. Results indicated the capacity of probiotic to remain in the gut for 63 days, and it was inhibited neither by autochthonous microbiota nor by prebiotics used. The feed additives tested for Nile tilapia did not cause a beneficial or adverse effect on growth or hematological variables evaluated. However, these treatments protected the fish from *A. hydrophyla* infection, proved by higher survival rate, and relative protection levels. We concluded that probiotic PAS-TR and prebiotics MOS and KAP, combined or not as symbiotics, may promote immune protection and reduce the mortality rate of *A. hydrophyla* infection.

Keywords: Immunostimulants; Symbiotics; Nutrition; Hematology; *Aeromonas hydrophila*; Aquaculture.

Suplementação de prebióticos, probióticos e algas marinhas na dieta de tilápia juvenil

RESUMO

O objetivo deste estudo foi avaliar o uso de probióticos, mananoligossacarídeos (MOS) e algas marinhas na alimentação de juvenis de tilápia do Nilo (*Oreochromis niloticus*), analisando-se a taxa de crescimento dos peixes, os parâmetros hematológicos e imunológicos e a microbiota intestinal. Além disso, os peixes foram submetidos ao desafio experimental de infecção com Aeromonas hydrophila para avaliar a resposta imune. Juvenis de tilápia do Nilo (peso médio 8,86 ± 3,22 g) foram utilizados nos seis tratamentos com quatro repetições, durante 63 dias. Os tratamentos foram: controle com dieta basal; PAS-TR: dieta basal mais 0,04 g·kg^{-1} do probiótico (*Bacillus cereus* e *Bacillus subtilis*); MOS: dieta basal acrescida de 4,0 g·kg^{-1} do prebiótico manooligossacarídeo, 4,0 g·kg^{-1} do prebiótico *Kappaphycus alvarezii*. Mais duas dietas foram formuladas pela combinação de PAS-TR + MOS (4,0 +4,0 g·kg^{-1}) e PAS-TR + KAP (4,0 +4,0 g·kg^{-1}). Para o experimento de desafio, os peixes foram alimentados por 21 dias, infectados via injeção intraperitoneal com *A. hydrophila* (1 x 10^{6} UFC mL^{-1}), e a taxa de mortalidade foi registrada por 15 dias pós-infecção. Os resultados indicaram a capacidade do probiótico permanecer no intestino por 63 dias, e não foi inibido pela microbiota autóctone nem pelos prebióticos utilizados. Os aditivos alimentares testados para tilápia do Nilo não causaram efeito benéfico ou adverso no crescimento ou nas variáveis hematológicas avaliadas. No entanto, estes tratamentos protegeram os peixes da infecção por *A. hydrophyla*, comprovado pela maior taxa de sobrevivência e níveis relativos de proteção. Concluímos que o probiótico PAS-TR e os prebióticos MOS e KAP, combinados ou não como simbióticos, podem promover proteção imunológica e reduzir a taxa de mortalidade da infecção por *A. hydrophyla*.

Palavras-chave: Imunoestimulantes; Simbióticos; Nutrição; Hematologia; *Aeromonas hidrofila*; Aquicultura.

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INTRODUCTION

In general, fish disease outbreaks, deterioration of water quality, poor quality diet, intensive culture, and unbalanced ecosystem cause high economic losses in worldwide aquaculture (Kautsky et al., 2000). Chemicals (e.g., disinfectants, drugs, and antibiotics) have been used for disease prevention and control in aquaculture production. The overuse of antimicrobials must be considered a public health hazard due to their capacity to generate the spread of antimicrobial-resistant bacteria and other pathogens. Residues in aquaculture products and the environment (Miranda et al., 2018; Romero et al., 2012) and damage to gastrointestinal biota also raise concern. The gastrointestinal tract is responsible for vital physiological processes such as osmoregulation, food digestion, nutrient absorption, endocrine regulation, immune response, and elimination of toxic metabolites, in addition to functioning as a defense barrier against pathogens. The gastrointestinal tract is colonized by microorganisms that form commensal relationships with their hosts, maintaining the organism's homeostasis (Fuentes-Quesada et al., 2020).

The alternatives for antibiotics are suitable for prophylactic strategies for disease outbreak control, as, for example, the administration of prebiotics, probiotics, and immunostimulants through a fish diet. Prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the gut (Ganguly et al., 2013). Probiotics have been defined as bioactive, living microbial food/feed additives, which generally have a positive influence on the digestion and microflora of the gastrointestinal tract (Verschuere et al., 2000; Hoseinifar, et al., 2016; Wanka et al., 2018). Probiotics can persist in the digestive tract because of their tolerance to acid and bile salts (Cruz et al., 2012). Most bacteria used as probiotics in fish were originally isolated from milk, cheese, or other terrestrial sources (Newaj-Fyzul and Austin, 2015; Carnevali et al., 2017; Nguyen et al., 2017).

Studies carried out with tilapia fed probiotics, yeasts, and amino acids reported changes in intestinal morphology, promoting a greater increase in the absorption area of the intestinal mucosa, which may imply greater efficiency in the use of nutrients (De Rodriganez et al., 2009; Merrifield et al., 2010b; Mugwanya et al., 2022). The bacterial probiotic species *Bacillus subtilis* and *Bacillus cereus* inhibited, *in vitro*, the growth of *Aeromonas hydrophila* and were effective as growth promoters in Nile tilapia culture (El-Haroun et al., 2006). They increased the level of relative protection in an experimental infection with bacteria such as *A. hydrophila*, *Streptococcus iniae*, *Pseudomonas fluorescens*, *Vibrio alginolyticus*, and *Pseudomonas putida* (Aly et al., 2008b; Bernard et al., 2013). The addition of probiotics to Nile tilapia feed increased the percentage of relative survival of fish, development of villi, epithelial lining cells and goblet cells of the intestinal mucosa, alteration of protein levels, phagocytic capacity, plasma lysozyme, phagocytic index and mean corpuscular hemoglobin concentration (Telli et al., 2014; Tachibana et al., 2020).

Mannan oligosaccharide (MOS) is a prebiotic supplemented in food. Culjak et al. (2004) reported that the addition of 0.6% of MOS to the diet of *Cyprinus carpio* juveniles for 46 days increased growth, enhanced protein absorption, and increased survival rate. The addition of *Saccharomyces cerevisiae* and MOS in the diet for *Oreochromis niloticus* resulted in a significant increase in total proteins and globulins in fish supplemented compared to the basal diet (Abu-Elala et al., 2013). In a previous study, MOS and probiotic DBA® (*Bifidobacterium* sp., *Lactobacillus acidophilus*, and *Enterococcus faecium*) promoted better protection against *A. hydrophila* (Cavalcante et al., 2020).

Kappaphycus alvarezii is a widespread marine red alga originating from the Philippines and used as a raw material to produce biopolymers and carrageenan, a hydrocolloid used as a thickening and stabilizing agent in food, drugs, and cosmetics (Doty, 1987). Phytochemical studies confirmed the presence of a high content of proteins, steroids, carotenoids, vitamins, and minerals in this species of red alga (Rajasulochana et al., 2009; Nagarani and Kumaraguru, 2012). The inclusion of cooked *K. alvarezii* meal at 6% in the diet for Asian seabass (*Lates calcarifer*) increased the growth rate and crude protein percentage of whole-body composition (Shapawi et al., 2015). In addition, the extract of this alga inhibited *in vitro* the growth of bacterial species *Staphylococcus aureus*, *Micrococcus leutues*, *Escherichia coli*, and fungi *Aspergillus flavus*, *Aspergillus fumigates* and *Candida albicans* (Prabha et al., 2013).

Combining prebiotics and probiotics as symbiotics may lead to synergistic effects that enhance the nutritional value and/or absorption of fish diets. Indeed, prebiotics provide an adequate substrate to enable and adapt probiotic bacteria to the host's intestinal microbiota, promoting their multiplication and functional action (Amenyogbe et al., 2020). This study aimed to verify the immunostimulant and growth rate improvement of probiotic MOS, and marine algae for Nile tilapia juveniles (*O. niloticus*). For this purpose, we tested the addition of one probiotic PAS-TR (*Bacillus cereus* and *Bacillus subtilis*); two prebiotics, MOS (4 $g \cdot kg^{-1}$) and *K. alvarezii* (KAP) (4 $g \cdot kg^{-1}$); and symbiotics composed of PAS-TR + MOS (4 +4 $g \cdot kg^{-1}$) and PAS-TR + KAP (4 +4 $g \cdot kg^{-1}$) in fish feed. Performance

and experimental challenge assays were conducted to evaluate the growth, hematological parameters, gut microbial diversity, immune response, and protection against *A. hydrophila* infection.

MATERIAL AND METHODS

Ethical approval

All procedures of study are in accordance with the ethical standards established by the Ethics Committee of the Fisheries Institute (Protocol no. 06/2018).

Two experiments were run in parallel. The first experiment was designed to investigate the juvenile growth performance, and the second one was designed to check the challenge test with *A. hydrophila*.

Growth performance experiment

Experimental design

Two hundred and forty juvenile Nile tilapia reverted males (lineage GIFT) (8.84 ± 1.29 g and 7.91 ± 0.36 cm) were distributed into 24 aquaria (volume: 40 L, density of 10 fish per aquaria). A recirculation system with continuous aeration supply and water temperature control (26°C) was used. The water quality parameters of dissolved oxygen (OD), pH, and temperature were monitored daily by a multiparameter probe (YSI) and total ammonia by a kit (Alcon). The used tanks were distributed into six groups of treatments and four replicates for each group.

Fish were fed *ad libitum* four times a day for 63 days. During this period, with intervals of 21 days, animals from each experimental unit were fasted for 24 hours, then randomly selected and anesthetized using clove oil solution (0.3 $mL \cdot L^{-1}$) for biometric measures (weight and length) to calculate the following variables: weight gain (WG), WG = [(final weight) - (initial weight)]; specific growth rate: SGR = 100 x [(ln final weight - ln initial weight)/period], apparent diet consumption (DC), and apparent feed conversion ratio (FCR), FCR = [(feed intake)/(weight gain)].

Experimental diets

The basal diet was formulated according to the nutritional requirements of Nile tilapia (Cavalcante et al., 2020) (Table 1) and extruded (Ferraz, Brazil). The prebiotics were included after the extrusion process. The probiotics were included after the extrusion process to guarantee the survival and efficacy of the bacterial species. The lyophilized commercial product (PAS-TR) was diluted in soybean oil (2% of feed weight), according to Dias et al. (2012), and sprinkled on feed, which was maintained at 4°C. We tested the addition of one probiotic PAS-TR (*B. cereus* var. *toyoi* 4,0x10^{12} UFC and *B. subtilis* 4.0x10^{12} UFC); two prebiotics, MOS (4 $g \cdot kg^{-1}$) and KAP (4 $g \cdot kg^{-1}$); and symbiotics composed by PAS-TR + MOS (4 +4 $g \cdot kg^{-1}$) and PAS-TR + KAP (4 +4 $g \cdot kg^{-1}$) in fish feed.

The viability of probiotic species in the final form of the prepared diet was performed by dilution of 1 g of diets supplemented with probiotic in 9 mL of sterile water. Serial dilution up to 10-8 fold was made, and 0.1 mL of each dilution was cultivated on tryptone soy agar (TSA) medium in duplicate. The plates were incubated at 30°C for 24 h for later counting of *Bacillus* spp.

Organs and blood sampling

After the experimental period (63 days), individuals from each treatment were randomly selected and sedated with clove oil (0.3 $mL \cdot L^{-1}$) for blood sampling from the caudal vein with a non-heparinized syringe and killed by medullar dissection to collect aseptically the intestine (anterior fraction) and liver. The blood samples were centrifuged with a microtube centrifuge (Hettch Lab Technology, Germany) to collect the serum.

Then, the microbiology–probiotic viability (*Bacillus* sp. colonies counts) and denaturing gradient gel electrophoresis (DGGE), hematology (hematimetric measures, erythrocyte, leucocyte, and thrombocyte counts), immunology (phagocyte activity, total protein, and albumin level, lysozyme, and gene expression), and hepatic histopathology analyses were performed.

Microbiology analysis

Probiotic viability

A *Bacillus* sp. selective media, TSA (Irianto and Austin, 2002), was used for colony forming unit (CFU) counting to compare the concentration of *Bacillus* in diets and foregut. The intestine samples (n = 24) were weighted and macerated inside the test tubes. The volume of sterile saline solution (0.85%) was adjusted to obtain the proportion of 1:10 and added to each tube. After homogenization, 10-fold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were prepared, and 100 µL of each solution was seeded in *Bacillus* sp. selective media. The plates were incubated at 30°C for 72 hours, and subsequent CFU counting.

Study of intestinal microbiota by denaturing gradient gel electrophoresis

Extracted intestinal contents from Nile tilapia (n = 6 samples per treatment) were preserved in FTA cards. The DNA extraction of samples was performed according to the protocol described by Aljanabi and Martinez (1997).

The DGGE patterns of intestinal microbiota were determined by DNA amplification using the 16Sr DNA bacterial domain-specific primers 968-GC-F (5'GA-ACGCGAAGAACCTTAC-3') and 1401-R (5'- CGGT-GTGTACAAGACCC-3'). The PCR

Table 1. Experimental diets formulation and composition: control, prebiotics manan oligosaccharide (MOS, Actigen) and *Kappaphycus alvarezii* (KAP), PRO (commercial product PAS-TR; *Bacillus subtilis* and *Bacillus cereus*), symbiotics PRO + MOS (PAS-TR and MOS) and PRO + KAP (PAS-TR and KAP).

Ingredient (%)	Control	MOS	KAP	PRO	PRO+MOS	PRO+KAP
Feather meal	4.00	4.00	4.00	4.00	4.00	4.00
Ground corn	22.74	22.34	22.34	22.71	22.31	22.31
Ground viscera	15.00	15.00	15.00	15.00	15.00	15.00
Soybean meal	18.05	18.05	18.05	18.05	18.05	18.05
Corn gluten meal	3.50	3.50	3.50	3.50	3.50	3.50
Wheat bran	8.56	8.56	8.56	8.56	8.56	8.56
Rice grits	7.00	7.00	7.00	7.00	7.00	7.00
Meat meal	10.00	10.00	10.00	10.00	10.00	10.00
Fish meal	3.00	3.00	3.00	3.00	3.00	3.00
Blood meal	4.00	4.00	4.00	4.00	4.00	4.00
Salt	0.20	0.20	0.20	0.20	0.20	0.20
Dicalcium phosphate	0.32	0.32	0.32	0.32	0.32	0.32
Soybean oil	2.00	2.00	2.00	2.00	2.00	2.00
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10
L-Lysine	0.22	0.22	0.22	0.22	0.22	0.22
L-Threonine	0.10	0.10	0.10	0.10	0.10	0.10
DL-Methionine	0.26	0.26	0.26	0.26	0.26	0.26
Antioxidant	0.05	0.05	0.05	0.05	0.05	0.05
Antifungal	0.40	0.40	0.40	0.40	0.40	0.40
Premix[1]	0.50	0.50	0.50	0.50	0.50	0.50
PAS-TR[2]	0.00	0.00	0.00	0.03	0.03	0.03
MOS (Actigen)	0.00	0.40	0.00	0.00	0.40	0.00
K. alvarezii	0.00	0.00	0.40	0.00	0.00	0.40
			Percent composition (%)			
Dry matter	95.45	94.76	94.99	95.54	94.31	94.83
Crude protein	38.87	39.37	38.89	38.96	39.12	39.22
Crude fiber	6.00	5.85	6.60	5.07	4.89	5.42
Ether extract	3.83	4.16	4.37	4.54	4.16	4.34
Ash	10.43	10.29	10.35	10.36	10.39	10.37

[1]Premix: vit A 12,000 IU; vit D_3 3,000 IU; vit E 150 mg; vit K_3 15 mg; vit B_1 20 mg; vit B_2 20 mg; vitB_6 17.50 mg; vit B_{12} 40 mg; vit C 300 mg; nicotinic acid 100 mg; pantothenic acid 50 mg; biotin 1 mg; folic acid 6 mg; antioxidant 25 mg; Cu 17.50 mg; Fe 100 mg; Mn 50 mg; Zn 120 mg; I 0.80 mg; Se 0.50 mg; Co 0.40 mg; inositol 125 mg; choline 500 mg; [2]PAS-TR®: *B. subtilis,* 4 x 10^{8+}UFC·g^{-1} and *B. cereus,* 4 x 10^{8} UFC·g^{-1}.

conditions and mixture applied in this test were described by Tapia-Paniagua et al. (2010). The amplicons were separated by the DGGE process using the Dcode TM system (Bio-Rad Laboratories, Hercules, Canada) (Muyzer et al., 1993).

For electrophoresis, 8% polyacrylamide gels (37:5:1 acrylamide--bisacrylamide) (Sigma-Aldrich) (dimension of 20 x 20 x 0.01 cm) were prepared, containing 30 to 55% denaturing gradient of urea and formamide. The PCR products were added into each gel lane in aliquots of 13 µL. The gels were run in TAE buffer (20 mM Tris acetate [pH 7.4], 10 mM sodium acetate, 0.5 mM Na_2- EDTA) for the first 10 minutes at 200V and then 16 hours at 85V, at a constant temperature of 60°C.

At the end of DGGE, the gels were stained with AgNO3, following the protocol of Sanguinetti et al. (1994), and images were obtained by scanning with ChemiDoc (Bio-Radio) and analyzed by Image Lab Software (Bio-Radio). The dendrogram was constructed with DGGE patterns clustering by unweighted pair group method with arithmetic mean (UPGMA).

Phagocytic activity

The phagocytic activity was performed *in vivo* according to the protocol developed by Telli et al. (2014). Briefly, 48 fish (n = 8 per treatment) were randomly selected, anesthetized with clove oil (0.30 mL·L^{-1}), and injected with 1 mL of yeast (*S. cerevisiae*, type II, Sigma, USA, at concentration 11,000 cells·mm^{-3}) solution into the coelomic cavity. After 4 hours of incubation, animals were re-anesthetized and killed by medullar dissection. The cavity was washed with 1 mL of saline solution (0.70%), and the fluid was collected using a Pasteur pipette into tubes and centrifuged at 1,500 x g for 5 minutes, discharging the supernatant.

The obtained pellets were examined on glass slides under a phase contrast microscope (400 x) with a green filter, and 100 phagocytes were counted to calculate the phagocytic capacity (PC=number of phagocytes that phagocyted yeast/number of phagocytes observed), and phagocytic index (PI=number of yeasts ingested/number of phagocytes observed).

Hematological analysis

Blood samples were drawn from 48 fish (n = 8 per treatment), and red blood cells (RBC) were immediately counted in a Neubauer chamber. The hematocrit percentages (Ht) were determined by the microhematocrit method, and hemoglobin level (Hb) by the cyanmethemoglobin method, according to Hrubec and Smith (1998; 2010) and Ranzani-Paiva et al. (2023). The hematimetric indices mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated. The number of leukocytes and thrombocytes was determined in blood smears stained with May-Grünwald-Giemsa, according to the method of Hrubec and Smith (2010).

Lysozyme activity

The lysozyme activity was evaluated in 48 fish (n=8 per treatment), following the method described by Kim and Austin (2006), adapted to a microplate reader. The serum samples were diluted into PBS (0.05M), and aliquots of 100 µL were pipetted into 96 microplate wells. Each well was added with 100 µL of *Micrococcus lysodeikticus* (Sigma, United States of America) solution at the concentration of 0.4 mg·mL^{-1} of PBS. The microplates were incubated and read at 540-nm absorbance at time points 0, 5, and 10 minutes, with microplate reader (EON). In this assay, the PBS was considered a blank solution. The lysozyme unit was considered as the amount of serum that reduces the absorbance by 0.001 min^{-1}.

Total protein, albumin, and globulin contents

The concentrations of total protein (TP), albumin, and globulin were analyzed using specific commercial kits (Labtest, Minas Gerais, Brazil), following the manufacturer's instructions.

Challenge with Aeromonas hydrophila

For the experimental infection assay, 180 Nile tilapia males (mean weight 8.86 ± 3.22 g) were distributed into 18 aquaria (volume 40 L, density of 10 fish per experimental unit), with individual aeration, and temperature control. For *A. hydrophila* infection, the 50% lethal dose (LD) was previously set at 1.26 x 10^8 CFU·mL^{-1}.

For 21 days, fish were fed *ad libitum* with experimental diets three times a day. Thereafter, fish were infected via intraperitoneal injection of 0.10 mL of *A. hydrophila*. Animals were observed for 15 days to detect possible pathological alterations and mortality (Aly et al., 2008a). The relative protection level (RPL) was calculated by Eq. 1 (Newman and Majnarich, 1982):

$$\text{RPL (\%)} = 100 - (\text{\% of treatment mortality} / \text{\% of control mortality}) \times 100 \quad (1)$$

Hepatic histopathology

For the morphometric analysis, liver fragments were fixed in Bouin, dehydrated in an increasing ethanol series (70, 80, 90, 95, and 100%), with changes every 30 minutes, and embedded in methacrylate glycol (Historesin, Leica). After inclusion, 3-µm cuts were made and subjected to the following methods: toluidine / borate blue for morphometric analysis. Two histological slides were prepared from each animal. The preparations were analyzed under a light microscope, model Nikon Eclipse e400, Coolpix 5000, using a 40x objective. Morphometry was performed using the AxioVision image analysis software. Ten random fields were selected from each histological slide, and, with the aid of a 100-square grid, the coincident points on hepatocytes with and without cytoplasmic micro and macrovesicles were counted (Engelman et al., 2000).

Statistical analysis

Data on growth performance, microbiology, hematology, and immunology variables were tested by analysis of variance (ANOVA) and Dunnett's test ($p<0.05$) using Statistica 7 software.

RESULTS

Growth performance variables

During 63 days of feeding, no mortality rate related to the probiotic, prebiotics, and symbiotic supplementation was observed. The results of growth performance variables showed no significant difference ($p < 0.05$) between the treatments (Table 2).

Probiotic viability

The CFU counting of *Bacillus* species in experimental diets and fish gut (Table 3) was significantly different between the control and fish fed probiotics (PRO), fish fed PRO+MOS and fish fed PRO+KAP. The addition of *B. subtilis* and *B. cereus* in diets resulted in a higher concentration of this genus on the fish gut, regardless of the inclusion of MOS and KAP.

Denaturing gradient gel electrophoresis analysis

The dendrograms constructed according to the DDGE patterns of the intestinal microbiota of experimental groups (Fig. 1) indicated the main interference of *B. subtilis* and *B. cereus* oral administration with these results. The animals receiving the control and prebiotics (MOS and KAP) diets showed similarities in microbiota composition and were different from probiotic (PRO) and symbiotic groups (PRO+MOS and PRO +KAP).

Hematological analysis

The probiotic, prebiotic, and symbiotic diet feeding for 63 days caused no effect on hematimetric variables, leukocyte counts, and thrombogram values (Tables 4 and 5).

Phagocytic activity

The phagocytic capacity values ranged between 83 and 90, whereas the phagocytic index ranged between 2.19 and 2.26. For both phagocytic parameters, no significant difference was detected between the control and all the treatments (Fig. 2).

Lysozyme activity, total protein, albumin, and globulin contents

The lowest lysozyme activity (4.78 ± 0.98 units·mL^{-1}) was observed in the experiment of fish fed PRO, and the highest value (7.23 ± 1.40 units·mL^{-1}) in fish fed MOS. The total protein value ranged from 3.34 ± 0.18 to 3.52 ± 0.10 g·dL^{-1} for all the treatments. The concentration of albumin in all the experiments ranged from 0.80 ± 0.04 to 1.09 ± 0.10 g·dL^{-1}. For globulin, values between 2.44 ± 0.12 and 2.62 ± 0.12 g·dL^{-1} were registered. Table 6 lists the values for all the experiments. The addition of prebiotics, probiotic, or symbiotics in diets for

Table 3. *Bacillus* sp. counting (CFU·g^{-1}) of experimental diets and gut samples of Nile tilapia (*Oreochromis niloticus*) fed experimental diets for 63 days ($p < 0.05$).

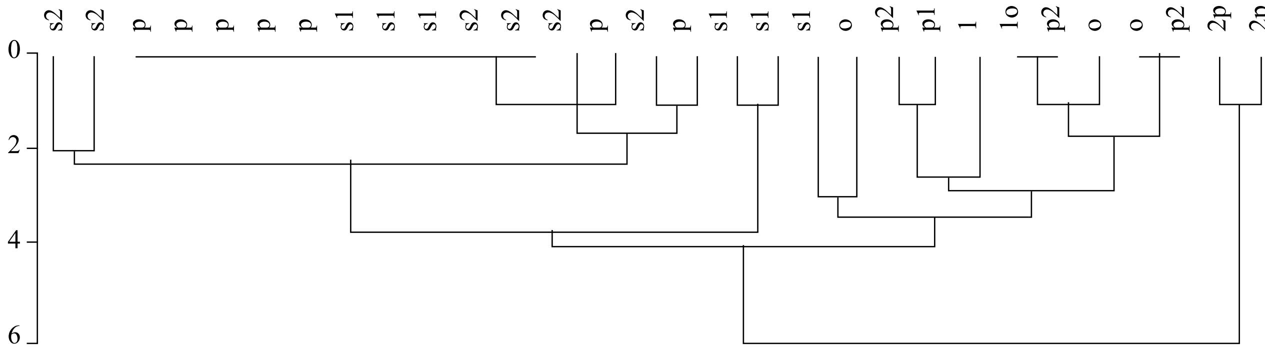
Treatments*	Diets	Gut
Control	1.1×10^{1} a	1.9×10^{2} a
MOS	0.9×10^{1} a	1.4×10^{3} ab
KAP	1.3×10^{1} a	1.7×10^{3} ab
PRO	3.5×10^{5} b	5.6×10^{6} b
PRO+MOS	2.9×10^{5} b	4.8×10^{6} b
PRO+KAP	3.9×10^{5} b	5.8×10^{6} b

*Different letters in the same column indicate significant differences by Dunnett's test ($p<0.05$); control: basal diet; MOS: prebiotics mannan oligosaccharide (Actigen); KAP: *Kappaphycus alvarezii*; PRO: commercial product PAS-TR (*B. subtilis* and *B. cereus*).

Table 2. Means and standard errors of growth performance variables: weight gain (WG), specific growth rate (SGR), diet consumption (DC), and feed conversion rate (FCR) of Nile tilapia (*Oreochromis niloticus*) fed experimental diets for 63 days ($p < 0.05$).

Treatments	WG (g)	SGR (% day^{-1})	DC (g)	FCR
Control	21.52 ± 7.66	2.51 ± 0.24	23.03 ± 7.17	1.19 ± 0.16
MOS	16.68 ± 6.16	1.91 ± 0.23	21.06 ± 5.74	1.44 ± 0.24
KAP	21.77 ± 3.39	2.10 ± 0.20	24.58 ± 2.66	1.16 ± 0.08
PRO	20.89 ± 2.88	2.17 ± 0.15	23.23 ± 3.21	1.12 ± 0.03
PRO + MOS	21.11 ± 4.34	2.39 ± 0.29	22.80 ± 3.74	1.11 ± 0.09
PRO + KAP	22.09 ± 8.39	2.46 ± 0.11	25.33 ± 7.25	1.35 ± 0.26

Control: basal diet; MOS: prebiotics mannan oligosaccharide (Actigen); KAP: *Kappaphycus alvarezii*; PRO: commercial product PAS-TR (*B. subtilis* and *B. cereus*).



Control: basal diet; MOS: prebiotics mannan oligosaccharide (Actigen); KAP: *Kappaphycus alvarezii*; PRO: commercial product PAS-TR (*Bacillus subtilis* and *B. cereus*).

Figure 1. Denaturing gradient gel electrophoresis patterns of Nile tilapia fed experimental diets: control, prebiotics MOS (p1) and KAP (p2), probiotic PRO (p), symbiotics PRO+MOS (s1) and PRO+KAP (s2).

Table 4. Means and standard errors of red blood cell (RBC) counts and hematimetric variables: hematocrit percentage (Ht), hemoglobin level (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) of Nile tilapia (*Oreochromis niloticus*) fed experimental diets for 63 days ($p < 0.05$).

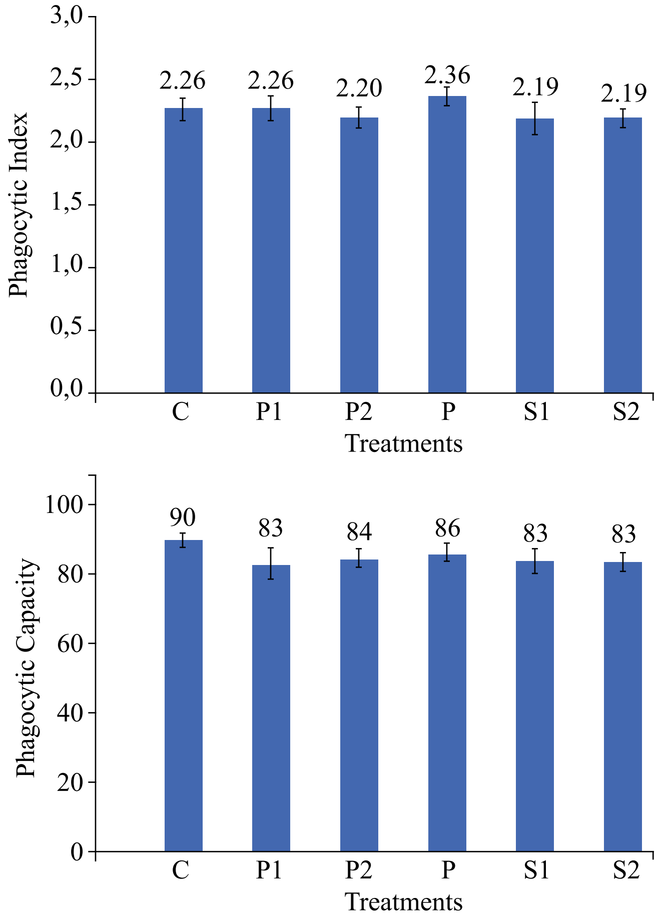
Treatments*	RBC (10^4 cells·µL^{-1})	Ht (%)	Hb (g·dL^{-1})	MCV (fL)	MCH (pg·cell^{-1})	MCHC (g·dL^{-1})
Control	132.50 ± 7.12	33.13 ± 1.22	6.43 ± 0.16	253.72 ± 12.47	49.53 ± 2.92	19.50 ± 0.47
MOS	131.63 ± 9.98	33.08 ± 0.99	7.09 ± 0.28	273.59 ± 16.57	55.21 ± 2.94	20.37 ± 0.79
KAP	118.19 ± 6.86	33.56 ± 0.90	6.64 ± 0.14	290.90 ± 18.44	57.58 ± 3.49	19.85 ± 0.41
PRO	118.56 ± 6.76	36.03 ± 1.47	6.61 ± 0.22	310.63 ± 21.48	56.73 ± 3.02	18.50 ± 0.80
PRO+MOS	113.56 ± 8.16	34.84 ± 1.21	6.71 ± 0.31	314.94 ± 18.52	60.27 ± 3.24	19.26 ± 0.63
PRO+KAP	110.00 ± 8.44	35.38 ± 1.37	7.51 ± 0.34	320.23 ± 15.10	68.07 ± 3.37	21.27 ± 0.54

Control: basal diet; MOS: prebiotics mannan oligosaccharide (Actigen); KAP: *Kappaphycus alvarezii*; PRO: commercial product PAS-TR (*Bacillus subtilis* and *B. cereus*).

Table 5. Means and standard errors of leukocyte, total and differential counts (leukogram) and thrombogram of Nile tilapia (*Oreochromis niloticus*) fed experimental diets for 63 days ($p < 0.05$).

Treatments	Leukocytes (cells·µL^{-1})	Lymphocytes (cells·µL^{-1})	Neutrophils (cells·µL^{-1})	Monocytes (cells·µL^{-1})	Thrombocytes (cells·µL^{-1})
Control	25,225.59 ± 2,966.43	44,296.29 ± 6,081.12	3,405.88 ± 887.43	2,194.57 ± 916.80	1,7761.13 ± 5,968.42
MOS	20,471.05 ± 2,710.33	38,474.39 ± 5,418.43	2,013.91 ± 346.74	1,255.25 ± 408.22	6,398.82 ± 2,458.27
KAP	23,421.92 ± 3,403.35	42,205.34 ± 6,154.83	3,898.79 ± 847.08	1,911.54 ± 508.03	12,886.28 ± 4,992.40
PRO	29,500.03 ± 4,804.29	54,801.50 ± 1,0182.16	3,034.06 ± 1,060.23	1,928.24 ± 451.65	9,222.41 ± 2,008.81
PRO+MOS	21,459.84 ± 3,551.43	38,040.55 ± 6,822.91	4,450.04 ± 798.61	1,876.14 ± 492.64	14,252.89 ± 1,454.11
PRO+KAP	23,762.73 ± 3,023.91	43,601.27 ± 6,102.31	3,515.45 ± 1,025.62	1,254.36 ± 248.98	12,522.76 ± 2,736.29

Control: basal diet; MOS: prebiotics mannan oligosaccharide (Actigen); KAP: *Kappaphycus alvarezii*; PRO: commercial product PAS-TR (*Bacillus subtilis* and *B. cereus*).



C: control (basal diet); p1: prebiotics prebiotics mannan oligosaccharide (MOS) (Actigen); p2: *Kappaphycus alvarezii* (KAP); p: probiotic PRO; s1 synbiotics PRO+MOS; s2: PRO+KAP.

Figure 2. Phagocytic activity (%) and phagocytic index (mean ± standard deviation) of macrophages of Nile tilapia (*Oreochromis niloticus*) fed experimental diets.

Nile tilapia had no significant effect on lysozyme activity, total protein, albumin, and globulin contents.

Challenge with Aeromonas hydrophila

During 15 days after infection with *A. hydrophila*, behavior alterations (erratic swimming, apathy), and clinical signs (scale loss, petechia, exophthalmia, fin hemorrhage, and erosion) were noted in the infected fish group.

Relative protection level

The experimental infection with *A. hyprophila* proved that feeding fish with prebiotic, probiotic, and symbiotic diets for 21 days enhanced survival and ensured effective protection against this pathogen. Compared to the control experiment, there was a significant difference for the groups KAP, PRO, PRO+KAP, and PRO+MOS. The percentage of RPL relative to the groups fed KAP, PRO+MOS, and PRO+KAP was higher than 50% (Table 7, Fig. 3). Comparing MOS and PRO+MOS

Table 7. Relative protection level of Nile tilapia fed experimental diets for 21 days ($p < 0.05$).

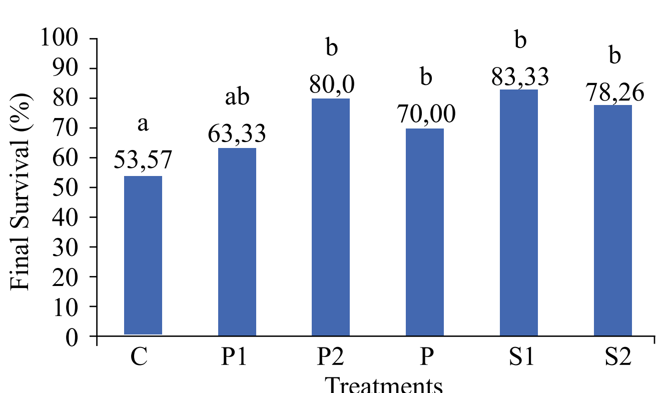
Treatments	Relative protection level (%)
MOS	21.02
KAP	56.92
PRO	35.39
PRO+MOS	64.10
PRO+KAP	53.18

Control: basal diet; MOS: prebiotics mannan oligosaccharide (Actigen); KAP: *Kappaphycus alvarezii*; PRO: commercial product PAS-TR (*Bacillus subtilis* and *B. cereus*).

Table 6. Means and standard errors of lysozyme activity, total protein, albumin, and globulin contents of Nile tilapia (*Oreochromis niloticus*) fed experimental diets for 63 days ($p < 0.05$).

Treatments*	Lysozyme activity (units·mL^{-1})	Total protein (g·dL^{-1})	Albumin (g·dL^{-1})	Globulin (g·dL^{-1})
Control	6.28 ± 1.13	3.45 ± 0.13	0.83 ± 0.05	2.62 ± 0.12
MOS	7.23 ± 1.40	3.46 ± 0.20	0.90 ± 0.10	2.54 ± 0.17
KAP	6.90 ± 1.66	3.34 ± 0.18	0.80 ± 0.04	2.54 ± 0.16
PRO	4.78 ± 0.98	3.52 ± 0.10	1.09 ± 0.10	2.44 ± 0.12
PRO+MOS	5.63 ± 1.05	3.46 ± 0.18	0.89 ± 0.06	2.57 ± 0.20
PRO+KAP	5.45 ± 1.39	3.50 ± 0.20	0.90 ± 0.08	2.60 ± 0.20

Control: basal diet; MOS: prebiotics mannan oligosaccharide (Actigen); KAP: *Kappaphycus alvarezii*; PRO: commercial product PAS-TR (*Bacillus subtilis* and *B. cereus*).



C: control (basal diet); p1: prebiotics prebiotics mannan oligosaccharide (MOS) (Actigen); p2: *Kappaphycus alvarezii* (KAP); p: probiotic PRO; s1 synbiotics PRO+MOS; s2: PRO+KAP; *letters indicate significant differences between means by Dunnett's test ($p < 0.05$).

Figure 3. Nile tilapia (*Oreochromis niloticus*) final survival of the control and treated groups 15 days post-infection with *Aeromonas hydrophila**.

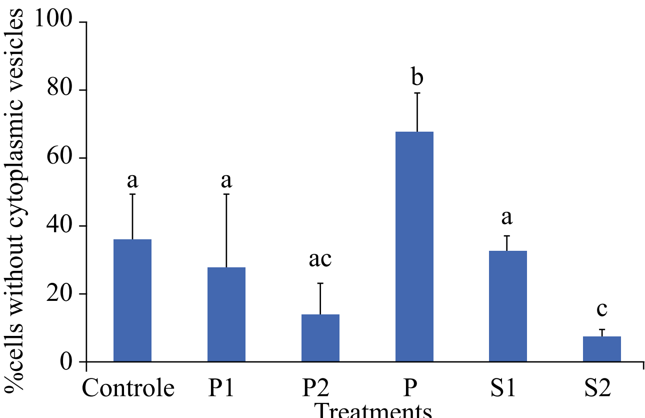


Figure 4. Means and standard deviations of the percentage of liver cells without micro and macrovesicles in Nile tilapia fed the control diet (C), diets supplemented with probiotic (P), with prebiotic 1 (P1), prebiotic 2 (P2), symbiotic 1 (S1) and symbiotic 2 (S2). Values with different letters are significantly different ($p < 0.05$) by Dunett's test.

groups, the additive effect of the combination PRO (*B. subtilis* and *B. cereus*) and MOS was evidenced in this assay.

Liver histopathology

The morphometric analysis revealed that animals supplemented with P2 (Probiotic: *K. alvarezii*) had a significantly higher number of liver cells without cytoplasmic micro and macrovesicles than animals treated with the control diet, P (PAS diet) -TR® (*B. subtilis* 4x108 UFC·g^{-1} and *B.cereus*), P1 (MOS - Actigen®), S1 (PAS-TR® + MOS) and S2 (PAS-TR® + *K. alvarezii*). Animals fed the S1 diet showed a significantly higher number of hepatocytes without micro and macrovesicles than animals of treatments P and S2 (Figs. 4 and 5).

DISCUSSION

The intake of probiotics could modify the composition of the intestinal microbiota, which is a key component in excluding potential invaders and maintaining the organism health (Tapia-Paniagua et al., 2010).

In the present study, the number of *Bacillus* sp. in the Nile tilapia diet ranged from 2.9x10^{5} to 3.9x10^{5} CFU·mL^{-1} in all experiments in which the diet was supplemented with probiotic (PRO, PRO+MOS, and PRO+KAP) (Table 4). The number of *Bacillus* sp. in the gut was between 4.8x10^{6} and 5.8x10^{6} CFU·mL^{-1}. These results demonstrate that *Bacillus* sp. colonized the fish gut, but it did not affect the growth parameters. Moreover, results suggested no inhibition by adding prebiotics (MOS and KAP) to the *Bacillus* strains.

Marengoni et al. (2015) showed that probiotics *B. cereus* var. *toyoi* and *B. subtilis* C-3102, added individually in combination at 0.5% in tilapia feed, colonized fish intestines without negatively affecting the intestinal bacterial microflora. These authors found no significant change in growth rates (GPM, TBI, and CAA) after 127 days of the experiment. Other studies reported that adding *B. subtilis* to the Nile tilapia fingerlings diet (1x10^{7} CFU·g^{-1}) enhances weight gain, specific growth rate, and feed conversion rate (Aly et al., 2008a; Nguyen et al., 2017). On the other hand, Dias et al. (2018) showed that the *B. cereus* probiotic added at a high concentration of 3.9 x 10^{6}CFUg^{-1} diet and 3.3 x 10^{8} CFUg^{-1} improved weight and length gains in tambaqui (*Colossoma macropomum*) only after 60 days. However, the same fish added with 4.2 × 10^{4} showed no significant difference from the control.

Our results, together with other works, suggest that the beneficial effect of probiotic depends on the concentration and species of bacteria, the fish species, and the feeding period and that the advantage probably starts with the colonization of the gut followed by other interaction mechanisms between bacterial species and the intestine physiology/microbiota of fish species that need to be more investigated. Other studies showed that the effect of probiotic supplemented in fish feed depends on the age, size, water quality, strategy, and method of probiotic administration (Doan et al., 2016; Ridha and Azad, 2016; Doan et al., 2017; Dias et al., 2018; Tachibana et al., 2020).

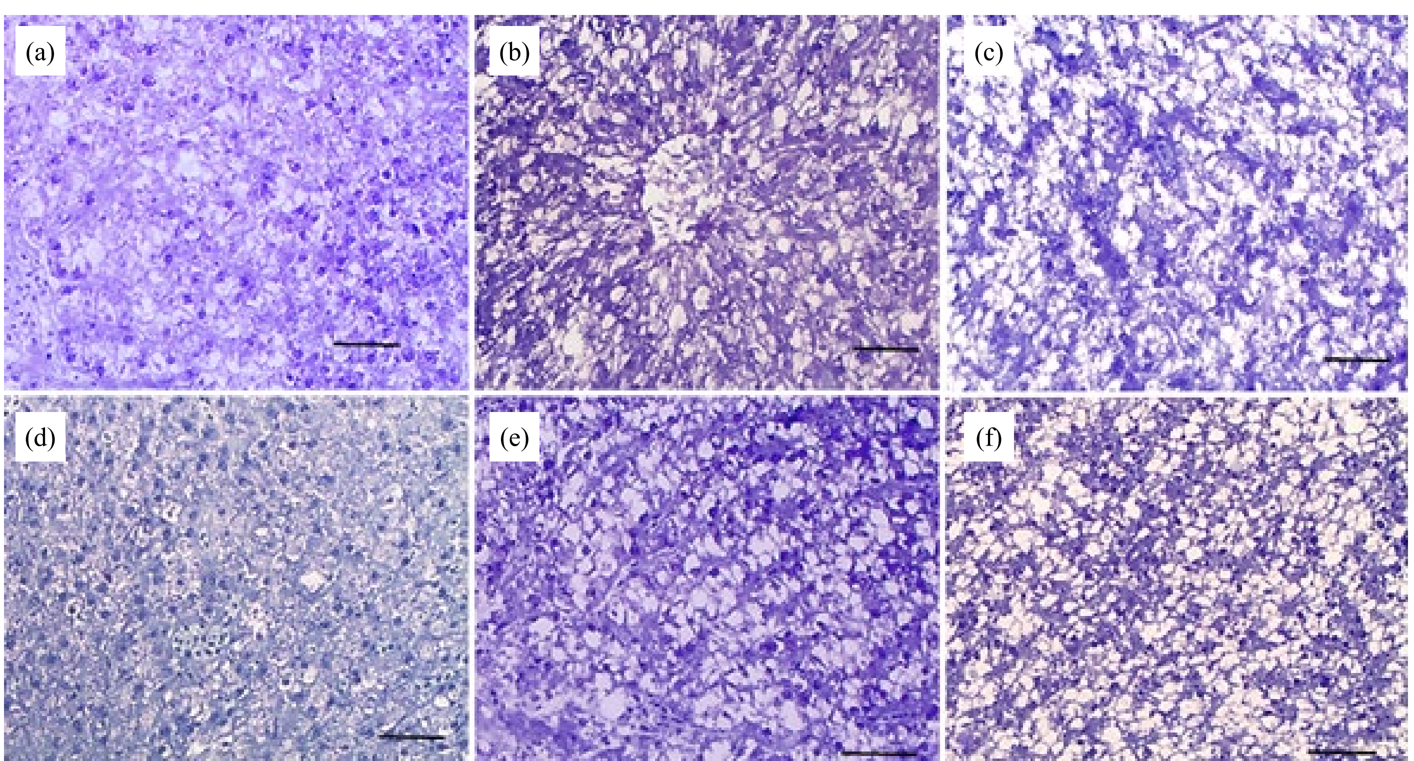


Figure 5. Photomicrographs showing the hepatic tissue of *Oreochromis niloticus*, mainly consisting of polyhedral hepatocytes, with a lateralized nucleus and cytoplasm with varied vacuolization: (A) hepatic tissue, with 65% of vacuolated hepatocytes (control); (B) liver tissue, with 75% of vacuolated hepatocytes (PAS-TR (*Bacillus subtilis* $4x10^8$ CFU·g^{-1} and *B. cereus* $4x10^8$ CFU·g^{-1}); (C) liver tissue, with 85% of vacuolated hepatocytes (mannan oligosaccharide–MOS; Actigen; (D) liver tissue, with 35% of vacuolated hepatocytes (*Kappaphycus alvarezii*); (E) liver tissue, with 65% of vacuolated hepatocytes (PAS-TR + MOS); (F) liver tissue, with 90% of vacuolated hepatocytes (PAS-TR® + *K. alvarezii*) 50-µm scale bar Staining: toluidine / borate blue.

The differences in results found in studies related to probiotic supplementation in fish diet could be also attributed to experimental conditions. Indeed, aquatic organisms are more dependent on environmental factors (dissolved oxygen, temperature, pH, and environmental microflora), probiotic proliferation, and function in the intestinal tract of the host (Das et al., 2008; Mehrim, 2009). For fish fed probiotics, the enhancement of growth parameters could be due to the increased activity of some enzymes, specifically phosphohydrolase, and glycosidase, which indicates that these probiotics are properly functioning after passage through the stomach (Nguyen et al., 2017). Rahman et al. (2022) investigated the effects of commercial symbiotics containing *Lactococcus lactis* (1×10^{11} CFUkg^{-1}) and starch on growth performance, phenotypic traits, and digestive enzyme activities of monosex Nile tilapia and reported a significant increase in amylase and protease activities in the intestine with increasing symbiotics supplementation.

For probiotic viability in the fish intestine, we found in this study that probiotic population was higher in animals fed supplemented diets than in control fish. This could explain the competence of these bacteria to adhere and colonize the fish intestine.

Recovering the probiotic *B. subtilis* from the intestine of fish fed probiotic-supplemented diet was proved even after 84 days (Telli et al., 2014; Giatsis et al., 2016). *B. subtilis* is classified as a transitory bacterium in the gastrointestinal tract, unable to attach to the intestinal epithelium, but it assists in the multiplication and colonization of lactic acid producers (Giatsis et al., 2016). In general, the genus *Bacillus* inhabiting animal intestines can produce antimicrobial substances. Their sporulation capacity confers them the advantage of heat tolerance and longer shelf-life (Geng et al., 2012; Raida et al., 2003). Interestingly, for larvae of tilapia, Giatsis et al. (2016) demonstrated that *B. subtilis* was present in the gut during the seven days of probiotic application. Although *B. subtilis* was no longer detected in the guts of fish exposed to the probiotic after day 7, the gut microbiota of the

exposed tilapia larvae remained significantly different from that of the control treatment.

Fish fed supplemented diets did not show any significant differences ($p < 0.05$) in lysozyme, total proteins, albumin, globulins, phagocytic capacity, and phagocytic index. We observed an improvement in serum globulin values in fish fed symbiotic diets (prebiotics +probiotics) (S1 and S2) compared to treatments fed the two immunostimulants (P, P1, and P2) separately, but this increase did not reach a significant level.

Wang et al. (2008) also showed that the addition of a probiotic (*Enterococcus faecium*) to the water of Nile tilapia fingerlings fed a basal diet, for 40 days, did not affect the levels of total proteins, albumin, and globulin. However, the inclusion of the *S. cerevisiae* probiotic and the MOS prebiotic in the fish diet for two months caused a significant difference in total proteins and globulins, which indicates an enhancement of the immune system since globulins are the main fraction of immunologically active proteins (Abu-Elala et al., 2013). The increase in plasma protein concentration is primarily due to the albumin and globulin fractions, which are responsible for the transport of nutrients, maintenance of blood osmotic balance, and improvement of animal defense mechanisms (Thomas, 2000; Dias et al., 2018).

Supplementation of fish diets with probiotics was tested to enhance fish states without any eventual pathogenic effect on the host. Studies carried out with high doses and for a long period of exposure to the additive often do not have positive responses. These strategies are suggested to cause tolerance of the immune system, which could be induced for a moment and then return to the normal state. A previous study (Tachibana et al., 2020) demonstrated that cyclic delivery of probiotic to Nile tilapia results in a significant difference in immune system parameters only after a 14-day fed period. This corroborates Merrifield et al. (2010a), who reported that the short-term cyclic delivery of the probiotic may be a viable alternative for fish immunostimulation.

Lysozyme contributes to the innate defense and mechanisms of host phagocytosis, being present in phagocytic granules of neutrophils, monocytes, macrophages, and epithelial cells (Cole et al., 2002; Lopera et al., 2008). In the present study, the levels of lysozyme remained without a statistical difference ($p > 0.05$) between all treatments tested. Telli et al. (2014) revealed no change in lysozyme levels between normal and stressed Nile tilapia fed probiotics.

The phagocytic activity of macrophages *in vivo* did not show any significant difference between the treatments tested here. The phagocytic capacity determines the number of active macrophages among the total number of macrophages observed. The phagocytic index is the number of foreign microorganisms, in this case, yeast *S. cerevisiae*, each active macrophage was able to phagocytize. In addition, some prebiotics induce the growth of commensal bacteria that inhibit the adhesion of pathogenic microorganisms by competition for the same glycoconjugates found on the surface of epithelial cells, altering the pH of the intestine, and promoting increased mucus production, production of short-chain fatty acids, and induction of cytokine release (Akhter et al., 2015).

Dias et al. (2012) observed better results of PC in matrinxãs (*Brycon amazonicus*) fed *B. subtilis*, but there was no statistical difference for PI. Telli et al. (2014) observed that fish fed *B. subtilis* and reared in high stocking density showed higher values of PI ($p < 0.05$), but there was no statistical difference for PC. The difference between these studies could be related to fish size, immune system maturity, and farming conditions.

In general, hematological parameters indicate the health status of the organism at a given moment, and the blood elements are related, quantitatively and proportionally, to the functional status of the fish. In this study, no statistical difference in hematological parameters was detected between fish fed supplemented and normal diets. Diet supplementation with prebiotics and probiotics did not affect the plasma glucose rate, leukogram, thrombogram, and complete blood count.

Fish under stress conditions respond physiologically with an increase in glycemia (hyperglycemia) (Urbinati and Carneiro, 2001). In the present study, the plasma glucose results showed that the animals were not under stress, maintaining their normal levels for the species.

In the present study, there was a slight increase in RBC in fish fed the diet supplemented with MOS compared to fish fed other diets. In contrast, Abu-Elala et al. (2013) found that RBC and Hb values were significantly higher in Nile tilapia fed a diet supplemented with 2 $g \cdot kg^{-1}$ of MOS for 60 days than in the control. In an experiment with matrinxãs (*B. amazonicus*) receiving a diet supplemented with *B. subtilis* for 84 days, there were no changes in RBC and Ht compared to the control. However, these parameters showed a difference after 42 days (Dias et al., 2012). Aly et al. (2008b) revealed that an increase in Ht values after feeding with probiotics reflects the safe use of these additives and their efficacy for improving health status. In our experiment, we suggested that there was a response peak that probably occurred before 63 days of feeding.

Our results for VCM, HCM, and CHCM shape corroborate other studies on supplemented probiotics in fish food (Telli et al., 2014). For tambaqui supplemented with *B. cereus*

(10^4, 10^6, and 10^8 CFU/g), Dias et al. (2018) reported no difference in hemoglobin levels, hematological indices (VCM, HCM, and CHCM), leukocytes, lymphocytes, monocytes, or basophils. The hematological parameters analyzed in this study reflect the valuable fish health status.

Dias et al. (2012) reported the same results for fish fed *B. subtilis* and *B. cereus*. This may be explained by immune system responses that did not interact with these bacteria as pathogens. Neutrophils are the first cell defense line in the injury site to limit the disease spread and initiate the immune response (Li et al., 2019). These cells possess a high phagocytic capacity (Doggett and Harris, 1989). Basophils and eosinophils occur in very low numbers in fish blood, not passing 1.5% of the total leukocytes, appearing in higher amounts when the animals show some allergic reaction or infection by parasites (Ranzani-Paiva et al., 2023).

Thrombocytes and leukocytes are cells of different lineages. Every component of the immune system has its own inherent protective value. The final combination of these components is likely to be related to a satisfactory immune response (Whyte, 2007). Thrombocytes participate in fish hemostasis and blood coagulation. The interspecific variation and the methodological differences used in thrombocyte count are responsible for the variability in the number of these cells in fish blood (Tavares-Dias and Oliveira, 2009). The variation in the number of leukocytes was expected (Ranzani-Paiva et al., 2023). For fish, this is a very common situation that leads to difficult interpretations of the leukocyte count. However, there was no statistical difference among treatments values or among them and the control; it is considered that the fish maintained homeostasis throughout the experiment.

The supply of probiotics in the fish diet can produce immunostimulation (Jatobá et al., 2011) and cause an alert/preparation for possible infections. Therefore, it would be recommended to provide the probiotic before high-stress situations or periods of disease incidence. In this study, no change in the number of leucocytes was found with the supply of probiotics *B. subtilis* and *B. cereus* in the diet. An increase in the number of leukocytes was also expected in fish fed diets containing the probiotics and prebiotics. The stimulation of the defense cells is suggested to occur in other periods and/or dosages of administration of immunostimulants, specifically for *O. niloticus*. Possibly, the alterations can occur in very short periods after the beginning of the feeding, not allowing the detection of the changes by the methodology used.

For the infection experiment with *A. hydrophila*, various clinical signs were observed in the control group, which confirms the pathogenic character of this bacteria. The fish fed the control diet had a survival rate of 53.57% after the challenge. This percentage was significantly lower ($p < 0.05$) than in fish fed diets supplemented with prebiotics and/or probiotics. However, there was no significant difference between fish groups fed supplemented diets (P1, P2, P, S1, and S2). Thus, probiotics and prebiotics used in this study probably stimulate a nonspecific immune response against *A. hydrophila*. Similar results were obtained by Salinas et al. (2005) after dietary administration of *Lactobacillus delbrüeckii* and *B. subtilis*, alone or in combination, to gilthead seabream for three weeks. Aly et al. (2008a) reported that rainbow fed the probiotics *B. subtilis* and *L. acidophillus* presented low mortality rates after the challenge with *A. hydrophila* and *P. fluorescens*.

The analysis of liver histology of infected fish showed that fish fed the diet containing the prebiotic KAP had the best hepatoprotective effect in tilapia after *A. hydrophila* infection. The satisfying results of the survival rate registered for this group confirmed that a preserved liver plays a key role in modulating the innate immune system during the infectious and inflammatory process. This provides adequate conditions for fish to overcome possible disease outbreaks caused by opportunistic etiologic agents (Belo et al., 2014; Castro et al., 2014; Paniagua et al., 2014; Lin et al., 2019).

CONCLUSIONS

Prebiotics and probiotics in the diet for Nile tilapia improved non-specific immunity, demonstrated by better survival rates compared to the control diet, providing better protection to fish when challenged with *A. hydrophila*. These results show the supplemented diet for 21 days stimulated the immune system and enhanced fish defense against potential pathogenic agents. The inclusion of probiotics and prebiotics can be used as immunostimulants in fish diets.

CONFLICT OF INTEREST

Nothing to declare.

DATA AVAILABILITY STATEMENT

Data will be available upon request from the author.

AUTHOR’S CONTRIBUTION

Conceptualization: Ranzani-Paiva MJT, Tachibana L; **Formal Analysis:** Oshiro E, Dias DC; **Investigation:** Oshiro O, Dias DC, Cavalcante RB, Telli G, Alarcon MF, Ishikawa CM, Natori MM; **Resources:** Ranzani-Paiva MJT, Tachibana L; **Supervision:** Ranzani-Paiva MJT; **Validation:** Moriñigo MA,

Tapia S; **Data curation:** Petesse ML, Hamed SB; **Writing – original draft:** Oshiro E; **Writing – review & editing:** Ranzani-Paiva MJT; **Final approval:** Ranzani-Paiva MJT.

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