Original Research Article

Synergistic effects of prebiotics and probiotics on the performance and physiology of juvenile Nile tilapia (*Oreochromis niloticus*)

ABSTRACT

The objective of this study is to evaluate the effects of the inclusion of prebiotics, probiotics, and synbiotics on the productivity and physiological parameters of Nile tilapia juveniles. The experiment was carried out in the Aquaculture Area of the Federal University of Grande Dourados (UFGD), for a period of 60 days. A total of 300 animals with an initial average weight of 11.3±0.32 g and an initial average length of 8.5±0.57 cm were distributed in a completely randomized design in 15 experimental units, with three replications in each treatment, for 60 days. The treatments were named Control (additive-free), PROB (4 g.kg-1), composed of a mixture of probiotics such as Bacillus subtilis, PREB MOS (4 g.kg-1), SIMB I (2 g.kg-1 of probiotic + 2 g.kg-1 of prebiotic), and SIMB II (4 g.kg-1 of probiotic + 4 g.kg-1 of prebiotic). The inclusion of additives improved feed conversion, protein efficiency rate, and protein retention compared to the control treatment. The SIMB II treatment provided the highest intestinal quotient and plasma glucose index. The development of liver cells was influenced by the inclusion of additives, with reduced height and width of hepatocytes. There were no differences between treatments in histological evaluations of the midgut and in enzymatic analysis of the liver and intestine. Multivariate analysis showed that the control group was completely different from the others. The use of MOS additives and probiotics improved both the development and health status of Nile tilapia juveniles (Oreochromis niloticus) throughout their growth. The use of the SIMB II dose (4 g.kg-1 of probiotic + 4 g.kg-1 MOS) is recommended.

Keywords: Biotechnology, non-pathogenic bacteria, additives, gut health

1. INTRODUCTION

Aquaculture production has contributed significantly to supplying the demand for food of protein origin. Nile tilapia is largely responsible for this expansion*. O.niloticus* is the third most produced fish species in the world (8.3% of production) due to its zootechnical performance characteristics. Fish nutrition is one of the main factors responsible for this expansion in production, however, among various inputs, the cost of feed represents almost 70% of the total production costs [1], reducing profitability, thus food quality and efficiency are necessary for successful production, having a direct impact on water quality, survival and performance [2].

The inclusion of additives in the animal diet is promising, as they act directly on animal health and on nutrient absorption efficiency, such as probiotics, defined as live microorganisms that produce useful effects on the host by modifying the associated patterns or the community of microorganisms, promoting improved growth, better use of food and nutrients, reducing disease and developing immune responses in addition to the microbiological quality of the exposed environment [3, 4].

Prebiotics are non-digestible food compounds that beneficially affect the host by selectively stimulating the proliferation or activity of populations of beneficial bacteria, which act by modifying intestinal morphology, allowing a greater density of microvilli and a low exposure to pathogenic bacteria, as they derive from the cell wall of yeasts, which bind to pathogens, thus blocking colonization in the intestine and increasing nutrient absorption capacity [5].

The combined use of prebiotics and probiotics can act in three ways: additivity, synergism, or potentiation. It allows an increase in the action of probiotic bacteria through prebiotics due to the action of this compound in increasing the activity of probiotic bacteria and in improving growth metabolism and its activation [6]. Studies have shown that supplementation of probiotics with prebiotics rapidly improves growth, feed utilization, digestive enzyme activities, disease resistance, health status and gastric morphology of aquaculture species [7].

Although studies have addressed the effects of additives on aquaculture nutrition, demonstrating the benefits promoted to production, the type of additive, as well as the inclusion dose is a key factor for beneficial effects on host animals. Therefore, the objective of this study was to verify the action of different dosages of prebiotic MOS, mix of probiotics with *Bacillus subtillis* and symbiotic in two doses, on the growth performance and physiological parameters of juvenile Nile tilapia.

2. MATERIAL AND METHODS

2.1 Statement of ethics and experimental design

The experiment was carried out it the Aquicultural Area of the Federal University of Grande Dourados (UFGD), Dourados, Mato Grosso do Sul, Brazil. The Ethics Committee on Animal Use of the Federal University of Grande Dourados (CEUA/UFGD) approved the experimental procedures of this study, under protocol no. 28/2020.

A total of 300 Nile tilapia juveniles (11.3±0.32 g and 8.5±0.57 cm) were distributed in a re-circulation system; consisting of fifteen 2,000-liter water tanks with constant aeration and renewal of 10% of water a day. The experimental design was completely randomized, with five treatments and three replications (n =15), totaling 20 fish per experimental unit.

The water quality parameters; dissolved oxygen temperature (YsiEcoSense DO200A), pH (Hanna Waterproof Portable pH/ORP Meter), and electrical conductivity (TDS & EC Meter) were measured once a week with portable digital potentiometers. The averages obtained for water quality variables were temperature: 24.3±3.76ºC, dissolved oxygen: 7.55±1.53 mg L-1, pH 7.27 ± 0.37, and electrical conductivity: 113.0±2.0 µS.cm-1, within the expected range for the species during the experimental period [8].

2.2 Experimental diets and feeding management

The animals fed on a commercial extruded feed containing 32% crude protein (Table 1), to which different levels of additives were added. The treatments consisted of: PROB = 4 g of

probiotic (*Bacillus subtillis, Bifidobacterium bifidum, Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus lactis, Lactobacillus plantarum, Pediococcusacidilactici*, all at a concentration of 5x109 CFU/g per kg-1 of feed – Probiotic MultAqua® Biomart – Martinópolis, Brazil; PREB = 4 g of prebiotic (crude protein max 30%, moisture max 8%, crude fiber max 3%, ash max 6%, carbohydrates 55%, of which: 25% are mannanoligosaccharides (MOS) and 30% are β-glucans) per kg-1 of feed – Prebiotic ActiveMOS® - Biorigin, LençóisPaulista, Brazil; SIMB I = symbiotic (2 g.kg-1 probiotic + 2 g.kg-1 MOS); and SIMB II = symbiotic (4 g.kg-1 probiotic + 4 g.kg-1 MOS), in addition to a control diet (without inclusion of additives).

Additives were manually incorporated into the feed to ensure the survival of bacterial species [9]. Additives were homogenized in soybean oil (2% of feed weight) and stored under refrigeration. This procedure was performed once a week. Fish were fed four times a day (7:00 am, 10:00 am, 1:00 pm, and 4:00 pm) until apparent satiation.

Table 1. Assurance levels of base diet used for the inclusion of additives in commercial extruded feed for Nile tilapia juveniles

Nutrient	**Assurance level**
Moisture (max)	100 g/Kg
Crude Protein (min)	320 g/Kg
Ethereal Extract (min)	40 g/Kg
Fiber Matter (max)	50 g/Kg
Mineral Matter (max)	86.5 g/Kg
Calcium (max)	22 g/Kg
Calcium (min)	16 g/Kg
Phosphorus (min)	13.5 g/Kg
Sodium (min)	3.7 g/Kg
Cobalt (max)	0.20 mg/Kg
Copper (min)	10 mg/Kg
Iron (min)	50 mg/Kg
Iodine (min)	1 mg/Kg
Manganese (min)	70 mg/Kg
Selenium (min)	0.2 mg/Kg
Zinc (min)	50 mg/Kg
Vitamin A (min)	10,000.00 µi/Kg
Vitamin D3 (min)	3,200.00 µi/Kg
Vitamin E (min)	12 µi/Kg
Vitamin K3 (min)	3.4 mg/Kg

Vitamin B1 (min)	2 mg/Kg
Vitamin B2 (min)	5 mg/Kg
Vitamin B6 (min)	6 mg/Kg
Vitamin B12 (min)	20 mg/Kg
Vitamin C (min)	250 µg/Kg
Choline Chloride (min)	210 mg/Kg
Niacin (min)	45 mg/Kg
Folic Acid (min)	2 mg/Kg
Pentatonic Acid (min)	13 mg/Kg
Biotin (min)	0.1 mg/Kg
Lysine (min)	15.5 mg/Kg
Methionine (min)	1,500.00 mg/Kg
Threonine (min)	12.4 mg/Kg
Tryptophan (min)	4,000.00 mg/Kg

Centesimal composition determined in laboratory (%) after the inclusion of additives

	Dry matter	Mineral matter	Crude Protein	Ether extract
Control	94.76	8.26	30.76	11.53
Probiotic (4 $g.kg^{-1}$)	95.84	8.36	28.75	10.19
Prebiotic (4 $g.kg^{-1}$)	94.59	8.31	28.69	8.68
Symbiotic I (2 $g.kg^{-1}$ PROB + 2 $g.kg^{-1}$ MOS)	95.89	8.22	28.76	10.90
Symbiotic II (4 $g.kg^{-1}$ PROB + 4 $g.kg^{-1}$ MOS)	96.17	8.24	27.75	8.72

Probiotic: (Bacillus subtilis, Bifidobacterium bifidum, Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus lactis, Lactobacillus plantarum, Pediococcusacidilactici, all at a concentration of $5x10^9$ CFU/g - MultAqua®, Biomart - Martinópolis, Brazil). Prebiotic Mannanoligosaccharide (ActiveMOS® - Biorigin, LençóisPaulista - Brazil).

2.3 Productive performance and somatic indexes

After the experimental period, all fish were captured and stunned with eugenol at a dose of 100 mg.L-1 [10] to carry out the final biometry, by which the parameters of productive performance were evaluated. Three fish from each experimental unit were stunned until deep anesthesia for blood collection. They were slaughtered to remove organs (liver,

intestine, and visceral fat). These organs were intended for somatic, proximate composition, enzymatic, oxidative stress, and histological analyses.

2.4 Analysis of proximate composition (fish and feed)

For proximate analysis, approximately 100 g of whole fish from each experimental unit and 20 g of feed per treatment were weighed according to the methodology of [11]

2.5 Oxidative stress

The SOD enzyme analysis was performed by auto-oxidation of pyrogallol, which is inhibited in the presence of SOD [12]. Absorbance readings were performed at 420 nm, considering that 0.1 IU inhibits 50% of pyrogallol auto-oxidation. CAT activity was evaluated by decreasing the absorbance of H2O2 at 230 nm [12]. One unit of CAT was defined as the amount of enzyme required in 1.0 µmol of H2O2.min-1 of oxidation, and the molar absorptivity used was (H2O2) ελ230 = 0.071 mM.cm-1.

2.6 Parameters of hepatic metabolism

The supernatant, obtained after homogenization and centrifugation of the material, was collected for enzymatic analysis by spectrophotometry (BIOPLUS S200 semiautomatic spectrophotometer) using appropriate wavelengths for each test [13]. The analyses of albumin, aspartate aminotransferase, and alanine aminotransferase were performed using commercial kits (Gold AnáliseDiagnóstica®) according to the manufacturer's instructions. The method specification was performed by reading in a spectrophotometer, performed using a semiautomatic equipment BioPlus S-200.

2.7 Digestive parameters

In the evaluation of the activity of the protease enzyme, the evaluation method was followed according to the total proteolytic activity evaluated by casein hydrolysis [14]; for the digestive enzyme lipase, the methodology of [15] performing some adjustments [16] was used. To determine the concentration of free glucose, the protocol proposed by [17] was followed. Amylase activity was determined using the Gold Analyze Diagnostica kit with modified methodology by [18], consisting of incubating the supernatant in the presence of starch and a phosphate buffer at pH 7.0. In the presence of iodine, the soluble starch had a bluish color. By the action of amylase, hydrolysis of starch occurred, progressively disappearing the blue color. Values were expressed in percentage of Amylase Unit (AU)

2.8 Plasma Biochemistry

For plasma biochemical evaluation, samples from three animals were separated per experimental unit. Blood collection was performed by caudal puncture using disposable syringes containing heparin. The material was centrifuged at 2,500 rpm for five minutes. Then, the analysis of plasma glucose (mg/dl), triglycerides (mg/dl), and total protein (mg/dl) was performed by means of colorimetric evaluations with the aid of Gold AnáliseDiagnostica analysis kits and determined in a spectrophotometer (BioPlus S-200).

2.9 Intestinal and liver histology

For histological analysis, a fraction of the midgut and a portion of the liver were collected from three animals per box. The collected samples were placed in 10% formalin solution for

fixation, stored for 24 hours, and then replaced for 70% alcoholic solution to be better handled in histological processing.

After tissue adhesion to slides, these were stained with hematoxylin-eosin and analyzed under an optical microscope [19]. For analysis, the histological sections were observed under a microscope using a 10X objective for the intestine and 40X for the liver to capture the observation fields. Image Pro-Plus version 4.5 image analysis system was used. Six slides were used per treatment. Seven sections each were photographed for the liver and 25 villi for the intestine, and then the number of goblet cells was counted.

2.10 Liver integrity

For the analysis of hepatic integrity, morphological changes were qualitatively evaluated using the injury index of [20], according to the formula: Bernet = Σ importance factor (w) x score (α). Three importance factors were used: (1) mild injury, (2) moderate injury, and (3) irreversible damage, which leads to partial or total organ loss. For each histopathological alteration, scores (α) were evaluated. Scores ranged from 0 to 6, depending on the degree of alteration: (0) no alteration, (2) little occurrence, (4) moderate occurrence, (6) serious injury occurrence. To determine lesions, a table was developed for the study, indicating the main histopathological lesions found.

2.11 Statistical analyses

All data were submitted to the Shapiro-Wilk normality and Levene homogeneity test. The zootechnical performance data, proximate composition, and enzymatic analyses were submitted to ANOVA analysis. When statistical differences were observed, the Tukey test was applied at 5% of significance. The computational statistical program used was R [21]. For the analysis of liver integrity, the data were submitted to Kruskal-Wallis at 5% significance.

Using the same program, Pearson's correlation analysis was performed to verify possible correlations between the selected variables [22]. Coefficients ranging from 1 to 0.7 positive or negative are considered strong, 0.3 to 0.7 positive or negative are considered moderate, and 0.3 to 0 positive or negative are considered weak.

Using Pearson's correlation data between measurements of final weight, hepatosomatic index, visceral fat, intestinal quotient, weight gain, feed conversion, protein retention, protein efficiency ratio, amylase, lipase, protease, triglycerides, glucose, height of the villi, width of the villi, area of the villi, height of the hepatocytes, width of the hepatocytes, and area of the hepatocytes, it was decided to perform a multivariate analysis. To perform multivariate analysis, taking into account that the data set contains quantitative and qualitative variables, an extension of the principal components analysis (PCA) method was used, called Factor Analysis of Mixed Data (FAMD), which is used for the analysis of mixed data [23].

3. RESULTS

3.1 Productive performance and somatic indexes

The variables final weight, final length, survival, weight gain, specific growth rate, uniformity, hepatosomatic index, and visceral fat showed no significant differences between the

inclusions of additives (p>0.05). However, feed conversion, protein efficiency rate, and intestinal quotient were positively affected by the inclusion of additives in the diet, significantly differing from the control treatment (p<0.05) (Table 2).

Table 2. Productive performance of Nile tilapia juveniles fed on probiotic, prebiotic, and symbiotic diets.

	Treatment					
Variable	**Control**	**PROB (4 g.kg^{-1})**	**PREB (4 g.kg^{-1})**	**SIMB I**	**SIMB II**	**p-value**
IW (g)	11.40±0.47	11.30±0.40	10.80±0.50	11.6±0.27	10.90±0.56	0.27
FW (g)	104.98±6.02	97.74±1.29	101.90±2.78	109.92±6.54	104.11±3.34	0.08
SUR (%)	93.33±2.89	96.67±5.77	100	88.33±7.64	98.33±2.89	0.07
FC	1.67±0.15b	1.20±0.10a	1.15±0.03a	1.22±0.08a	1.15±0.07a	<0.001
WG (g)	93.62±6.12	86.43±1.12	91.06±2.73	98.33±6.30	93.21±3.33	0.08
SGR (%day^{-1})	3.70±0.12	3.59±0.06	3.74±0.08	3.75±0.07	3.76±0.10	0.21
PER (%)	1.96±0.19a	2.91±0.22b	3.04±0.09b	2.85±0.18b	3.14±0.18b	<0.001
UNI (%)	68.03±9.95	70.93±6.44	66.67±2.89	68.18±6.95	62.81±4.86	0.67
HSI (%)	1.64±0.25	1.77±0.28	1.60±0.14	1.49±0.29	1.66±0.14	0.68
VFI (%)	2.27±0.78	2.01±0.36	2.00±0.19	1.90±0.37	2.13±0.46	0.88
IQ	6.21±0.26b	6.41±0.23ab	6.56±0.06ab	6.43±0.17ab	6.73±0.06a	0.04

Caption: PROB = Probiotic 4 g.kg^{-1}; PREB = Prebiotic 4 g.kg^{-1}; SIMB I = Symbiotic (2 g.kg^{-1} probiotic + 2 g.kg^{-1} prebiotic), and SIMB II = Symbiotic (4 g.kg^{-1} probiotic + 4 g.kg^{-1} prebiotic). FW = Final weight (g); FL = Final length (cm); SUR = Survival (%); FC = feed conversion; WG = weight gain (g); SGR = Specific growth rate (% day^{-1}); PER = Protein efficiency rate (%); UNI = Uniformity (%); HSI = hepatosomatic index (%); VFI = Visceral fat index (%); IQ = Intestinal Quotient.

3.2 Analyses of proximate composition

There was no change in the proximate parameters between the food treatments tested. However, protein retention increased significantly in animals fed on a diet containing the inclusion of additives (p<0.05) in relation to the control diet (Table 3).

Table 3. Centesimal composition of Nile tilapia juveniles fed on probiotic, prebiotic, and symbiotic diets.

	Treatment					
Composition	**Control**	**PROB**	**PREB**	**SIMB I**	**SIMB II**	**p-value**

(%)		(4 g.kg-1)	(4 g.kg^{-1})			
Moisture	75.64±3.11	73.99±1.49	74.73±3.79	73.69±1.07	74.45±0.85	0.87
Protein	20.37±2.95	19.61±1.39	19.37±2.53	19.89±3.57	19.93±2.07	0.99
Lipid	6.43±2.55	6.86±1.37	7.73±1.14	6.57±0.97	6.75±1.34	0.86
Ash	2.60±0.73	2.51±0.40	3.03±0.66	3.01±0.80	2.98±0.98	0.84
RT	38.60±3.06b	54.79±4.52a	56.42±8.15a	61.47±1.89a	64.00±1.66a	0.002

Caption: PROB = Probiotic 4 g.kg^{-1}; PREB = Prebiotic 4 g.kg^{-1}; SIMB I = Symbiotic (2 g.kg^{-1} probiotic + 2 g.kg^{-1} prebiotic), and SIMB II = Symbiotic (4 g.kg^{-1} probiotic + 4 g.kg^{-1} prebiotic). RT= body protein retention.

3.3 Oxidative stress

The oxidative enzyme variables catalase and superoxide dismutase did not show significant differences compared to the control diet (p>0.05) (Table 4).

Table 4. Metabolites of Nile tilapia juveniles fed on probiotic, prebiotic, and symbiotic diets.

	Diet					
	Control	**PROB (4 g.kg^{-1})**	**PREB (4 g.kg^{-1})**	**SIMB I**	**SIMB II**	**p-value**
Oxidative enzymes mmol/min/mg protein						
CAT	7.41±1.52	6.24±0.66	7.58±2.08	6.48±0.71	4.21±0.84	0.06
SOD	1.33±0.39	1.61±0.11	1.22±0.40	1.08±0.17	1.34±0.35	0.36

Caption: PROB = Probiotic 4 g.kg^{-1}; PREB = Prebiotic 4 g.kg^{-1}; SIMB I = Symbiotic (2 g.kg^{-1} probiotic + 2 g.kg^{-1} prebiotic), and SIMB II = Symbiotic (4 g.kg^{-1} probiotic + 4 g.kg^{-1} prebiotic). CAT = Catalase; SOD = Superoxide dismutase.

3.4 Parameters of hepatic metabolism

The hepatic parameters of albumin, aspartate aminotransferase, and alanine aminotransferase metabolism of juvenile Nile tilapia were not significantly affected by the inclusion of additives in the diet (p>0.05) (Table 5).

Table 5. Variables of hepatic metabolism of Nile tilapia juveniles fed on probiotic, prebiotic, and symbiotic diets.

	Diet					
	Control	**PROB (4 g.kg-1)**	**PREB (4 g.kg^{-1})**	**SIMB I**	**SIMB II**	**p-value**
Hepatic metabolism IU/mg protein						
ALB	4.06±0.56	4.05±1.39	3.41±0.62	4.71±1.86	3.90±1.35	0.79
AST	17.32±6.45	15.12±3.04	18.51±3.29	19.40±8.23	16.09±2.15	0.84

ALT	20.53±5.20	25.19±12.60	20.12±9.35	21.06±12.20	15.88±4.92	0.82

Caption: PROB = Probiotic 4 g.kg^{-1}; PREB = Prebiotic 4 g.kg^{-1}; SIMB I = Symbiotic (2 g.kg^{-1} probiotic + 2 g.kg^{-1} prebiotic), and SIMB II = Symbiotic (4 g.kg^{-1} probiotic + 4 g.kg^{-1} prebiotic). ALB = Albumin; AST = Aspartate aminotransferase; ALT = Alanine Aminotransferase.

3.5 Digestive parameters

The digestive enzymes analyzed in this study did not show significant differences compared to the control treatment (p>0.05) (Table 6).

Table 6. Digestive parameters of Nile tilapia juveniles fed on probiotic, prebiotic, and symbiotic diets.

	Diet					
	Control	**PROB (4 g.kg^{-1})**	**PREB (4 g.kg^{-1})**	**SIMB I**	**SIMB II**	**p-value**
Digestive enzymes (IU/mg)						
Amylase	90.50±16.90	78.09±33.20	60.78±29.50	81.71±28.80	77.94±25.10	0.74
Lipase	3.03±1.34	3.31±1.54	2.90±1.18	1.79±1.45	1.81±1.35	0.54
Protease	239.04±30.31	196.83±24.58	241.14±23.95	217.60±37.60	208.79±12.90	0.27

Caption: PROB = Probiotic 4 g.kg-1; PREB = Prebiotic 4 g.kg-1; SIMB I = Symbiotic (2 g.kg-1 probiotic + 2 g.kg-1 prebiotic), and SIMB II = Symbiotic (4 g.kg-1 probiotic + 4 g.kg-1 prebiotic).

3.6 Plasma Biochemistry

For the plasma metabolites evaluated, only glucose showed significant differences between diets, in which the diet containing probiotic (4 g.kg-1 of 5×109 CFU) and the diet with symbiotic II (4 g. kg-1 probiotic + 4 g.kg-1 prebiotic) had the highest plasma glucose levels in relation to the other treatments (p<0.05), as Table 7 shows.

Table 7. Plasma metabolites of Nile tilapia juveniles fed on probiotic, prebiotic, and symbiotic diets.

	Diet					
	Control	**PROB (4 g.kg-1)**	**PREB (4 g.kg^{-1})**	**SIMB I**	**SIMB II**	**p-value**
Plasma metabolites (mg/dl)						
Protein	3.10±0.78	3.55±1.12	3.92±1.60	5.79±0.26	4.25±1.63	0.14
Triglyceride	62.72±16.30	103.26±26.80	108.55±33.95	98.68±39.99	92.51±52.01	0.57

Glucose	67.44±23.20ab	83.80±26.50ab	86.70±7.51ab	49.33±11.01b	111.33±14.70a	0.02

Caption: PROB = Probiotic 4 g.kg^{-1}; PREB = Prebiotic 4 g.kg^{-1}; SIMB I = Symbiotic (2 g.kg^{-1} probiotic + 2 g.kg^{-1} prebiotic), and SIMB II = Symbiotic (4 g.kg^{-1} probiotic + 4 g.kg^{-1} prebiotic).

3.7 Intestinal and liver histology

For the histological variables of the intestine, there were no significant differences between the control and the other treatments with the inclusion of additives (p>0.05).

Table 8. Intestine of Nile tilapia juveniles fed on probiotic, prebiotic, and symbiotic diets.

	Diet					
	Control	**PROB (4 g.kg^{-1})**	**PREB (4 g.kg^{-1})**	**SIMB I**	**SIMB II**	**p-value**
Villus height (µm)	203.33±56.60	185.10±22.00	242.42±54.20	214.45±13.60	241.54±88.40	0.65
Villus width (µm)	77.69±10.70	63.43±9.96	73.95±1.19	78.83±15.20	67.84±10.00	0.37
Villus area (µm^2)	14,595.36±2.98	10,980.28±2.83	17,464.84±3.27	17,320.84±3.18	16,878.57±8.76	0.46
Thickness (µm)	42.51±9.66	40.41±6.96	41.50±1.78	42.00±5.63	41.24±8.41	0.99
Goblet cells (µm)	12.65±2.83	7.97±1.60	12.35±1.44	9.12±0.83	10.90±1.56	0.10

Caption: PROB = Probiotic 4 g.kg-1; PREB = Prebiotic 4 g.kg-1; SIMB I = Symbiotic (2 g.kg-1 probiotic + 2 g.kg-1 prebiotic), and SIMB II = Symbiotic (4 g.kg-1 probiotic + 4 g.kg-1 prebiotic).

3.8 Liver histology

Only hepatocyte height and width variables showed significant differences between treatments. The inclusion of additives significantly reduces these histological parameters (p<0.05), as Table 9 shows.

Table 9 - Liver of Nile tilapia juveniles fed on probiotic, prebiotic, and symbiotic diets.

	Diet					
	Control	**PROB (4 g.kg^{-1})**	**PREB (4 g.kg^{-1})**	**SIMB I**	**SIMB II**	**p-value**
Hepatocyte height (µm)	8.76±0.21a	7.75±0.22b	7.59±0.15b	7.39±0.41b	8.26±0.54ab	0.003

Hepatocyte width (µm)	8.03±0.08a	7.48±0.05b	7.38±0.07b	7.11±0.15b	7.48±0.41b	0.003
Hepatocyte area (μm^2)	52.01±5.32	50.59±4.15	51.18±9.26	47.41±5.03	57.45±3.43	0.38
Hepatocyte number	56.33±5.77	60.33±17.00	55.00±5.29	56.83±15.1	52.32±9.71	0.93

Caption: PROB = Probiotic 4 g.kg-1; PREB = Prebiotic 4 g.kg-1; SIMB I = Symbiotic (2 g.kg-1 probiotic + 2 g.kg-1 prebiotic), and SIMB II = Symbiotic (4 g.kg-1 probiotic + 4 g.kg-1 prebiotic).

3.9 Liver integrity

The liver integrity data reported in this study did not show significant differences compared to the control treatment (p>0.05) (Figure 1).

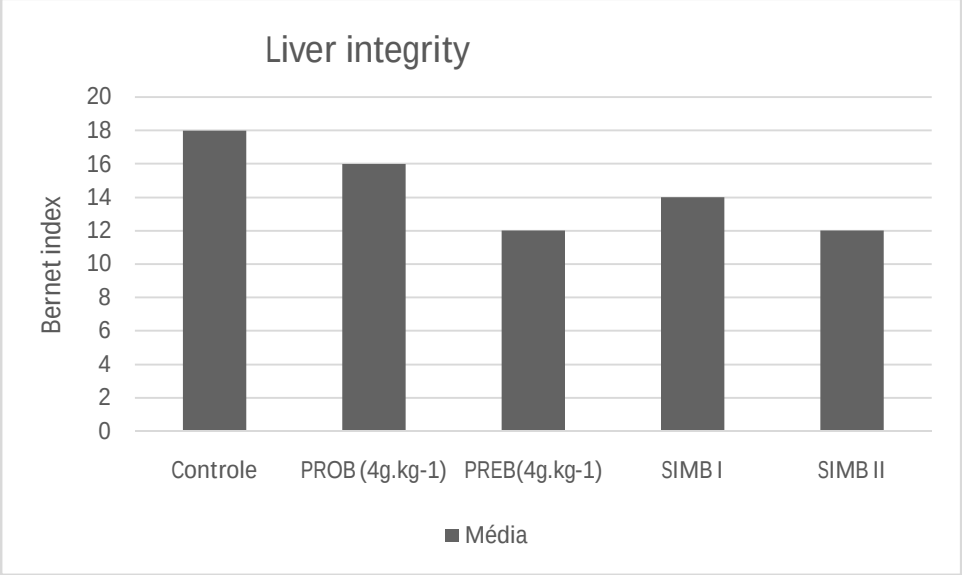

Fig.1. Index of histopathological lesions of Nile tilapia fingerlings (*Oreochromis niloticus*) in the treatments PROB = Probiotic 4 g.kg-1; PREB = Prebiotic 4 g.kg-1; SIMB I = Symbiotic (2 g.kg-1 probiotic + 2 g.kg-1 prebiotic); and SIMB II = Symbiotic (4 g.kg-1 probiotic + 4 g.kg-1 prebiotic).

3.10 Pearson Correlation Performance x histology

The correlation data obtained from the variables performance and histology show that there is a strong positive correlation between the factors FW with WG and SGR and a moderate positive correlation with villus width, with a moderate negative correlation attributed to the HSI parameter. In the HSI item, only a moderate negative correlation with WG was obtained, and for IQ there is a strong and positive correlation with the variables PR and PER and a moderate negative correlation for FC. For the parameter WG, there is a strong and moderate positive correlation with the variables SGR and villus width.

The FC showed a strong and negative correlation with the variables PR and PER and a strong and moderate positive correlation with the width of hepatocytes and villi, respectively. Regarding PR, there was a strong positive correlation with PER and a moderate negative correlation with hepatocyte width and height, while PER had a moderate negative correlation with hepatocyte height. Villus height showed a strong positive correlation with villus area and villus width and a moderate positive correlation with villus area. For hepatocyte data, hepatocyte height showed a positive correlation with feed conversion and a negative correlation with PR and PER (Table 10).

Table 10-Pearson's correlation between performance and histological variables

	HSI	VF	IQ	WG	FC	SGR	PR	PER	Villus height	Villus width	Villus area	Hepatocyte height	Hepatocyte width	Hepatocyte area
FW	-0.54	-0.11	0	1	0.03	0.7	0.1	-0.06	-0.22	0.54	0.07	-0.18	-0.3	-0.24
HSI		0.27	0.16	-0.54	-0.01	-0.41	-0.08	0.07	-0.13	-0.41	-0.27	0.43	0.38	0.55
VF			0.01	-0.09	0.33	0.02	-0.07	-0.23	-0.06	-0.03	-0.12	0.2	0.18	0.37
IQ				0.02	-0.63	0.14	0.7	0.7	0.34	-0.21	0.3	-0.18	-0.46	0.22
WG					0	0.75	0.12	-0.03	-0.19	0.55	0.1	-0.18	-0.31	-0.22
FC						-0.22	-0.73	-0.99	-0.03	0.2	0	0.63	0.74	-0.03
SGR							0.2	0.24	0.14	0.45	0.28	-0.14	-0.34	-0.02
PR								0.76	0.17	-0.24	0.1	-0.52	-0.63	0.02
PER									0.08	-0.26	0.02	-0.57	-0.72	0.13
Villus He										0.18	0.91	0.06	0	0.08
Villus width											0.53	-0.07	0.06	-0.21
Villus area												-0.01	-0.05	-0.05
Hepatocyte He													0.89	0.52
Hepatocyte width														0.41

Values in red are significant at 5%. Caption: final weight (FW); hepatosomatic index (HSI); visceral fat (VF); intestinal quotient (IQ); weight gain (WG); feed conversion (FC); specific growth rate (SGR); protein retention (PR), protein efficiency ratio (PER).

3.11 MFA Multivariate Analysis

According to multivariate analysis, it is possible to verify that when analyzing five dimensions, there is an explanation of 74.2% of data variation. Dimension 1 presents the greatest explanation.

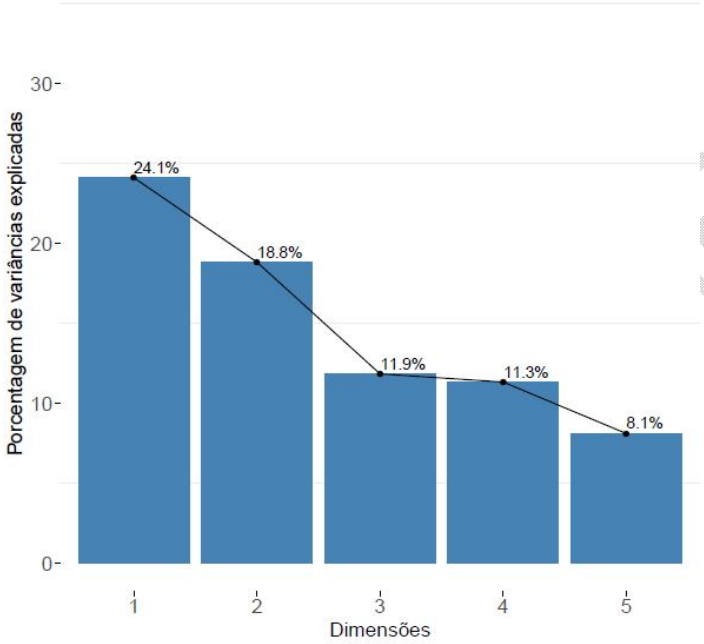

Fig. 2. Percentage of variances among the five dimensions analyzed.

When all variables are analyzed together, it is possible to notice the effects of additives. There is a clear separation between the experimental units that received the control treatment (in green), distributed on the left part of the graph, and the experimental units that received some of the additives, grouped on the right part of the graph (Figure 3).

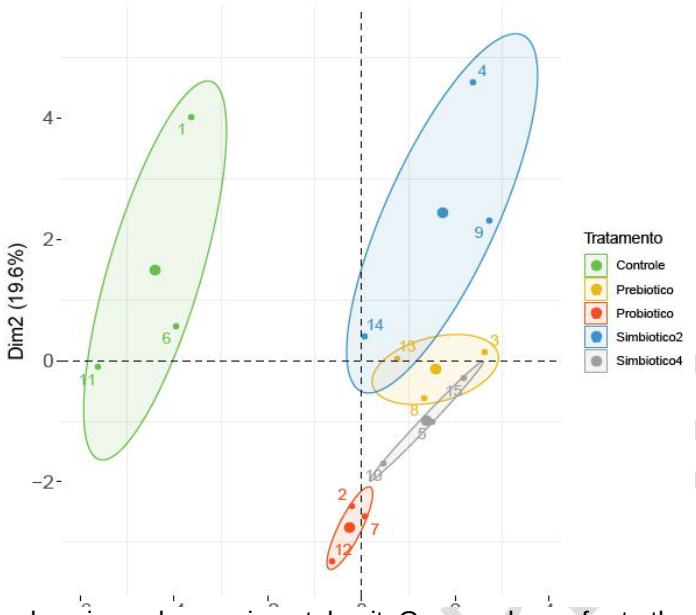

Fig. 3. Means of values in each experimental unit. Green colors refer to the control treatment (no additives), yellow colors refer to the treatment with prebiotic inclusion (4 $g.kg^{-1}$), red values refer to the treatment with probiotic inclusion (4 $g.kg^{-1}$), blue colors refer to the group of fish fed with symbiotic (2 $g.kg^{-1}$ prebiotic + 2 $g.kg^{-1}$ probiotic), and gray colors refer the group of fish fed on symbiotic diet (4 $g.kg^{-1}$ of prebiotic + 4 $g.kg^{-1}$ of probiotic).

4.DISCUSSION

4.1. Productive performance and somatic indexes

Zootechnical additives have been used in animal diets at different stages of cultivation as a way to provide better zootechnical indexes and animal health [24]. In the present study, the variables FC and PER of fish fed on the additives PROB, PREB, SIMB I, and SIMB II showed significant improvements compared to the control diet. This confirms the hypothesis that the use of additives in animal nutrition allows a greater use of food due to the proliferation of beneficial bacteria in the intestinal microbiota, which act by converting the protein consumed into weight gain.

[25] state that the inclusion of *Bacillus* in the diet of Nile tilapias leads to actions at the cellular level, where microorganisms and their exoenzymes play a significant role in the digestion process, increasing the intestinal enzymatic activity [26] and stimulating the production of endogenous enzymes [27]. In turn, this can increase the digestibility of food and, consequently, the use of nutrients, thus reducing the feed conversion and increasing the protein efficiency rate, results also demonstrated in the present study.

Although the inclusion of additives in the nutrition of Nile tilapia juveniles did not change the proximate composition of the carcass, there was a beneficial action in the retention of carcass protein in relation to the control diet. This validates the hypothesis that the additives

allow the fish to use the protein supplied in the diet more efficiently, transforming it into muscle, a fact also demonstrated by [28] for the same species.

According to [29, 30] the effects on zootechnical performance (weight gain, feed conversion, nutrient retention) of animals receiving feed supplemented with additive may be attributed to the improvement of digestive activity as a whole due to the increase in the synthesis of vitamins, cofactors, and enzymatic activities, which favors digestion, absorption of nutrients, and consequently weight gain. This statement can be confirmed with the improvement in productive performance variables (FC, PER and IQ). It also shows that the supplementation of prebiotics, probiotics, and symbiotics in this study at the concentrations proposed here did not cause negative impacts on carcass quality and could be incorporated into aquaculture nutrition, since the quality of fish carcasses directly affects profitability.

The intestinal quotient (IQ) is an important aspect to assess the animal's diet, as it is the result of the division between the length of the intestine and the standard length of the fish [31]. In the present study, the highest IQ was obtained in the SIMB II treatment, differing from the control treatment. This can be explained by the intense activity of beneficial bacteria in the intestine, allowing a better use of nutrients and a greater availability of carbohydrates, causing an increase in intestine length and glucose absorption [32].

4.2 Plasma Biochemistry

Blood biochemical parameters are useful, low-cost, and practical tools to assess the health status of fish [33]. Glucose is a carbohydrate that produces energy in the body of fish [34]. An increase in glucose content in the bloodstream points to a greater consumption of energy and to a higher metabolic response, in addition to being used to determine the use of nutrients provided in the diet [35].

In the present study, the glycemic level of juvenile tilapia was high in the diet containing symbiotic II (4 g.kg-1 of probiotic + 4 g.kg-1 of prebiotic) with a greater inclusion of additives. These values are close to those [36] found, who, supplementing the tilapia diet with a dose of 0.25 g/kg of yeast (Saccharomyces cerevisiae), obtained a value of 196.3 ± 9.8 g dL-1 of plasma glucose. However, all other treatments are still within the ideal range of homeostasis for the species, which varies from 14.1 to 92.1 g dL-1 [37].

4.3 Liver histology

The liver is responsible for the metabolism of nutrients. This is a response to the nutritional status of fish; changes in this organ, such as adaptations, injury or cell death may result in an unbalanced diet and/or exposure to chemical substances [38]. In this way, the histomorphometric analysis of hepatocytes becomes an essential tool as a biomarker of the metabolite state of animals [39].

The inclusion of all levels of additives in the diets of juvenile tilapia promotes a reduction in the morphometric parameters height and width of hepatocytes in relation to the control diet. This fact may be related to the loss of materials stored in the cytoplasm and to changes in activity, which reduce the overload of the liver during metabolic processes and consequently reduce tissue damage [40].

Such data confirm the hypothesis that the use of additives improves the nutritional status of fish due to the positive role played by microorganisms in the digestive system, thus improving host health and liver activity.

4.4 Pearson Correlation Performance x histology

Correlation analysis allows us to understand how much an animal performance factor is related to the other parameters of the animal organism. In the present study, there was a large proportion of correlations for final weight. A positive correlation with villus width is beneficial since a greater nutrient absorption surface promotes an improvement in PER and, consequently, in the WG of the animal, not overloading the liver. This is confirmed by the negative correlation with HSI due to the good use of nutrients provided in the diet which, therefore, reduces the overload on the liver. This hypothesis was confirmed by the positive correlation of HSI with hepatocyte area.

Regarding intestinal quotient, there was a strong and positive correlation with the parameters protein retention and protein efficiency rate, showing that in animals with larger intestines there is a better use of dietary protein, transforming it into muscle. The greater length of intestines implies an increase in the surface area for absorption of nutrients available in the diet [41], which is stated in the negative correlation of IQ with FC.

FC is a key parameter to analyze the efficiency of the diet provided to the animals and the use of nutrients. In the present study, there is a negative correlation between FC with PR and PER. This is because the increase in feed conversion leads to a lower use of nutrients; thus, the animals would have to consume more food to obtain the same final weight as other fish that received diets with additives. This corresponds to an overload on the liver, as it is a fundamental organ of fish metabolism. This response is in agreement with that verified in the analysis of liver integrity in control treatment fish, which, as observed, obtained a greater number of alterations, such as loss of cell limit and plasma vacuolization. This can be confirmed by the positive correlation to height and width of hepatocytes, altering the morphophysiology of the liver.

4.5 Multivariate Analysis

The multivariate analysis makes it possible to verify all parameters studied here by grouping them into just one outcome. The analysis revealed that there is a separation between the groups studied, but mainly a separation between the control group and the other groups containing additives.

The separation of the control group from the others is mainly due to the quality parameters of representation of the variables. Feed conversion, protein efficiency rate, protein retention, and width of the hepatocytes have greater importance in the quality of data, and it is precisely where the control group had the worst results. Among the group of additives, the PROB treatment (4 g.kg-1) distanced itself the most among the groups that received additives. This is explained by the contribution of the variables FW and WG as the main ones for dimension two. Among fish that received additives, this treatment presented the lowest values; thus, this separation happened, as Figure 4 shows.

The use of additives in animal nutrition is an efficient and viable alternative for maximizing production. However, the selection of these compounds must be taken into account since by using symbiotics, if an inefficient combination occurs, it may cause physiological problems and microbial diversity [42]. The structural characteristics of prebiotics should be evaluated according to their interaction with probiotic bacteria through assays in vitro and in vivo [43]. In this experiment, the inclusion of both additives alone and in combination provided beneficial effects to production, which indicates a good interaction between the strains used for supplementation.

5. CONCLUSION

The present study demonstrated that the dietary administration of MOS additives and probiotics has a positive effect on growth performance, feed utilization and protein deposition and on liver histology. The results of this study confirm that supplementing the diet of tilapia with additives can be an effective alternative to ensure the improvement of intestinal physiology responses. The use of SIMB II dose (4 g.kg-1 of probiotic + 4 g.kg-1 of MOS) is recommended.

ETHICAL APPROVAL (WHEREEVER APPLICABLE)

All authors hereby declare that "principles of laboratory animal care" (nih publication no. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

The experiment was carried out it the aquicultural area of the Federal university of grandeDourados (UFGD), Dourados, Mato Grosso do Sul, Brazil. The ethics committee on animal use of the Federal university of Grande Dourados (CEUA/UFGD) approved the experimental procedures of this study, under protocol no. 28/2020

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