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Lactobacillus rhamnosus improves feed intake, condition factors, hepatic and intestinal histomorphometric indexes of dourado *Salminus brasiliensis*

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Abstract

We herein investigated *Lactobacillus rhamnosus* as a probiotic, paraprobiotic and their synbiotic combination in the diet of dourado *Salminus brasiliensis*. Two hundred and forty juvenile *S. brasiliensis* with average weight and length of 6.78 ± 1.65 g and 8.97 ± 0.42 cm were randomly distributed in 16 experimental units (300 L) under four treatments: diet without additive (control), 0.02% probiotic (*L. rhamnosus* 10^8 CFU), 2.0% paraprobiotic (inactive *L. rhamnosus* 10^{10} CFU) and synbiotic (probiotic + paraprobiotic), all in quadruplicate, for 45 days. Then, zootechnical performance, hemato-biochemicals (pre-and post-challenge), intestinal and hepatic histomorphometric analyses were performed, in addition to a bacterial challenge with *Aeromonas hydrophila*. The feed intake was significantly lower ($p < 0.05$) in the probiotic group. The allometric condition factors were significantly higher ($p < 0.05$) in fish from the paraprobiotic and synbiotic groups. No significant differences ($p > 0.05$) were observed in the blood count between the supplemented groups and the control group. However, significant differences ($p < 0.05$) were observed in the blood count between the pre- and post-challenge periods. Fish in the synbiotic groups had higher total villi height and villi height when compared to fish in the probiotic and control group. The serosa were significantly ($p < 0.05$) thicker in the intestines of fish from the probiotic and synbiotic groups. Goblet cells were significantly numerous ($p < 0.05$) in fish from the synbiotic group. In conclusion, the use of 0.02% probiotic, 2.0% paraprobiotic, and synbiotic proved to be a promising practice due to their action on productive performance, enabling weight gain similar to that of control group with lower feed intake. In addition to improving intestinal immune-histomosphometric parameters, possibly promoting an improved condition in facing intestinal pathogens.

Keywords Lactobacilli-derived · Fish farming · Functional diet · Sustainability

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Highlights
- Feed intake of *Salminus brasiliensis* was significantly lower in the probiotic group.
- Allomeric condition factors of *S. brasiliensis* were significantly higher in paraprobiotic and synbiotic groups.
- Symbiotic and probiotic groups showed intestinal improvements.
- Goblet cells were significantly higher in *S. brasiliensis* from synbiotic group.

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Introduction

Even with the predominance of Nile tilapia (*Oreochromis niloticus*) in Brazilian fish farming, the native Brazilian species also increased by 1.8% in statistics, reaching the mark of 267,060 tons in 2022 (PeixeBR 2023). It is not surprising that indigenous fish species contribute to the advancement of Brazilian aquaculture, given that in recent years, the emphasis of production and national research has given priority to native species (Saint-Paul 2017; Valenti et al. 2021) and that more than 30% of total production is currently derived from native species (PeixeBR 2023). This supports the notion that enhancing species diversification can enhance aquaculture (Oboh 2022). Within this scenario emerges the dourado (*Salminus brasiliensis*).

Dourado *S. brasiliensis* belongs to the Characidae family and has a wide geographic distribution, with great potential for fish farming characterized by high weight gain and accelerated growth in the nursery phase, spending less time in this phase (30% shorter) than most species (Della Flora et al. 2010). However, the growth performance of *S. brasiliensis* can be negatively affected by the stress of frequent handling in cage systems (Braun et al. 2010), while nutritional strategies with adequate inclusion of vitamin A in the diet (Koch et al. 2018) or vegetable-based diets supplemented with swine liver hydrolysate (Lorenz et al. 2022) increase the immunomodulation activity (Koch et al. 2018) and the growth performance of the species (Lorenz et al. 2022).

Research results such as those mentioned above boost the use of potentially immunostimulating bioproducts in fish feeding as a strategy to enhance defense mechanisms in the prevention of stress and resistance against infectious diseases (Vallejos-Vidal et al. 2016; Hoseini et al. 2020). In this context, probiotics stand out as dietary supplements to improve the immune response and zootechnical performance (Navarrete and Tovar-Ramírez 2014; Akhter et al. 2015; Hoseinifar et al. 2018).

Research has already reported that non-viable counterparts of probiotic organisms, known as paraprobiotics (Choudhury and Kamilya 2019; Cuevas-González et al. 2020), inactivated probiotics or ghost probiotics (Taverniti and Guglielmetti 2011; Almada et al. 2016; Piqué et al. 2019), when administered in adequate amounts and frequency, also provide benefits to the animal. On the other hand, synbiotics are a combined mixture of food additives that can confer a synergistic and potentiated effect of benefits to the host and have been highlighted as immunostimulants in research in several areas of animal production, such as broiler chickens (Soomro et al. 2019; Abd El-Hack et al. 2020), ruminants (Arowolo and He 2018; Raabis et al. 2019; Mahesh et al. 2021) and fish (Elumalai et al. 2020; El-Saadony et al. 2021; Oliveira et al. 2022).

In this diverse universe of beneficial microorganisms, there are those of the genus *Lactobacillus*, –belonging to the group of gram-positive lactic acid bacteria (BAL), nonsporeforming (Tamang 2014)– which can adapt and colonize the intestinal epithelium in different environmental conditions (Pfeiler and Klaenhammer 2007). In fish diets, the use of *Lactobacillus rhamnosus* was related to improvements in intestinal morphology and immunity, and disease resistance in Nile tilapia (Pirarat et al. 2011; Xia et al. 2018), while in *Pargus major* improves the immune system and physiological responses (Dawood et al. 2017). On the other hand, dietary supplementation of *L. rhamnosus* for *Oncorhynchus mykiss* was efficient in controlling infections by *Vibrio anguillarum*, *Flavobacterium psychrophilum*, and *Aeromonas salmonicida* (Nikoskelainen et al. 2003).

The genus *Aeromonas* are the main causing agents for diseases frequently found in fish farms, where the species *A. hydrophila*, which are freshwater, facultatively anaerobic

bacterium, cause disease outbreaks with high mortality rates (Austin et al. 2016; Tavares-Dias and Martins 2017; Semwal et al. 2023) and growth impairments (Carraschi et al. 2011), becoming a limiting factor for the development of aquaculture.

Species with a high productive potential, such as the dourado *S. brasiliensis*, are in evidence in Brazilian aquaculture due to their great economic, social and environmental importance (Della Flora et al. 2010). In view of this purpose, the aim of the present study was to investigate the probiotic, paraprobiotic and combined effects of dietary supplementation with *L. rhamnosos* on growth performance, hematology, intestinal and hepatic histology and resistance of *S. brasiliensis* after bacterial challenge with *A. hydrophila*.

Material and methods

Experimental design

Two hundred and forty healthy juvenile dourado *S. brasiliensis* with average initial weight and length of 3.49 ± 0.20 g and 7.32 ± 0.12 cm obtained from the commercial fish farm *Projeto Pacu,* Terenos, Mato Grosso do Sul, Brazil, were randomly distributed in 16 circular experimental units (300 L) with constant aeration and continuous water-flow, housed in a greenhouse, totaling 15 fish per tank. The fish went through a pre-assay acclimatization period for three weeks and were fed a commercial diet (*Laguna* carnivorous fish / Socil® crude protein 450 g kg^{-1}; ethereal extract 120 g kg^{-1}; crude fiber 45 g kg^{-1}; mineral matter 140 g kg^{-1}; calcium (Min.) 15 g kg^{-1}; calcium (Max.) 25 g kg^{-1}; phosphorus 10 g kg^{-1}; moisture 120 g kg^{-1}; vitamin C 600 mg kg^{-1}) three times daily at a rate of 5%. During this period, no clinical signs such as erratic swimming, opacity of the cornea, lesions on the body or any other signs indicating diseases were observed.

After the acclimatization period, the fish had an average weight and length of 6.78 ± 1.65 g and 8.97 ± 0.42 cm and were submitted to four established treatments: feed supplemented with (manufacturer recommended inclusions) 0.02% of probiotic (*L. rhamnosus* 10^8 colony forming unit—CFU); 2.0% of paraprobiotic (inactive strains of *L. rhamnosus* 10^{10} CFU); synbiotic supplementation (0.02% probiotic + 2.0% paraprobiotic), and feed without additive (control group); all in quadruplicate according to Ruiz et al (2020).

The additives were added in an oily medium (soybean oil) and sprinkled directly on the commercial feed described above (*Laguna pescado* carnivores / Socil®) and then placed to dry at room temperature according to Nakandakare et al. (2018). For the control diet, only soybean oil was added to the feed. The probiotic Rham GB® (lyophilized microbial culture of *L. rhamnosus*) and the paraprobiotic Neoimuno® HealthCare (inactive strains of *L. rhamnosus*) were donated by the company GABBIA Biotecnologia®. The experimental diets were analysed by the company for the microbiological presence of the probiotic *L. rhamnosus* in MRS agar medium. After 48 h in a bacteriological incubator at 37° C, the analyses of the plates were performed. The microbiological analyzes for the presence of *L. rhamnosus* showed control diet = absence; probiotic diet = *L. rhamnosus* 2.50×10^7 CFU g^{-1}; paraprobiotic diet = absence; and synbiotic diet (viable + inactive) = *L. rhamnosus* 2.35×10^7 CFU g^{-1}.

Fish were fed twice daily (8:00 am and 4:00 pm) until apparent satiation for 45 days, and the amounts of feed provided were calculated. Monitoring of water quality variables were performed daily and mean values in the morning remained at pH 6.90 ± 0.64, dissolved oxygen 6.19 ± 0.77 mg L^{-1} and temperature 25.03 ± 0.87 °C; while in the afternoon

they remained with pH 7.52 ± 0.61, dissolved oxygen 5.14 ± 0.93 mg L^{-1} and temperature 28.37 ± 0.87 °C. Ammonia, nitrite and nitrate remained undetectable. Whenever necessary, the experimental units were siphoned to remove feces and leftover feed.

Zootechnical performance

After 45 days of supplementation, the fish fasted for 24 h and were subsequently anesthetized with Eugenol (50 mg L^{-1}) for biometry. With the biometric data, the zootechnical indexes were calculated for weight gain (WG) = (final weight—initial weight); length gain (LG) = (final length—initial length); apparent feed conversion (FCR) = (feed intake (g) / weight gain); protein efficiency ratio (PER) = ((weight gain / protein intake) × 100), specific growth rate (SGR) = ((*ln* Final weight/Initial weight/days) × 100). Survival rates (SR) (initial fish number—final fish number) were calculated after 45 days of dietary supplementation as well as on the seventh post-challenge day, according to Nunes et al. (2020). The allometric condition factors were calculated by the formula K = W/L*b* where, where *b* is estimated by the weight-length equation (W = *a*L*b*; where W is the weight and L is the total length and *a* and *b* are estimates of the correlation parameters), after logarithmic transformation and adjustment by the method of least squares of the data (Lima-Junior et al. 2002).

Hemato-biochemicals analyses

Three fish from each experimental unit, 12 per treatment, were removed from the units and used in blood collections for complete blood count analysis before the bacterial challenge. The fish remaining in the experimental units were challenged with the bacterium *A. hydroplila* and 24 h post-challenge, aliquots of blood from three fish per experimental unit were collected again for complete blood count. Blood collections were performed by puncturing the caudal vessel with insulin syringes coated with anticoagulant solution HEMSTB (EDTA K2 15 g dL^{-1}). Hematocrit (Ht) was determined by the microhematocrit method (Goldenfarb et al. 1971). Hemoglobin (Hb) levels were determined by spectrometry according to the cyanmethemoglobin method (Collier 1944). The number of erythrocytes (Er) was performed in in Neubauer's chamber after diluting in formalin citrate solution (1:200). From these data, the hematimetric indexes were calculated: mean corpuscular volume (MCV) = (Ht × 10/Er); mean corpuscular hemoglobin concentration (MCHC) = (Hb × 100/Ht); and mean corpuscular hemoglobin (MCH) = Hb/10 × Er (Wintrobe 1934). Blood smears were made in duplicate from each animal, the slides air-dried and stained with May Grünwald-Giemsa-Wright (Tavares-Dias and Moraes 2004). Differential leukocyte counts (lymphocytes, neutrophils, monocytes, eosinophils, granulocytic cells or granular leukocyte PAS (LG-PAS) and basophils) were performed under an optical microscope (1000×) with the aid of a digital differential counter.

For blood glucose analysis (glucose oxidase [GPO]) the roche accu chek active glucose measuring device was used. Leukocyte activities were determined according to the methodology described by Biller et al. (2013). This method consists of determining the reactive oxygen species (ROS) produced by the respiratory burst through a colorimetric assay based on the reduction of the nitroblue tetrazolium (NBT) reagent, which gives rise to precipitates of insoluble material with a dark blue color inside the phagocyte, called formazan granules (Klein et al. 1990). Fifty microliters of blood added to 50 µL of 0.2% nitroblue tetrazolium (NBT) solution were used. The NBT solution was prepared in buffer (PBS) and incubated for 30 min at 25 °C. After the incubation period, 50 µL of the solution were diluted in 1000 µL (1 mL) of

N-dimethyl formamide (DMF) and centrifuged at 3000 *g* for 5 min. The reading of the samples was determined by spectrophotometry at a wavelength of 540 nm.

Intestinal and hepatic histomorphometric analyses

At the end of the dietary supplementation period, two fish from each experimental unit (n = 32) were anesthetized with Eugenol (50 mg L^{-1}) and euthanized by spinal cord transection and submitted to a longitudinal ventral incision to expose the intestine and liver. Tissue fragments from the liver and posterior portion of the medial intestine of approximately three cm were fixed in 10% buffered formalin. The samples were then washed and dehydrated in increasing series of ethyl alcohol, clarified in xylol and embedded in paraffin at 60 °C to be cut in 4 µm sections and stained with Hematoxylin–Eosin (H & E) according to Humason (1972).

Subsequently, the slides were prepared in Entellan® media and the histological sections were photomicrographed using an opticam 14.0 camera and the variables were analyzed using the Motic Images Plus 2.0 ML program. For intestinal histomorphometry, the total villi height (µm), villi height (µm), villi width (µm), epithelium thickness (µm), lamina propria (µm), submucosa (µm), layer muscle (µm), serosa (µm) and number of goblet cells per villi (n = 40 per region). For liver histomorphometry, 50 hepatocytes were randomly sampled to measure the area (μm^2) and perimeter of the cytoplasm (µm); area (μm^2), perimeter (µm) and diameter (µm) of the nucleus; nucleus area/cytoplasm area ratio (Nacr = nuclear area/cytoplasm area × 100); nucleus perimeter/cytoplasm ratio (Npcr = nuclear perimeter/cytoplasm perimeter × 100); hepatocyte nuclear volume (NV (μm^3) = $4/3.\pi.r^3$, where r^3 = diameter/2); and the circularity of the hepatocyte nucleus (CN = $p^2/4.\pi.a$, where p^2 = nuclear perimeter and a = nuclear area) according to Rodrigues et al. (2017).

Aeromonas hydrophila challenge

The *A. hydrophila* strain (Strain KJ561021) used for the challenge was provided by the Laboratory of Microbiology and Parasitology of Aquatic Organisms at CAUNESP/Jaboticabal-SP. After sterilization of the culture medium (BHI, agar) in an autoclave at 121 °C for 20 min, the *A. hydrophila* strains were plated and incubated for 24 h in a bacteriological incubator at 28 °C. After the growth of bacterial colonies, the washing procedure was performed with phosphate-bufferid saline (PBS) in a centrifuge at 4000 g for 20 min, 3 times. Then the bacteria were resuspended in 20 ml PBS and the reading performed in a spectrophotometer (625 nm). The established concentration for the challenge was 0.8×10^6 CFU and 0.1 mL of the solution were inoculated intraperitoneally in each fish (n = 40 per treatment) according to Rodrigues et al. (2021).

Statistical analyses

Data were analysed for normality and homogeneity using the Shapiro–Wilk and Bartlett tests, respectively. Growth performance and histomorphometric data were submitted to analysis of variance (one-way ANOVA). A Two-way ANOVA was used on blood parameters with two independent variables in a 4 × 2 factorial scheme, (4 treatments × 2 collection times). The parameters immature leukocytes, monocytes, neutrophils, leukocytes, thrombocytes and respiratory activity of leukocytes were transformed into log and square root because they did not present the assumptions of normality and homogeneity. Means were

compared by Duncan's test ($p < 0.05$). All tests were performed using the statistical program R version 3.4.3 (Packages ExpDes.pf).

Results

Zootechnical performance

As in the control group, supplementation had no effects on selection or rejection of feed. After 45 days of supplementation, survival was 100% in all treatments. The feed intake was significantly lower ($p < 0.05$) for fish in the probiotic group when compared to the other groups; however, feed intakes were equal between the control and synbiotic groups. The allomeric condition factors were significantly higher ($p < 0.05$) in fish from the paraprobiotic and synbiotic groups, while the lowest condition factor was found in fish from the probiotic group. In the other zootechnical indexes, no significant differences were found between the groups (Table 1).

Hemato-biochemical parameters

There were no interactions between the factors in the hematological analyses. Separately, no significant differences ($p > 0.05$) were observed in the blood count between the supplemented groups and the control group. However, significant differences ($p < 0.05$) were observed in the blood count between the pre-challenge and post-challenge periods. Hemoglobin, hematocrit, number of neutrophils, lymphocytes, leukocytes, immature leukocytes, monocytes and thrombocytes decreased significantly ($p < 0.05$) after exposure to *A.*

Table 1 Zootechnical performance indexes of dourado (*S. brasiliensis*) fed for 45 days with a diet containing 0.02% probiotic (*L. rhamnosus*), 2.0% paraprobiotic (inactive *L. rhamnosus*), synbiotic (0.02% probiotic + 2.0% paraprobiotic), and control group (diet without additive)

	Treatments				
Zootechnical indexes	*Control*	*Probiotic*	*Paraprobiotic*	*Synbiotic*	*CV*
Initial weight	6.78 ± 0.32	6.83 ± 0.48	6.79 ± 0.25	6.24 ± 1.22	5.73
Initial length	9.02 ± 0.16	8.98 ± 0.27	9.08 ± 0.09	8.79 ± 0.65	1.63
Final weight	25.41 ± 7.75	25.91 ± 6.77	28.72 ± 8.42	25.47 ± 7.41	28.47
Final length	13.66 ± 1.39	13.85 ± 1.06	14.22 ± 1.28	13.71 ± 1.34	8.92
Weight gain (g)	18.94 ± 2.10	19.10 ± 2.67	22.13 ± 3.30	19.71 ± 1.84	14.70
Length gain (cm)	4.7 ± 0.38	4.88 ± 0.45	5.20 ± 0.50	5.01 ± 0.4	9.01
Specific growth rate (% day^{-1})	2.92 ± 0.24	2.95 ± 0.24	3.20 ± 0.25	3.19 ± 0.19	8.71
Protein efficiency ratio (%)	42.09 ± 4.67	42.46 ± 5.94	49.17 ± 7.34	43.81 ± 4.09	14.70
Apparent feed conversion	1.26 ± 0.14	1.08 ± 0.10	1.01 ± 0.15	1.15 ± 0.07	12.49
*Feed intake (g)**	0.52 ± 0.0^{a}	0.45 ± 0.02^{c}	0.49 ± 0.02^{b}	0.50 ± 0.02^{ab}	3.93
*Condition factor**	0.008 ± 0.0^{b}	0.004 ± 0.0^{c}	0.011 ± 0.0^{a}	0.011 ± 0.0^{a}	5.81
Survival rate (%)	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.00	0.0

Values are expressed as mean ± standard deviation. Different letters on the same line indicate significant difference between Treatments ($p < 0.05$) by Duncan's test. CV = coefficient of variation. (*) Significant

hydrophila (Table 2). The other hematimetric indexes (MCH, MCV and MCHC) showed no significant difference ($p > 0.05$) neither between treatments nor between collection times (Fig. 1).

Blood glucose levels did not show significant differences ($p > 0.05$) between the treatments at the end of the supplementation period. However, glucose levels reduced significantly ($p < 0.05$) in the post-challenge period (Fig. 1). Likewise, the respiratory activity of leukocytes showed a significant increase ($p < 0.05$) only after exposure to *A. hydrophila* (Fig. 1). Survival rates decreased after bacterial infection, but no significant differences ($p > 0.05$) were observed between groups (Fig. 1).

Hepatic and intestinal histomorphometric analysis

Significant differences ($p < 0.05$) were observed in the hepatic and intestinal histomorphometric indexes. Fish in the paraprobiotic and synbiotic groups had higher total villus height and villus height when compared to fish in the probiotic group. Serosa were significantly ($p < 0.05$) thicker in the intestines of fish from the probiotic and synbiotic groups; however, the paraprobiotic and control group were the same as the synbiotic group. The number of goblet cells was significantly higher ($p < 0.05$) in fish from the synbiotics group.

Regarding liver tissue, the cytoplasmic areas of hepatocytes in fish from the paraprobiotic group were significantly larger ($p < 0.05$) than in the other groups. Cytoplasmic perimeters were significantly smaller ($p > 0.05$) in fish in the synbiotic group when compared to fish in the probiotic and paraprobiotic groups, and equal to the fish in the control group. Hepatocyte perimeter/nucleus/cytoplasm ratio was significantly higher ($p < 0.05$) in fish from the synbiotic group. The other variables showed no difference ($p > 0.05$) (Table 3).

Discussion

Nutrition based on functional diets has been shown to be fundamental for improvements in the aquaculture production sector, where the use of prebiotics, probiotics, paraprobiotics and synbiotics help to improve the health status of animals, the aquatic environment, and consequently reduce the incidence of disease outbreaks (Amenyogbe et al. 2020; El-Saadony et al. 2021).

Feed intake in fish can be regulated by several factors related to the rearing environment, and the better use of feed ensures better growth performance with less waste generation (Houlihan et al. 2001). Herein, although no significant differences were observed in weight gain and final weight, we found that dietary supplementation with *L. rhamnosus* in different forms (probiotic, paraprobiotic and synbiotic) were able to reduce feed intake in juvenile dourado *S. brasiliensis*, where the fish in the probiotic and paraprobiotic groups, even ingesting smaller amounts of feed, reached the same productive indexes as the fish in the control group. This benefit may have been achieved by the presence of probiotic bacteria in the intestine, which can improve the balance of the intestinal microbiota and improve the absorption and utilization of nutrients. (Amenyogbe et al. 2020; Naiel et al. 2021).

Likewise, the condition factor is seen as an important productive indicator (Owatari et al. 2022a) and in the paraprobiotic and synbiotic groups, the highest condition factor index can indicate the condition of the fish, which were in an accelerated growth phase, directing all the nutritional benefits of ingested feed for body building (Gomiero et al. 2010). Considering that the largest inputs resulting from fish farming are related to feed

Table 2 Hematological parameters of dourado (*S. brasiliensis*) fed for 45 days with a diet containing 0.02% probiotic (*L. rhamnosus*), 2.0% paraprobiotic (inactive *L. rhamnosus*), synbiotic (0.02% probiotic + 2.0% paraprobiotic), and control group (diet without additive)

Treatments								
Parameters	Control	Probiotic	Paraprobiotic	Synbiotic	p-value	Interaction	Pre-challenge	p-value (sample time)
Hemoglobin (g dL^{-1})	13.14 ± 3.74	12.19 ± 3.53	11.81 ± 3.48	11.65 ± 3.53	0.453	*ns*	12.35 ± 3.61^{A}	0.001
Hematocrit (%)	35.9 ± 9.00	30.00 ± 9.00	32.80 ± 8.80	27.18 ± 8.50	0.357	*ns*	31.86 ± 8.71^{A}	0.018
Erythrocytes (× 10^{6} μL^{-1})	3.10 ± 1.10	2.77 ± 1.13	2.58 ± 1.12	2.49 ± 1.12	0.81	*ns*	2.72 ± 1.12	0.085
Lymphocytes (× $10^{3}\mu L^{-1}$)	79.90 ± 51.91	80.80 ± 46.08	85.47 ± 44.33	51.13 ± 50.73	0.995	*ns*	77.86 ± 52.83^{A}	0.001
Immature leukocyte (x$10^{3}\mu L^{-1}$)	27.80 ± 26.36	18.40 ± 30.11	20.15 ± 31.82	19.52 ± 27.44	0.971	*ns*	22.55 ± 28.09^{A}	0.005
Monocytes (× 10^{3} μL^{-1})	4.80 ± 7.08	7.95 ± 6.86	3.26 ± 5.61	7.14 ± 8.26	0.644	*ns*	5.99 ± 8.26^{A}	0.001
Neutrophil (× 10^{3} μL^{-1})	3.91 ± 2.32	1.97 ± 1.88	3.54 ± 1.88	3.29 ± 2.32	0.614	*ns*	3.08 ± 2.29^{A}	0.001
Leukocytes (× 10^{3} μL^{-1})	17.01 ± 13	11.69 ± 10.49	12.95 ± 15.18	9.68 ± 11.26	0.653	*ns*	12.24 ± 13.74^{A}	0.001
Thrombocytes (× 10^{3} μL^{-1})	28.26 ± 23.31	18.58 ± 14.10	31.35 ± 24.70	23.81 ± 22.72	0.662	*ns*	24.90 ± 20.06^{A}	0.001
Parameters							***Post-challenge***	
Hemoglobin (g dL^{-1})	10.02 ± 2.55	9.00 ± 2.60	8.79 ± 2.73	9.34 ± 2.75	0.453	*ns*	9.41 ± 2.63^{B}	0.001
Hematocrit (%)	29.66 ± 9.16	26.9 ± 8.73	21.87 ± 9.75	25.6 ± 9.61	0.357	*ns*	26.58 ± 9.06^{B}	0.018
Erythrocytes (× 10^{6} μL^{-1})	2.31 ± 0.81	2.17 ± 0.83	2.34 ± 0.87	2.27 ± 0.82	0.81	*ns*	2.29 ± 0.79	0.085
Lymphocytes (× $10^{3}\mu L^{-1}$)	7.60 ± 4.74	7.17 ± 5.54	7.38 ± 4.57	7.95 ± 4.34	0.995	*ns*	76.70 ± 4.36^{B}	0.001
Immature leukocyte (x$10^{3}\mu L^{-1}$)	4.88 ± 3.29	2.64 ± 2.99	2.24 ± 3.13	2.37 ± 3.46	0.971	*ns*	3.05 ± 3.36^{B}	0.005
Monocytes (× 10^{3} μL^{-1})	0.18 ± 0.20	0.28 ± 0.18	0.13 ± 0.18	0.18 ± 0.26	0.644	*ns*	0.26 ± 0.25^{B}	0.001
Neutrophil (× 10^{3} μL^{-1})	0.34 ± 0.27	0.34 ± 0.28	0.23 ± 0.24	0.23 ± 0.24	0.614	*ns*	0.29 ± 0.26^{B}	0.001
Leukocytes (× 10^{3} μL^{-1})	2.33 ± 1.57	1.21 ± 1.05	11.17 ± 10.76	1.35 ± 1.06	0.653	*ns*	1.51 ± 1.25^{B}	0.001
Thrombocytes (× 10^{3} μL^{-1})	3.7 ± 2.33	1.17 ± 1.14	2.39 ± 2.31	4.20 ± 3.38	0.662	*ns*	2.79 ± 2.31^{B}	0.001

Values are expressed as mean ± standard deviation. Different capital letters in the same column represent significant differences ($p < 0.05$) between the sampling periods (pre- and post-challenge) by Duncan's test

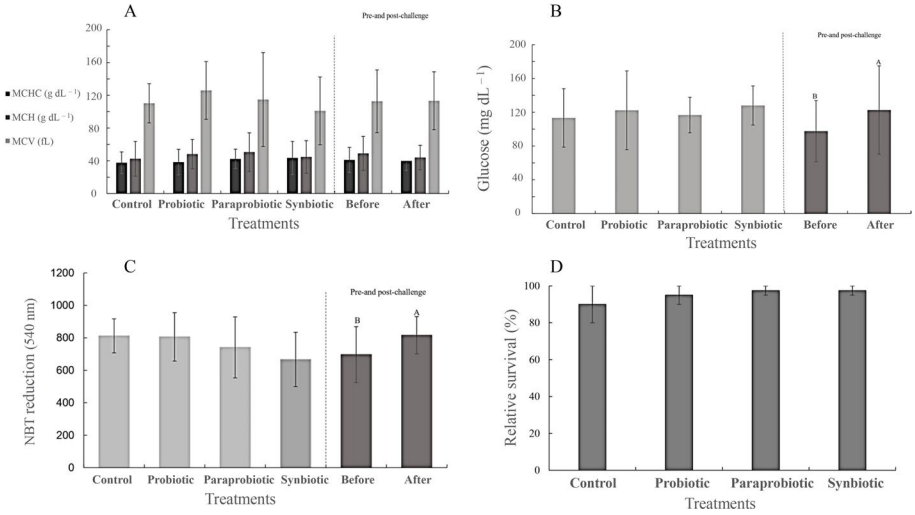

Fig. 1 In (**A**) Hematimetric indexes of mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV); in (**B**) plasma glucose levels; in (**C**) leukocyte respiratory activity (nitroblue tetrazolium (NBT) reduction) of dourado (*S. brasiliensis*) fed for 45 days with a diet containing 0.02% probiotic (*L. rhamnosus*), 2.0% paraprobiotic (inactive *L. rhamnosus*), synbiotic (0.02% probiotic + 2.0% paraprobiotic), and control group (diet without additive). In (**D**) survival rate of juvenile dourado (*S. brasiliensis*) up to 7 days after infection by *A. hydrophila*. Values are expressed as mean ± standard deviation. Different capital letters represent significant differences ($p < 0.05$) between the sampling periods (pre-and post-challenge) by Duncan's test

expenses (Castilho-Barros et al. 2020), we can infer that *L. rhamnosus* can be a viable alternative to generate savings in the productive sector.

Recently, Noshair et al. (2023) carried out an evaluation of the dietary supplementation of the probiotic *L. rhamnosus* on the growth performance of *O. niloticus* and found that the probiotic was able to significantly improve weight gain, protein efficiency rate, specific growth rate and condition factor in all probiotics-supplemented groups. On the occasion Noshair et al. (2023) conducted the experiment for 12 weeks, which brings us a huge suspicion that these indexes can also be achieved with dourado *S. brasiliensis* in trials with a longer duration.

Hematological assessments of fish in experimental aquaculture environments are usually related to diagnostics to decipher the health status of fish in response to nutritional changes or exposure to pathogens (Fazio 2019). Dawood et al. (2016) evaluated the effects of isolated or combined dietary supplementation of *L. rhamnosus* and *L. lactis* on the immune responses of sea bream *Pagrus major* and found that, compared to the control group, fish fed with probiotics in both forms, presented a significant increase in the hematocrit, guaranteeing a better state of health of the fish.

According to Satake et al. (2009) the hematocrit values of *S. brasiliensis* under experimental conditions can vary between 36 and 45% and despite that, in the present study the values remained below this reference, both before and after the bacterial infection. Likewise, reduced values for hematocrit were observed by Koch et al. (2018) in juvenile *S. brasiliensis* supplemented with different doses of vitamin A and exposed to *A. hydrophila*. The decrease in hematocrit was related to the reduction in erythrocytes

Table 3 Intestinal and hepatic histomorphometric parameters of dourado (*S. brasiliensis*) fed for 45 days with a diet containing 0.02% probiotic (*L. rhamnosus*), 2.0% paraprobiotic (inactive *L. rhamnosus*), synbiotic (0.02% probiotic + 2.0% paraprobiotic), and control group (diet without additive)

Treatments					
Intestine	Control	Probiotic	Paraprobiotic	Synbiotic	CV
Histomorphometric indexes					
Total villi height (μm)*	$716.66 \pm 82.66b^{c}$	671.51 ± 51.77^{c}	783.13 ± 86.82^{ab}	868.2 ± 52.85^{a}	24.01
Villi height (μm)*	$625.23 \pm 75.50b^{c}$	549.70 ± 65.01^{c}	695.60 ± 65.87^{ab}	770.81 ± 64.52^{a}	26.09
Villi width (μm)	145.58 ± 9.71	147.57 ± 18.14	130.49 ± 5.87	151.31 ± 10.94	26.09
Epithelium thickness (μm)	56.52 ± 6.34	56.77 ± 8.75	53.34 ± 1.82	58.25 ± 5.87	34.10
Lamina propria (μm)	51.73 ± 6.17	44.35 ± 9.35	34.61 ± 2.60	48.21 ± 9.38	20.33
Submucosa (μm)	40.55 ± 3.82	44.03 ± 6.62	44.17 ± 2.54	42.65 ± 30.2	8.38
Muscle layer (μm)	57.47 ± 6.49	78.69 ± 19.82	61.25 ± 12.38	72.6 ± 12.94	24.48
Serosa (μm)*	36.62 ± 3.19^{b}	55.19 ± 10.53^{a}	34.47 ± 29.59^{b}	44.13 ± 6.28^{ab}	22.47
Goblet cell per villi*	25.00 ± 1.48^{b}	22.00 ± 2.04^{b}	20.00 ± 4.36^{c}	28.20 ± 3.92^{a}	47.32
Hepatocytes					
Nuclear circularity (0–1)	1.00 ± 0.004	1.00 ± 0.007	0.99 ± 0.010	0.99 ± 0.10	0.92
Core area (μm^2)	1.02 ± 0.14	1.00 ± 0.16	1.03 ± 0.15	0.97 ± 0.12	16.04
Cytoplasm area (μm^2)*	9.03 ± 0.97^{b}	9.16 ± 1.04^{b}	10.07 ± 0.88^{a}	7.80 ± 0.89^{b}	11.74
Core perimeter (μm)	3.55 ± 0.27	3.52 ± 0.28	3.59 ± 0.26	3.48 ± 0.22	8.02
Cytoplasm perimeter (μm)*	11.53 ± 0.71^{ab}	11.59 ± 1.27^{a}	12.11 ± 0.61^{a}	$10.460.75^{b}$	6.68
Nucleus area:Cytoplasm ratio	11.52 ± 1.06	11.25 ± 1.31	10.82 ± 1.31	12.92 ± 1.37	12.11
Core perimeter:Cytoplasm ratio*	31.00 ± 1.52^{b}	30.54 ± 1.34^{b}	29.80 ± 0.91^{b}	33.92 ± 3.08^{a}	7.20
Nuclear volume (μm^3)	0.80 ± 0.16	0.87 ± 0.16	0.71 ± 0.13	0.86 ± 0.07	16.22

Values are expressed as mean ± standard deviation. Different letters on the same line indicate significant difference between treatments ($p < 0.05$) by Duncan's test. CV = coefficient of variation. (*) Significant

after the bacterial challenge, but this reduction observed by the authors was not below the reference values ($1.95 \sim 2.33 \times 10^6$ μL^{-1}) described by Satake et al. (2009). In the present study the number of monocytes, immature leukocytes, thrombocytes and leukocytes also decreased, corroborating the findings of Claudiano et al. (2019) who observed a reduction in the number of leukocytes in *Piaractus mesopotamicus* between one hour and nine hours after infection by *A. hydrophila*.

Blood glucose and cortisol levels are used as good physiological indicators in response to stress in fish (Sopinka et al. 2016). In aquaculture, fish are often subjected to different stressors, including routine handling (Portz et al. 2006), which cause changes in plasma glucose concentrations and have a multifactorial impact on the organism (Sopinka et al. 2016; Bartoňková et al. 2017). Such glycemic changes occur within minutes, hours or even days after suffering an adverse stimulus (Langiano and Martínez 2009).

According to Sopinka et al. (2016) knowing the basal levels of plasma glucose of the species studied is of great relevance, as the values obtained establish parameters to be used as a reference in future research. In the present study, fish exposed to *A. hydrophila* showed different blood glucose levels before and after infection, remaining higher in the

post-challenge period, regardless of treatment. Cortisol is one of the hyperglycemic hormones reported as the main responsible, during the period of infection, for deregulating glycemia (Johar et al. 2021). When a pathological agent invades the organism, it automatically activates the immune system, triggering an innate response that includes the action of phagocytic cells (neutrophils, monocytes and eosinophils) (Mahoney and Macnulty 1992; Iwama and Nakanishi 1996; Roberts 2012) causing hyperglycemia. This information explains the increase in glucose during the infectious process caused by *A. hydrophila*.

The increase of respiratory activity of leukocytes was observed by Román et al. (2012) in *Sparus aurata* and *Dicentrarchus labrax* after dietary supplementation with inactivated probiotic (paraprobiotic) *Vagococcus fluvialis*; and by Muñoz-Atienza et al. (2015) in *Scophthalmus maximus* fed a mix of viable and inactivated lactic acid bacteria. However, in the present study, no change in the respiratory activity of leukocytes related to dietary supplementation was observed. On the other hand, during the bacterial challenge, the respiratory activity of leukocytes was increased, suggesting a physiological response in fish related to the stress suffered. Reactive oxygen species (ROS) are normally produced during respiratory metabolism; however, production is controlled by an antioxidant defense system that inhibits and/or reduces damage caused by the action of free radicals (Biller and Takahashi 2018), establishing the respiratory activity of leukocytes as an indicator of the activation of the innate immune response after infection by pathogens (Biller et al. 2013).

The mechanisms of action of paraprobiotics are still not well understood, however, studies reinforce the ability of this additive to modulate the immune system (Almada et al. 2016; Barros et al. 2019) and inhibit the growth of pathogens through the adhesion in the intestinal epithelium (Grześkowiak et al. 2014). Such information is corroborated by the findings of the present study, where the paraprobiotic potentiated the effects of the probiotic on intestinal histomorphometry, and on the number of goblet cells.

Goblet cells play an important role in maintaining homeostasis and act as the front line of innate host defense (Kim and Ho 2010; Bevins and Salzman 2011). They are responsible for various digestive enzymes and mucus secretion, predominantly mucins, which act as a bactericidal barrier against pathogenic microorganisms (McGuckin et al. 2015). In view of the facts, the synbiotic (viable and inactive *L. rhamnosus*) provided intestinal immunomodulation, with an increase in the length of the villi, and consequent increase in the amount of goblet cells. On the other hand, the probiotic *L. rhamnosus* promoted a thicker serosa, which probably secrete more serous fluid, promoting greater organ integrity (Veggetti et al. 1999). The modulation of intestinal morphology can consequently contribute to the improvement of zootechnical performance, explaining the best performance indexes in feed intake and condition factor in the paraprobiotic and synbiotic groups.

Finally, regarding hepatocytes, liver tissue morphology can be used as biomarkers of nutritional responses, metabolic conditions and fish health (Rašković et al. 2011; Rodrigues et al. 2017), and alterations such as glycogen depletion, inflammation and neoplasms can be interpreted as a response to stress (Köhler 1992; Teh et al. 1997). Herein, the histomorphometric parameters of hepatocytes showed significant differences between treatments. According to Watanabe & Tanaka (1982) size of hepatocytes is linked to the volume of stored lipid droplets, and according to Schott et al. (2019) in hepatocytes, lipid droplets have diameters ranging from 60 nm to more than 5 µm and may play an important role in cellular catabolic processes. Thus, the presence of lipid in the liver should not be directly related to physiological anomalies or diseases (steatosis), as these droplets are common in up to 5% of liver cells (Scorletti and Carr 2022). However, increased lipid droplets within hepatocytes, constant and for a long time, can be caused by inflammation (Owatari et al. 2022b).

Conclusion

In conclusion, in the present study, the use of 0.02% of probiotic (*L. rhamnosus* 10^8 CFU) and 2.0% of paraprobiotic (inactive strains of *L. rhamnosus* 10^{10} CFU) proved to be a promising practice due to their action on productive performance, enabling weight gain similar to that of control group with lower feed intake, as well as improving the condition factor with 2.0% paraprobiotic and symbiotic (0.02% probiotic + 2.0% paraprobiotic). In addition to improving intestinal immunohistomorphometric parameters (goblet cells/villi), possibly promoting an improved condition in facing intestinal pathogens.

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Data Availability The data related to this research are available upon prior request.

Declarations

Ethical Approval The study was approved by the Animal Ethics Committee (CEUA) of the State University of Mato Grosso do Sul – UEMS, Aquidauana, MS, Brazil, under Protocol Nº 034/2022 and followed all ethical principles in animal experimentation. All fish used in biological analyzes were previously anesthetized with Eugenol (50 mg L^{-1}) and euthanized by spinal cord transection.

Competing interests The authors declare there are no competing interests.

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