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Autochthonous Weissella cibaria paraprobiotics mitigate inflammatory impacts induced by Aeromonas hydrophila in tambaqui

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Abstract

Probiotics can improve intestinal colonization and pathogen resistance in fish; however, their dependence on live cells limits storage and production, making paraprobiotics a promising alternative. Thus, this study evaluated the effects of dietary supplementation with *Weissella cibaria* paraprobiotics on the growth performance and immunoinflammatory responses of juvenile tambaqui, *Colossoma macropomum*. A temperature of 70°C for 10 min was sufficient to promote complete *W. cibaria* inactivation, as no bacterial growth was observed post-treatment, without compromising cell morphological integrity. In contrast, higher temperatures caused evident structural alterations, such as increased surface roughness and cell wall rupture. Fish supplemented with the MIX treatment showed enhanced innate immune responses after coelomitis induction, as evidenced by increased serum lysozyme and myeloperoxidase activities. In addition, this treatment modulated the inflammatory response induced by *Aeromonas hydrophila*, resulting in greater recruitment of lymphocytes, neutrophils, and monocytes to the inflammatory focus, lower thrombocyte consumption, reduced reactive oxygen species (ROS) production, and consequently lower oxidative and inflammatory tissue damage. Histopathological analyses showed that inflammation induced morphological alterations in hepatocytes and hepatic vascularization; however, these changes were less intense in fish supplemented with the MIX paraprobiotic. Furthermore, MIX supplementation increased intestinal villus height without improving zootechnical performance or causing adverse hepatic, hematological, or biochemical alterations, supporting the clinical safety of the paraprobiotic. Overall, dietary supplementation with the MIX paraprobiotic containing heat-inactivated *W. cibaria* strains M14 and I10 improved innate immunity and mitigated the effects of the inflammatory response on tissues and leukogram parameters in juvenile tambaqui.

1. INTRODUCTION

The rapid expansion of intensive aquaculture has elevated stress levels in farmed fish, predisposing them to infectious diseases (Castro et al., 2014). Under intensive culture conditions, the incidence and severity of bacterial diseases have increased, with enteritis and systemic infections being significant causes of mortality and economic losses in fish farming (Lee et al., 2002; Marinho Neto et al., 2019). Among bacterial pathogens, *Aeromonas hydrophila* is considered one of the main etiological agents affecting freshwater fish, especially round fish such as tambaqui, where it causes enteritis, septicemia, and mortality (Cascón et al., 2000; Claudiano et al., 2019).

In this context, antibiotics are still widely used to control bacterial outbreaks in animal production; however, their continuous and often indiscriminate use promotes the selection of resistant bacteria, the dissemination of resistance genes, and the persistence of residues in aquatic environments, with impacts on animal, environmental, and human health (Lulijwa et al., 2020; Han et al., 2020). This concern has become even more relevant due to increasing international demands for antimicrobial use control, as evidenced by the European Union's decision to remove Brazil from the list of countries authorized to export certain animal products starting in September 2026. Therefore, there is a growing need for

sustainable, preventive, and/or remedial biotechnological alternatives aligned with the One Health concept, aimed at reducing antimicrobial use in animal production.

Probiotics have been widely investigated in aquaculture because of their ability to improve growth performance, disease resistance, and immune modulation, representing an alternative to antibiotic use (Banerjee & Ray, 2017). However, studies on tambaqui have demonstrated that commercial multispecies formulations do not always provide consistent benefits to Amazonian fish species, even when considered safe or associated with improvements in water quality (Costa et al., 2021; Dos Santos et al., 2025). In addition, the clinical safety of probiotic strains must be carefully considered, as some bacteria with probiotic potential can also harbor virulence factors. In this context, autochthonous strains emerge as a promising alternative because of their superior ecological adaptation to the host. Studies conducted by our group have demonstrated that autochthonous *Weissella* spp. improved larval development and resistance against *Aeromonas hydrophila* in tambaqui (Dos Santos et al., 2025).

Despite the benefits of probiotics in aquaculture, their application presents limitations related to cell viability during feed extrusion, storage stability, and the potential risk of transferring virulence and antibiotic resistance (Choudhury & Kamilya, 2019; de Almada et al., 2016; Zucko et al., 2020). In this context, paraprobiotics have emerged as a promising alternative; they are defined as inactivated microbial cells or their structural components capable of conferring health benefits to the host (Taverniti and Guglielmetti, 2011). Cell wall components, such as peptidoglycans, teichoic acids, and polysaccharides, play crucial roles in innate immune modulation through their interactions with host pattern recognition receptors (Teame et al., 2020). Different inactivation methods, including heat, UV radiation, formalin, and sonication, can preserve biologically active cellular structures while eliminating microbial viability (Siciliano et al. 2021).

The present study investigated the effects of dietary supplementation with paraprobiotics on immunological and antioxidant parameters, resistance to inflammatory responses induced by *Aeromonas hydrophila*, intestinal integrity, and zootechnical performance.

2. MATERIALS AND METHODS

2.1. Obtaining and preparation of probiotic strains

Four *Weissella cibaria* strains were previously isolated from intestinal and fecal samples of juvenile tambaqui from the Amazon River (Santarém, Pará, Brazil) and identified using polymerase chain reaction (PCR) and 16S rRNA gene sequencing. After *in vitro* assays for probiotic potential, including bile resistance, adhesion, antagonism, and biofilm formation, the strains I10 and M14 were selected because of their superior functional performance and antagonistic activity against *Aeromonas hydrophila* (Dos Santos et al., 2025).

The selected probiotic strains were subsequently cultured in MRS broth (De Man, Rogosa, and Sharpe KASVI®) and incubated for 24 h at 37°C in a bacteriological incubator (Ethik®). After the incubation

period, the cultures were centrifuged at 3,000 rpm (1,500 × g) for 15 min at 25°C to pellet the bacterial cells. The supernatant was discarded, and the pellet was washed three times with phosphate-buffered saline (PBS), followed by resuspension using a vortex mixer (Loccus®). Subsequently, the bacterial suspension was standardized by spectrophotometry at 560 nm, adjusting the concentration to 10^8 CFU/mL before heat inactivation

2.2. Bacterial heat inactivation and scanning electron microscopy (SEM)

Bacterial heat inactivation was performed as described by Barui et al. (2023). For this purpose, 1 mL of the bacterial concentrate was transferred to a 2 mL cryogenic tube and exposed to increasing temperatures (50, 60, 70, 80, 90, and 100°C) for different periods (5, 10, 15, and 30 min) in a water bath, with all treatments performed in triplicate. After heat treatment, the viability of the bacterial strains was evaluated by culturing them in 90 × 15 mm Petri dishes containing MRS agar and incubating them at 37°C for 24 h (Dos Santos et al., 2025).

For the bacterial cell morphology analysis using SEM, the samples were fixed in Karnovsky solution (1% paraformaldehyde and 3% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2) for 24 h at room temperature. Three successive washes were performed in 0.2 M cacodylate buffer (pH 7.2) for 10 min. For post-fixation, the samples were immersed in a solution containing 1% osmium tetroxide and 1.5% potassium ferrocyanide (1:1 ratio) for 2 h. After washing in the same buffer, the samples were dehydrated in a graded ethanol series (30%, 50%, 70%, 90%, and 100%) and immersed in each solution for 10 min.

After dehydration, the samples in 100% ethanol were subjected to critical-point drying using a Leica EM CPD300 (Leica Microsystems, Wetzlar, Germany) for 1 h and 30 min. Subsequently, the samples were mounted on carbon-coated conductive tape attached to stubs and gold-coated for 3 min using a Smart Coater sputter coater (JEOL Ltd., Tokyo, Japan). Image visualization was performed using a JEOL JSM-IT500HR scanning electron microscope (JEOL Ltd., Tokyo, Japan) (Souza, 2007).

2.3. Fish, experimental design and diets

A total of 300 juvenile tambaqui, *Colossoma macropomum* (26.53 ± 5.35 g), were used in this experiment. The fish were distributed into 20 polyethylene tanks (500 L capacity; 300 L working volume) equipped with constant aeration. All experimental procedures involving fish were approved by the Ethics Committee on Animal Use of the Federal University of Western Pará (CEUA/UFOPA No. 0220250327) and conducted in accordance with the principles established by the Brazilian College of Animal Experimentation.

Fish were distributed in a completely randomized design with four treatments and five replicates. Each experimental unit consisted of 15 fish maintained at a stocking density of 50 fish/m³, with a total of 20 experimental units. The treatments were as follows: M14 – diet supplemented with paraprobiotics from strain M14, heat-inactivated at 70°C for 10 min; I10 – diet supplemented with paraprobiotics from strain

I10, heat-inactivated at 70°C for 10 min; MIX – diet supplemented with a mixture of paraprobiotics from strains M14 and I10 at a proportion of 7.5 mL/kg of feed for each strain, totaling 15 mL/kg of feed, with both strains heat-inactivated at 70°C for 10 min; and CTL – control diet without paraprobiotic supplementation.

For dietary supplementation with paraprobiotics, the concentration and dose of the bacterial suspension for both strains, as well as diet preparation, followed the protocols described by Dos Santos et al. (2025). Each suspension had a cell density of 10^8 CFU/mL, and the applied dose was 15 mL/kg feed. The supplemented diets were prepared every 15 days by adjusting each bacterial suspension to the defined concentration, spraying it onto the feed pellets, and manually homogenizing the mixture before feeding the fish. The fish were fed at a feeding rate of 4% of the biomass of each experimental unit, divided into two daily meals, during the 60-day experimental period.

2.4 Growth performance and water quality parameters

At 15-day intervals, biometric measurements were performed on a sample of 15 fish from each experimental unit to adjust the feeding rate. At the beginning and end of the experimental period, all fish from each experimental unit were weighed using an electronic balance (0.01 g precision). Before weighing, the fish were anesthetized by immersion in a eugenol solution until they exhibited loss of equilibrium to minimize stress and facilitate handling.

At the end of the experimental period, fish biometrics were performed and the following growth performance indices were evaluated: (a) weight gain (g) = final weight – initial weight; (b) daily weight gain (g day^{-1}) = weight gain / experimental period (days); (c) final biomass (g) = sum of the final weight of all fish; (d) productivity (kg m^{-3}) = final biomass (kg) / volume (m^3); and (e) survival (%) = (final number of fish / initial number of fish) × 100.

Water quality parameters, including temperature (°C), pH, and dissolved oxygen (mg L^{-1}), were measured daily in the morning using a multiparameter probe (AKSO®, model AK88). Total ammonia (mg L^{-1}) and nitrite (mg L^{-1}) concentrations were evaluated every three days by spectrophotometry following the methodologies described by Verdouw et al. (1978) and Boyd and Tucker (1992). Water renewal was performed daily by replacing 30% of the working water volume in each experimental unit to maintain water quality.

2.5 Morphophysiological analyses before inflammatory challenge

At the end of the supplementation period (day 60), blood samples were collected via caudal vein puncture (Claudiano et al., 2019). Blood samples (n = 12) destined for hematological analyses were transferred into 1.5 mL microtubes containing 2 µL of EDTA, whereas samples without anticoagulant were centrifuged at 1,200 × g for 10 min at 4°C to obtain serum for biochemical analyses.

An aliquot of whole blood was used to determine hematocrit (Ht), hemoglobin concentration (Hb), mean corpuscular volume (MCV = Ht × 10 / erythrocytes), mean corpuscular hemoglobin (MCH = Hb × 10 / erythrocytes), and mean corpuscular hemoglobin concentration (MCHC = Hb × 100 / Ht), as described by Tavares-Dias et al. (1999). Total erythrocyte counts (RBC) were determined using the indirect method described by Paiva et al. (2013). Total and differential leukocyte and thrombocyte counts were performed indirectly from blood smears stained with a rapid panoptic kit, following the methodologies described by Claudiano et al. (2019) and Tavares-Dias et al. (1999).

Serum biochemical analyses (n = 12), including glucose, cholesterol, triglycerides, total protein, albumin, and alanine aminotransferase (ALT), were performed using an automated biochemical analyzer and commercial kits (Labtest®) (Brito et al., 2015).

At the end of the experiment, proximal intestine samples (3 cm segments from the pylorus) from 10 fish per treatment were fixed in 10% buffered formalin for 24 h, transferred to 70% ethanol, and processed according to routine histological procedures. The tissues were dehydrated, cleared, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). Villus height and width were measured from the apex to the beginning of the muscular layer and at the midpoint of the villus, respectively (Melo et al., 2013).

2.6 Coelomitis induced by heat-inactivated *Aeromonas hydrophila*

The *A. hydrophila* strain was obtained from the culture collection of the Animal Health Laboratory at the Federal University of Western Pará (LARSANA/UFOPA). The strain was reactivated and cultured according to Claudiano et al. (2013). Before the challenge, the bacterial cells were heat-inactivated at 70°C for 10 min. The inflammatory response was induced by coelomic inoculation of 0.5 mL of the heat-inactivated bacterial suspension at a concentration of 3×10^9 CFU mL^{-1} (Claudiano et al., 2019). For this purpose, 12 fish from each treatment (4 fish for experimental unit) were anesthetized with benzocaine (50 mg L^{-1}; Gomes et al., 2001) until they reached the surgical anesthesia stage, according to the scale proposed by Ross and Ross (2008). Subsequently, the fish were inoculated with the bacterial suspension into the coelomic cavity using sterile needles and syringes.

Challenged animals received 0.5 mL of the bacterial inoculum, whereas the negative control group received 0.5 mL of sterile saline (0.65% NaCl) (Claudiano et al., 2019). The fish were divided into five experimental groups: negative control (CTL–), positive control (CTL+), M14, I10, and MIX. The negative control consisted of fish injected only with sterile saline solution (0.65% NaCl) and not subjected to bacterial induction. The positive control consisted of fish challenged with heat-inactivated *A. hydrophila* and fed the non-supplemented diet. Treatments M14, I10, and MIX corresponded to groups fed diets supplemented with different paraprobiotics throughout the experimental period and subsequently subjected to the bacterial challenge with *A. hydrophila*.

2.7 Immunophysiological and histopathological analyses after coelomitis induction

At 12 h post-induction of the inflammatory response, new blood samples and fragments of the intestine and liver were collected from 12 fish per treatment for hematological, histopathological, and immunological analyses.

The leukogram was evaluated as described previously in Section 2.5. Serum lysozyme activity was determined using a turbidimetric assay according to Castro et al. (2014), with modifications described by Marzocchi-Machado et al. (1999). The oxidative burst of leukocytes was assessed using the nitroblue tetrazolium (NBT) reduction assay, as described by Claudiano et al. (2019). Myeloperoxidase (MPO) activity was determined in microplates following de Mello et al. (2024), using 3,3',5,5'-tetramethylbenzidine (TMB) and H_2O_2 as substrates, with absorbance measured at 450 nm. For antioxidant analyses, liver tissue was collected 12 h post-induction of the inflammatory response, homogenized in 50 mM Tris-HCl buffer (pH 7.4), and centrifuged at 10,000 × g for 15 min at 4°C; the supernatant was stored at − 80°C until analysis. The total protein concentration was determined using the Bradford method (1976) with bovine serum albumin (BSA) as a standard, and the absorbance was measured at 595 nm. Catalase (CAT) activity was evaluated according to Aebi (1974), based on H2O2 decomposition monitored at 240 nm.

For histopathological evaluation, liver and intestinal fragments from the tambaqui supplemented with paraprobiotics and subjected to the inflammatory challenge were collected, fixed in 10% phosphate-buffered formalin for 24 h, and transferred to 70% ethanol until histological processing (N = 10). The tissues were dehydrated in a graded alcohol series, cleared in xylene, and embedded in paraffin wax. Subsequently, the paraffin blocks were sectioned using a rotary microtome to obtain 3–5 µm thick sections, which were mounted on glass slides and stained with hematoxylin and eosin (H&E) (Marinho Neto et al., 2019).

2.8 Statistical analysis

Statistical analyses were performed using R software (version 4.4.2; R Core Team, 2024). Initially, data were subjected to the Shapiro–Wilk normality test and Levene's test for homogeneity of variances. Data with normal distribution were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test ($p < 0.05$). Data that did not meet the assumptions of normality were analyzed using the non-parametric Kruskal–Wallis test, followed by Dunn's post hoc test.

3. RESULTS

3.1. Inactivation of probiotic strains

Exposure of the I10 and M14 strains to 70°C for 10 min was sufficient to promote complete bacterial inactivation, as confirmed by the absence of bacterial growth after treatment (Fig. 1A–B). In contrast,

bacterial growth was observed after exposure to lower temperatures, such as 60°C for 15 min and 50°C for 30 min, indicating incomplete inactivation under these conditions (Fig. 1C–D).

Scanning electron microscopy (SEM) showed that I10 and M14 cells inactivated at 70°C for 10 min maintained their bacillary morphology and did not exhibit evident cell wall disruption (Fig. 2C–D), compared with the non-inactivated controls (Fig. 2A–B). In contrast, exposure to 100°C caused marked morphological alterations, including increased surface roughness, cell deformation, rupture, and the presence of cellular debris (Fig. 2E–F).

3.2 Growth performance and water quality

The analysis of physicochemical water parameters showed no significant differences among treatments ($p > 0.05$) for dissolved oxygen (5.60 ± 0.23 mg L^{-1}), temperature (28.34 ± 0.13°C), total ammonia (0.36 ± 0.05 mg L^{-1}), pH (6.61 ± 0.43 to 6.80 ± 0.57), and nitrite (3.38 ± 0.24 mg L^{-1}). No significant differences were observed among treatments for the final weight, weight gain, daily weight gain, final biomass, and productivity ($p > 0.05$; Table 1). The survival rate was 100% in all treatments.

Table 1
Means ± standard deviation of zootechnical parameters in juvenile *Colossoma macropomum* supplemented with paraprobiotics containing *Weissella cibaria* in the diet during the 60-day experimental period.

Parameter	**Treatment**			
	CTL	**M14**	**I10**	**MIX**
Final weight (g)	102.27 ± 6.48 A	101.00 ± 11.10 A	89.00 ± 11.30 A	90.70 ± 12.20 A
Weight gain (g)	76.07 ± 6.40 A	73.89 ± 11.22 A	62.47 ± 11.40 A	64.07 ± 12.16 A
DWG (g/day^{-1})	1.27 ± 0.11 A	1.23 ± 0.19 A	1.04 ± 0.19 A	1.07 ± 0.20 A
Final biomass (g)	1534 ± 97.25 A	1509 ± 167.06 A	1335 ± 170.02 A	1361 ± 183.72 A
Productivity (kg/m^{-3})	5.11 ± 0.32 A	5.03 ± 0.56 A	4.45 ± 0.57 A	4.54 ± 0.61 A
Survival (%)	100 ± 0.00 A	100 ± 0.00 A	100 ± 0.00 A	100 ± 0.00 A

DWG: daily weight gain; CTL: control treatment (diet without supplementation); M14: diet supplemented with the inactivated M14 strain; I10: diet supplemented with the inactivated I10 strain; MIX: diet supplemented with the combination of the inactivated I10 and M14 strains. Values are expressed as mean ± standard deviation. Different uppercase letters within the same row indicate significant differences among treatments ($p < 0.05$).

3.3. Morphophysiological analyses

Throughout the experimental period, no clinical or behavioral alterations were observed in fish in any of the treatment groups. After 60 days of supplementation with autochthonous paraprobiotics, no

significant changes were observed in most erythrogram variables between supplemented and non-supplemented fish, except for an increase in total erythrocyte counts in the I10 treatment compared with the control group ($p < 0.05$; Table 2). Leukogram analysis of blood smears revealed lymphocytopenia and neutrophilia in fish supplemented with paraprobiotics compared to the control group ($p < 0.05$; Table 2). In addition, fish supplemented with I10 showed a significant increase in thrombocyte counts ($9.93 \pm 3.53 \times 10^4$ cells μL^{-1}) ($p < 0.05$). No significant differences were observed in the serum biochemical parameters ($p > 0.05$; Table 2).

Table 2
Means ± standard deviation of hematological and biochemical parameters of juvenile *Colossoma macropomum* supplemented with paraprobiotics derived from *Weissella cibaria* strains in the diet during the 60-day experimental period.

Parameter	Treatment			
	CTL	**M14**	**I10**	**MIX**
Hematocrit (%)	30.93 ± 2.92 A	33.07 ± 2.19 A	34.00 ± 3.11 A	32.87 ± 2.92 A
Hemoglobin (g/dL)	9.37 ± 0.55 A	10.10 ± 1.24 A	9.41 ± 0.77 A	9.52 ± 0.74 A
MCH (pg)	50.59 ± 16.25 A	46.88 ± 10.74 A	42.18 ± 14.54 A	44.83 ± 7.92 A
MCV (fL)	165.42 ± 49.74 A	153.48 ± 32.16 A	142.99 ± 68.12 A	155.10 ± 22.85 A
MCHC (g/dL)	30.50 ± 1.79 A	30.62 ± 4.03 A	27.69 ± 2.20 A	29.03 ± 1.56 A
Erythrocytes ($x10^6/mm^3$)	1.97 ± 0.43 A	2.26 ± 0.56 A	2.42 ± 0.67 A	2.16 ± 0.38 A
WBC ($\times10^4\ \mu L^{-1}$)	9.48 ± 2.39 B	10.55 ± 2.48 AB	14.88 ± 3.76 A	10.27 ± 1.85 AB
Lymphocytes ($\times10^4\ \mu L^{-1}$)	7.65 ± 2.44 A	4.35 ± 1.65 B	3.67 ± 1.53 B	4.39 ± 0.94 B
Monocytes ($\times10^3\ \mu L^{-1}$)	4.63 ± 2.82 A	4.74 ± 3.46 A	7.43 ± 3.90 A	6.72 ± 3.31 A
Neutrophils ($\times10^4\ \mu L^{-1}$)	0.20 ± 0.10 B	0.79 ± 0.52 A	0.54 ± 0.47 A	0.49 ± 0.59 A
Thrombocytes ($\times10^4\ \mu L^{-1}$)	1.15 ± 1.22 B	4.94 ± 2.64 B	9.93 ± 3.53 A	4.72 ± 2.58 B
GLIC (g/dL)	125.40 ± 21.37 A	140.00 ± 32.95 A	130.00 ± 20.84 A	143.87 ± 12.85 A
TP (g/dL)	3.81 ± 0.93 A	3.37 ± 0.76 A	3.20 ± 0.56 A	3.25 ± 0.51 A
ALB (g/dL)	0.951 ± 0.27 A	1.00 ± 0.32 A	0.766 ± 0.21 A	0.782 ± 0.16 A
COL (mg/dL)	169 ± 32.3 A	128 ± 33.1 A	140 ± 20.2 A	150 ± 55.8 A
AST (U/dL)	106 ± 28.8 A	96.3 ± 22.9 A	124 ± 54.3 A	101 ± 16.2 A

MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; WBC: White Blood Cells; GLIC, glucose; TP, total proteins; ALB, albumin; COL, cholesterol; AST, aspartate aminotransferase. CTL: control treatment (diet without supplementation); M14: diet supplemented with the inactivated M14 strain; I10: diet supplemented with the inactivated I10 strain; MIX: diet supplemented with the combination of the inactivated I10 and M14 strains. Different uppercase letters within the same row indicate significant differences among treatments ($p < 0.05$).

Intestinal morphometric parameters showed significant differences ($p < 0.05$) in villus height and width between the groups (Table 3; Fig. 3C–D). Fish from the I10 treatment showed lower villus height (153 ±

49.2 µm) and width (70.8 ± 17.3 µm) than those in the CTL group. Histopathological evaluation of the liver and intestinal tissues (Fig. 3C–D) revealed no significant alterations among the analyzed groups.

Table 3
Means ± standard deviation of villus height and width in the intestine of juvenile *Colossoma macropomum* during the 60-day supplementation period with paraprobiotics derived from *Weissella cibaria*

Parameter	Treatment			
	CTL	**M14**	**I10**	**MIX**
Height (µm)	209 ± 60.2 A	247 ± 53.8 A	153 ± 49.2 B	207 ± 30.2 A
Width (µm)	97.7 ± 25.8 A	80.9 ± 18.1 AB	70.8 ± 17.3 B	94.7 ± 25.1 AB

CTL: control treatment (diet without supplementation); M14: diet supplemented with the inactivated M14 strain; I10: diet supplemented with the inactivated I10 strain; MIX: diet supplemented with the combination of the inactivated I10 and M14 strains. Values are expressed as mean ± standard deviation. Different uppercase letters within the same row indicate significant differences among treatments ($p < 0.05$).

3.4 Immunological responses and inflammatory challenge

Paraprobiotic supplementation improved the innate immunity of the supplemented fish (Fig. 4). Tambaqui supplemented with MIX and M14 strain showed increased serum lysozyme concentrations compared to the other treatments ($p < 0.05$; Fig. 4A). Regarding the concentration of reactive oxygen species (ROS) in blood leukocyte granules, a reduction was observed in the supplemented groups compared to the control groups ($p < 0.05$; Fig. 4B). Myeloperoxidase (MPO) activity was higher in fish supplemented with MIX and I10 than in those in the other treatments ($p < 0.05$; Fig. 4C). No significant differences were observed in catalase activity or total hepatic protein levels among the treatments ($p > 0.05$; Fig. 4D–E).

After induction of the inflammatory response, the fish hemogram showed significant differences, with lymphopenia and neutropenia in fish supplemented with M14, as well as monocytopenia in the M14 and MIX treatments compared with the positive control group ($p < 0.05$; Table 4). In addition, fish supplemented with M14 and MIX showed lower thrombocyte consumption compared with the positive control group (Table 4).

Table 4
Means ± standard deviation of the hematological parameters of juvenile *Colossoma macropomum* supplemented with *Weissella cibaria* paraprobiotics, 12 hours after sepsis induction by *Aeromonas hydrophila*.

Parameter	Treatment				
	CONTROL -	**CONTROL +**	**M14**	**I10**	**MIX**
Hematocrit (%)	27.25 ± 3.67 A	25.25 ± 3.14 A	26.09 ± 5.11 A	30.00 ± 4.52 A	28.17 ± 1.85 A
Hemoglobin (g/dL)	8.51 ± 0.63 A	8.41 ± 0.84 A	8.45 ± 0.91 A	8.47 ± 1.07 A	8.56 ± 0.474A
Erythrocytes ($x10^5/mm^3$)	1.98 ± 0.55 A	1.84 ± 0.41 A	1.69 ± 0.37 A	1.87 ± 0.34 A	1.77 ± 0.36 A
MCH (pg)	46.07 ± 12.61 A	47.38 ± 16.25 A	49.83 ± 8.64 A	47.08 ± 15.52 A	50.10 ± 9.32 A
MCV (fL)	147.67 ± 41.99 A	142.44 ± 30.53 A	159.84 ± 28.11 A	152.8 ± 33.28 A	164.99 ± 31.35 A
MCHC (g/dL)	31.66 ± 4.09 A	33.53 ± 3.99 B	31.26 ± 1.42 AB	28.44 ± 3.64 A	30.43 ± 1.59 A
WBC ($\times10^4$ μL^{-1})	9.89 ± 2.73 A	9.19 ± 2.03 A	8.47 ± 1.83 A	9.36 ± 1.69 A	8.93 ± 1.86 A
Lymphocytes ($\times10^4$ μL^{-1})	0.71 ± 0.29 B	1.65 ± 0.62 A	0.75 ± 0.37 B	1.91 ± 0.89 A	1.52 ± 0.81 A
Monocytes ($\times10^4$ μL^{-1})	0.9 ± 0.43 B	2.01 ± 2.82 A	0.63 ± 0.33 B	1.75 ± 0.87 A	0.79 ± 0.35 B
Neutrophils ($\times10^4$ μL^{-1})	0.83 ± 0.40 B	1.37 ± 0.6 A	0.79 ± 0.56 B	1.18 ± 0.69 A	1.58 ± 0.49 A
Thrombocytes ($\times10^4$ μL^{-1})	7.45 ± 2.35 A	4.16 ± 1.14 C	6.32 ± 1.32 A	4.53 ± 1.28 C	5.04 ± 1.17 A

MCH: mean corpuscular hemoglobin; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; WBC: white blood cells. CONTROL −: negative control; CONTROL +: positive control; M14: diet supplemented with the inactivated M14 strain; I10: diet supplemented with the inactivated I10 strain; MIX: diet supplemented with the combination of the inactivated I10 and M14 strains. Values are expressed as mean ± standard deviation. Different uppercase letters within the same row indicate significant differences among treatments ($p < 0.05$).

Histopathological evaluation revealed cytoplasmic vacuolization, hyperemia, and pyknotic nuclei in the liver tissue (Fig. 3A–B), with lower levels of tissue alteration observed after MIX treatment.

4. DISCUSSION

Our data demonstrated that the preparation of *Weissella cibaria* as a paraprobiotic is feasible and capable of influencing biological responses in tambaqui by modulating the inflammatory response and innate immunity, corroborating findings reported in other aquatic organisms, including fish and crustaceans (Barui et al., 2024; Neissi et al., 2024).

Paraprobiotic production requires controlled inactivation, as excessive processing may compromise cellular integrity (Barros et al., 2021; Barui et al., 2024). Among the available methods, thermal inactivation is preferred because of its simplicity and applicability under both laboratory and industrial conditions (Taverniti and Guglielmetti, 2011; Sionek and Gantner, 2025). In this context, the present study demonstrated that this treatment could eliminate bacterial viability without destroying cellular structures. Indeed, *Weissella cibaria* was completely inactivated after exposure to 70°C for 10 min, while maintaining a smooth and intact cell surface comparable to that of non-inactivated bacteria. These findings indicate that, despite the loss of viability, the cells preserved their external structural conformations. Similar results have been reported for *Lactobacillus paracasei* ET-22 and *Bifidobacterium lactis* BL-99 after heat treatment at 70°C (Sun et al., 2023), reinforcing that this temperature is sufficient to eliminate the viability of different bacterial strains.

Temperatures above 70°C promoted a loss of viability in *Weissella* spp.; however, they also caused evident morphological alterations, including cell wall roughness, rupture, and the presence of cellular debris. These findings corroborate the observations reported for *Bacillus amyloliquefaciens* COFCAU_P1 at 110°C for 10 min (Barui et al., 2024) and *Lactobacillus acidophilus* LA-5 at 95°C for 5 and 7 min (Barros et al., 2021).

Coelomitis induction using heat-inactivated *A. hydrophila* demonstrated that paraprobiotic supplementation modulated the innate immune response of tambaqui, as evidenced by increased serum lysozyme activity, enhanced myeloperoxidase (MPO) activity, and increased diapedesis of monocytes and lymphocytes toward the inflammatory focus. These effects may be related to the recognition of paraprobiotic cellular components, such as peptidoglycans, teichoic acids, polysaccharides, and surface proteins, by pattern recognition receptors (PRRs), particularly Toll-like receptors (TLRs). This interaction activates innate immune effector mechanisms involving lysozyme, leukocytes, and MPO (Shenderov, 2013; Nataraj et al., 2020; Chen et al., 2024; Zhang et al., 2024) and stimulates plasma exudation and leukocyte recruitment to inflamed tissues (Claudiano et al., 2013). Furthermore, the greater increase in MPO activity observed in the MIX treatment may be associated with complementary interactions between strains, as bacterial combinations can activate multiple immune pathways simultaneously (Nayak, 2010; Ringø et al., 2020). Increased lysozyme and MPO activities suggest a greater microbicidal and antimicrobial defense potential in supplemented fish, corroborating the findings reported in *Channa striata* and *Oreochromis niloticus* supplemented with heat-inactivated *Lactobacillus plantarum* L-137 (Hien et al., 2021; Van Nguyen et al., 2019).

Respiratory burst corresponds to the rapid production of reactive oxygen species (ROS) by phagocytic leukocytes and represents one of the first cellular responses against infectious agents (Nayak, 2010). In

the present study, higher ROS production was observed in the positive control group, whereas the supplemented treatments presented lower values, suggesting a modulatory effect of paraprobiotics on the inflammatory response. Although ROS are crucial for pathogen destruction, their excessive production may cause oxidative tissue damage and has been associated with high mortality rates in teleosts with A. *hydrophila*-induced sepsis (Claudiano et al., 2019). Similarly, Untsa et al. (2024) reported lower respiratory burst activity in *Osphronemus goramy* supplemented with probiotics after a challenge with *A. hydrophila*, suggesting that supplemented animals may better control infection, thereby reducing the need for intense oxidative cellular activation. This modulation of oxidative responses may explain the lower severity of hepatic lesions observed in the supplemented fish, characterized by reduced cytoplasmic vacuolization, hyperemia, and pyknotic nuclei during the inflammatory process (Supplementary Table 1).

Despite the beneficial effects observed in the inflammatory response, paraprobiotic supplementation did not promote significant differences in growth performance parameters, including final weight, weight gain, daily weight gain (DWG), final biomass, productivity, and survival ($p > 0.05$). These findings indicate that supplementation with heat-inactivated strains did not interfere with the growth of juvenile *C. macropomum* throughout the experimental period and maintained a performance similar to that of the control group. The absence of effects on growth performance has also been observed in previous studies involving autochthonous *Weissella* spp. probiotics in tambaqui juveniles (unpublished data) and corroborates the findings reported for Salmo salar supplemented with heat-inactivated *Lactiplantibacillus plantarum* L-137 and Solea senegalensis supplemented for 45 days with heat-inactivated *Shewanella putrefaciens* Pdp11 (Domínguez-Maqueda et al., 2021; Rocha et al., 2023).

Although small hematological differences were observed between supplemented tambaqui and the control group before the induction of inflammation with *A. hydrophila*, no clinical, behavioral, serum biochemical, or histopathological alterations were observed in the liver or intestine, thus demonstrating the clinical safety of the paraprobiotic preparation. Similar findings have been reported in other teleost species supplemented with paraprobiotics, reinforcing the low toxicity and safety of these preparations for aquaculture applications (Nofouzi et al., 2019; Barui et al., 2024; Dos Santos et al., 2025).

5. CONCLUSION

Dietary supplementation with the MIX paraprobiotic, composed of heat-inactivated autochthonous *Weissella cibaria* strains M14 and I10, improved innate immune responses and attenuated the inflammatory effects induced by *Aeromonas hydrophila* in juvenile tambaqui. This response was characterized by increased serum lysozyme and MPO activity, modulation of leukocyte recruitment, reduced excessive oxidative response, and lower hepatic histopathological damage. Although no improvement in zootechnical performance was observed, the absence of clinical, behavioral, biochemical, and histopathological alterations under basal conditions indicates the clinical safety of the preparation. Therefore, *W. cibaria* paraprobiotics, especially the MIX treatment, represent a promising prophylactic strategy to improve inflammatory resilience and fish health in tambaqui aquaculture.

Declarations

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Author contributions: Jonny B. Teixeira: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review and editing. Investigation. **Larissa A. F. Sampaio:** Methodology, Investigation, Visualization, Writing – review and editing. **Layana A. B. Pereira:** Methodology, Investigation, Visualization, Writing – review and editing. **Laine P. C. dos Santos:** Investigation, Visualization, Writing – review and editing. **Andrya L. Lopes:** Investigation. **Juliana N. Ferreira**: Investigation, Visualization. **Igor B. A. de Barros:** Investigation, Visualization, Visualization, Writing – review and editing. **Raimundo de Jesus T. Diniz:** Investigation. **Laura G. M. de Carvalho:** Investigation. **Humberto R. da Costa Neto:** Investigation. **Gleika T. J. dos Reis:** Investigation. **Camilly M. S. dos Santos:** Investigation. **Manoel P. M. Júnior:** Investigation. **Michelle M. S. Fugimura:** Project administration, Resources. **Luciano J. Vaz:** Investigation, Resources. **Gustavo S. Claudiano:** Supervision, Project administration, Funding acquisition, Conceptualization, Methodology, Formal analysis, Data curation, Validation, Resources, Writing – review and editing.

Data availability: The data that support the fundings of this study are available from the corresponding author upon reasonable request.

Animal rights and ethical Note: All procedures involving animals were conducted in accordance with the ethical principles for animal experimentation established by the Brazilian College of Animal Experimentation and were approved by the Ethics Committee on Animal Use of the Federal University of Western Pará (UFOPA; protocol No. 0220250327).

Consent for publication: Not applicable.

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Figures

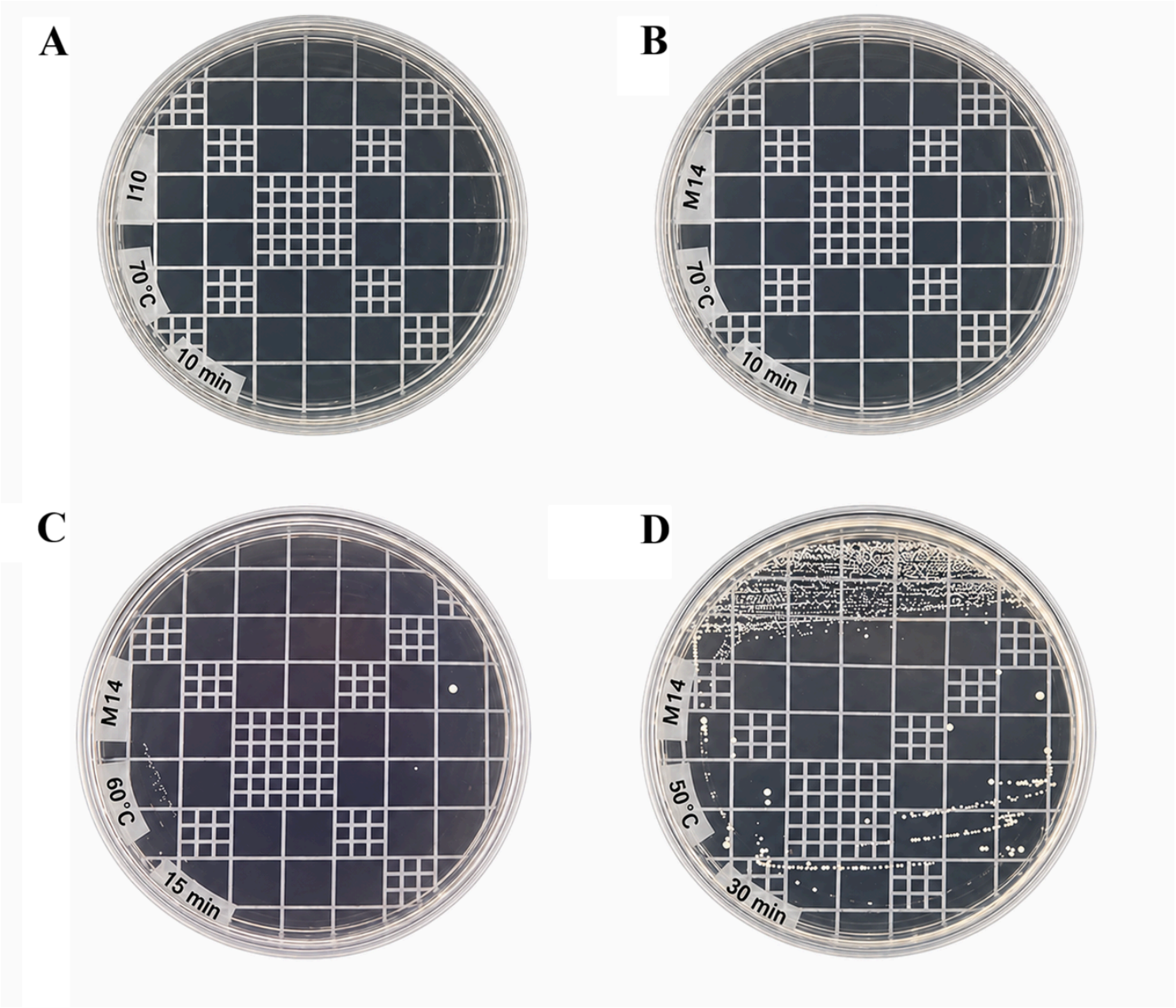


Figure 1

Viability of autochthonous *Weissella cibaria* strains after heat treatment at different temperatures and exposure times. Absence of bacterial growth was observed after treatment at 70 °C for 10 min in strains I10 (A) and M14 (B), indicating complete inactivation. Bacterial growth was observed after exposure of strain M14 to 60 °C for 15 min (C) and 50 °C for 30 min (D), indicating incomplete inactivation under these conditions.

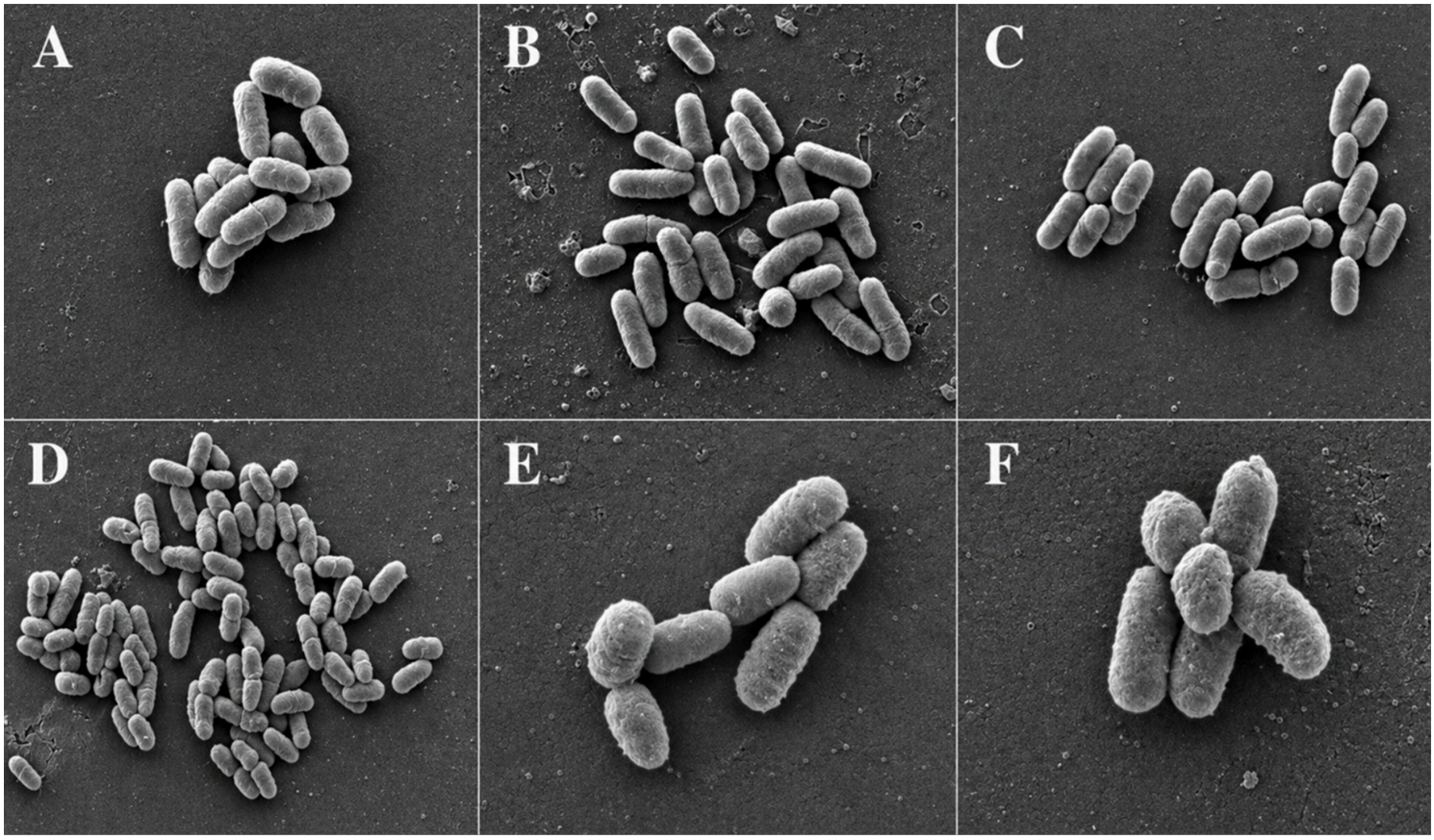


Figure 2

Scanning electron microscopy (SEM) of autochthonous *Weissella cibaria* strains subjected to heat treatment. (A) Non-inactivated M14 control; (B) non-inactivated I10 control; (C) M14 strain inactivated at 70 °C for 10 min; (D) I10 strain inactivated at 70 °C for 10 min; (E) M14 strain exposed to 100 °C for 10 min; and (F) I10 strain exposed to 100 °C for 10 min. Images show preservation of cellular morphology after treatment at 70 °C and marked structural damage after exposure to 100 °C.

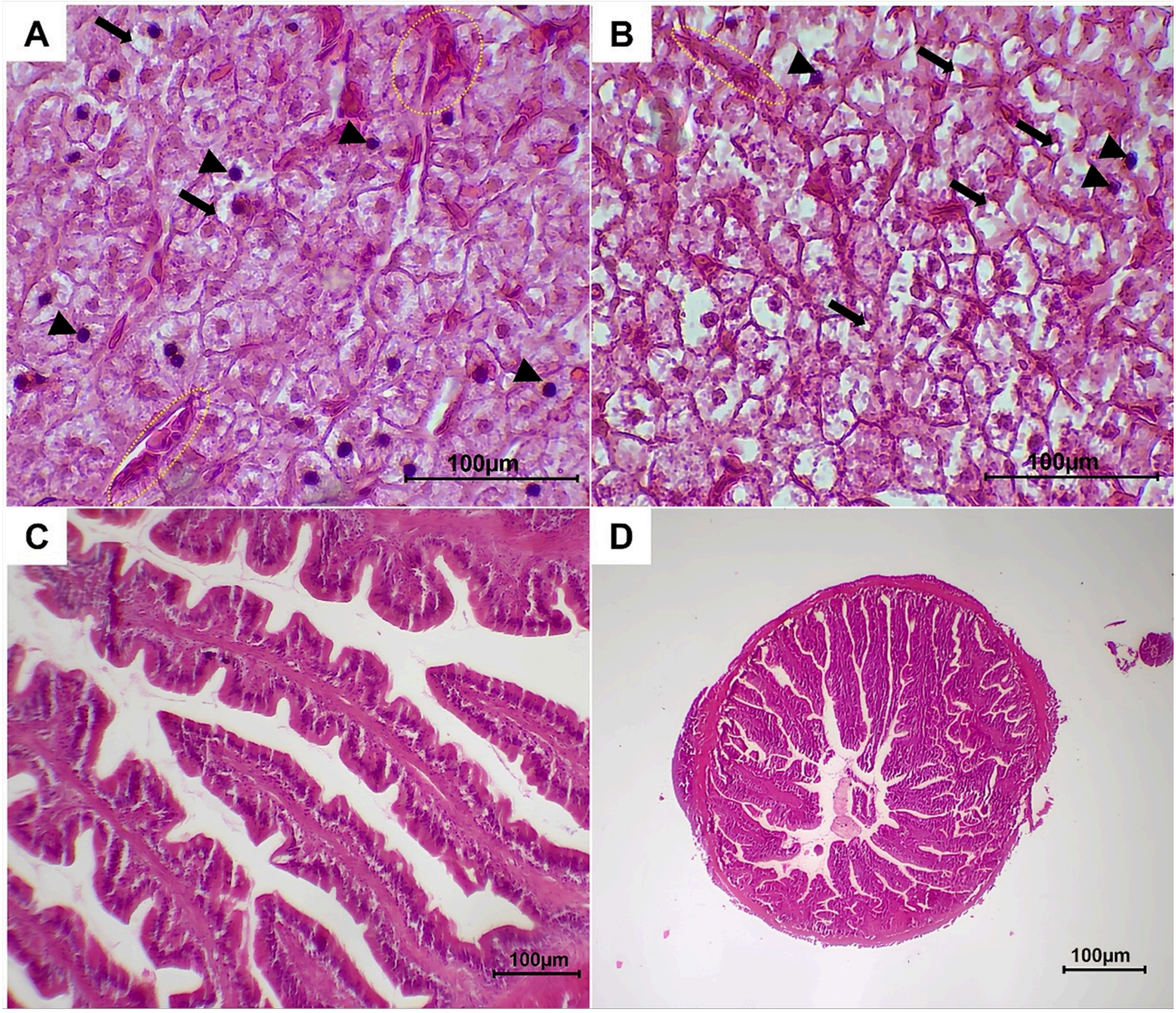


Figure 3

Histopathological and histomorphological evaluation of the liver and intestine in juvenile *Colossoma macropomum* supplemented with *Weissella cibaria* paraprobiotics for 60 days. (A) Liver of fish supplemented with the inactivated I10 strain before inflammation, showing hepatocytes with cytoplasmic vacuolization (arrows). (B) Liver of fish supplemented with the inactivated M14 strain after coelomitis induction, showing pyknotic nuclei (arrowheads) and hyperemia (yellow dotted circles). (C) Intestine of fish from the control treatment, diet without supplementation, showing intestinal villi in transverse section. (D) Fish intestine supplemented with the inactivated M14 strain, showing intestinal villi in longitudinal section. H&E staining. Scale bar = 100 µm.

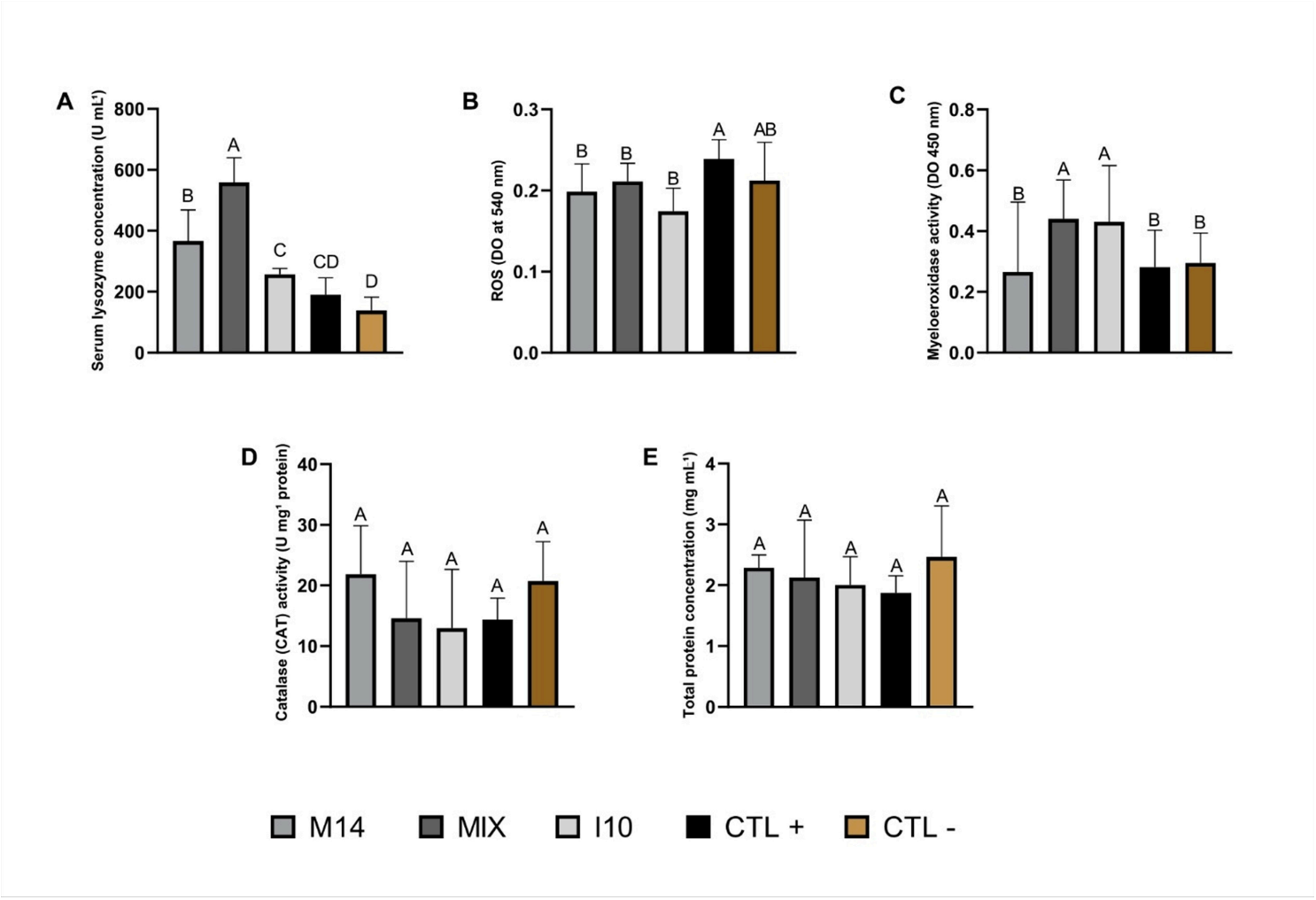


Figure 4

Mean ± standard deviation of serum lysozyme concentration (A), reactive oxygen species (ROS) production (B), myeloperoxidase activity (C), catalase activity (CAT) (D), and total protein concentration (E) in juvenile *Colossoma macropomum* supplemented with paraprobiotics derived from *Weissella cibaria* and challenged with *Aeromonas hydrophila*. M14: diet supplemented with the inactivated M14 strain; MIX: diet supplemented with the combination of the I10 and M14 strains; I10: diet supplemented with the inactivated I10 strain; Control +: positive control; Control −: negative control. Different uppercase letters indicate significant differences among treatments ($p < 0.05$).