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Autochthonous Weissella cibaria from Colossoma macropomum gut improves immunophysiological responses during aeromonosis

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Abstract

Antibiotic use in aquaculture poses increasing risks to environmental and public health, highlighting the need for effective alternatives for disease control. This study investigated whether dietary supplementation with autochthonous *Weissella cibaria* modulates host immunophysiological responses and influences the response to *Aeromonas hydrophila* infection in tambaqui (*Colossoma macropomum*). Juveniles were fed for 90 days with two probiotic strains at different feeding frequencies and subsequently subjected to experimental challenge. Probiotic supplementation did not affect growth performance or intestinal morphometry, but improved body composition, particularly in fish receiving strain I10, which showed higher protein deposition and lower carbohydrate levels. Under basal conditions, supplementation increased leukocyte populations and antioxidant activity without inducing clinical alterations, indicating enhanced immunophysiological readiness and physiological stability. Following A. hydrophila challenge, supplemented fish showed modulation of innate immune responses and improved hematological and biochemical profiles associated with septic status, although no significant differences in mortality were observed. Intestinal microbiota analyses revealed that community structure was driven mainly by infection rather than by probiotic supplementation, with a shift from the dominance of Fusobacteria and *Cetobacterium somerae* to a higher relative abundance of Proteobacteria and Enterobacteriaceae-related taxa. Notably, probiotic supplementation did not induce major taxonomic restructuring of the microbiota. These findings indicate that autochthonous W. cibaria acts primarily through host immunophysiological modulation rather than broad microbiota restructuring, supporting its potential application as a functional and sustainable strategy for health management in tropical aquaculture.

Introduction

Aquaculture is currently regarded as one of the fastest-growing food production sectors worldwide [1]. In Latin America, freshwater species of major aquaculture importance, including tambaqui, tilapia, and trout, occupy a strategic position, especially in Brazil, which is increasingly consolidating its role as a leading producer in the sector [1,2]. Nevertheless, the expansion of aquaculture production remains constrained by significant sanitary challenges, among which bacterial diseases are particularly relevant. In this context, species belonging to the genus *Aeromonas* are among the most impactful bacterial pathogens in fish farming, with *Aeromonas hydrophila* widely recognized as one of the primary etiological agents associated with septicemia outbreaks in cultured fish [3–5].

The widespread occurrence of aeromonosis in aquaculture has led to the intensification of antibiotic use, contributing to the emergence and dissemination of antibiotic-resistant bacteria, with detrimental consequences for aquatic ecosystems, animal health, and public health [6]. Consequently, probiotics have been increasingly investigated as functional feed additives in fish farming due to their potential to enhance digestive efficiency, host physiology, immune responses, and disease resistance [7–10]. Autochthonous probiotic strains represent a promising strategy, as host-derived microorganisms are

posited to be better adapted to the intestinal environment and more capable of interacting with the resident microbiota [8].

Among lactic acid bacteria, the probiotic potential of *Weissella* spp. has only recently been explored, showing promising results in both humans and animals [11]. The functional versatility of *Weissella* extends beyond simple colonization, encompassing significant contributions to host physiology through direct bacterial effects and indirect metabolite-mediated mechanisms. For instance, the immunomodulatory potential of *W. cibaria* JW15 has been shown to enhance natural killer cell activity in humans [12]. Recent studies have also revealed novel applications for *Weissella*-derived products, such as bacterial ghosts generated through sodium hydroxide treatment, which effectively stimulate macrophage activation and upregulate the expression of pro-inflammatory cytokines (IL-1β, TNF-α, and iNOS), suggesting their potential as immunotherapeutic agents [13].

These beneficial characteristics have positioned *Weissella* as a strong candidate for probiotic and biotechnological applications, particularly in fish, where positive effects on performance, survival after *Aeromonas* challenge, and immune responses have been reported in tambaqui larvae [8]. Given that intestinal colonization may be strongly influenced by host genotype and gut microbiome composition [14], strains specifically selected for the target population may exhibit superior colonization performance. Accordingly, *Weissella* spp. appear to be well adapted to tambaqui, as they have been isolated from both larvae and adult fish from the Amazon River [8].

However, the clinical relevance of *Weissella* is nuanced by reports of opportunistic pathogenicity, with *W. confusa* identified as a causative agent of neonatal sepsis in veterinary medicine [15]. This dual nature underscores the complexity of host–microbe interactions and highlights the need for rigorous strain-level characterization [11]. Despite substantial progress in *Weissella* research, several critical knowledge gaps remain, including challenges in accurate strain identification, variability in probiotic efficacy, and an incomplete understanding of the molecular mechanisms underlying host–microbe interactions in adult fish under septic conditions. Thus, the present study aimed to evaluate the effects of dietary supplementation with autochthonous *W. cibaria* strains on the physiological and immunological parameters, intestinal microbiota structure, and the response of *Colossoma macropomum* to *A. hydrophila* challenge.

Results

Growth performance, intestinal histomorphometry, proximate composition hematological, biochemical parameters, innate immunity and antioxidants.

After 90 days of supplementation, no significant differences ($p > 0.05$) were observed among treatments for weight gain, biomass gain, specific growth rate, or productivity (Table 1). No mortality was recorded in any of the groups during the experimental period.

Table 1

Mean ± standard deviation of zootechnical parameters of juvenile *Colossoma macropomum* supplemented with diets containing probiotic *Weissella cibaria* strains for 90 days. WG, weight gain; FCR, feed conversion ratio; SGR, specific growth rate; PROD, productivity; SUR, survival. CTL, control; M14-3F, diet supplemented with strain M14 and provided three times daily; M14-1F, diet supplemented with strain M14 and provided once daily; I10-3F, diet supplemented with strain I10 and provided three times daily; and I10-1F, diet supplemented with strain I10 and provided once daily. Different letters within columns indicate significant differences according to Tukey's test ($p < 0.05$).

Parameter	**Treatment**				
	CTL	**M14-3F**	**M14-1F**	**I10-3F**	**I10-1F**
WG (g)	135.88 ± 9.39 A	131.76 ± 24.48 A	112.55 ± 14.09 A	133.37 ± 31.64 A	127.43 ± 26.80 A
Biomass Gain (Kg)	2.04 ± 0.14 A	1.98 ± 0.37 A	1.69 ± 0.21 A	2.00 ± 0.47 A	1.91 ± 0.40 A
FCR	0.95 ± 0.04 A	0.97 ± 0.08 A	1.06 ± 0.04 A	0.96 ± 0.19 A	1.03 ± 0.12 A
SGR (%/day)	2.66 ± 0.10 A	2.69 ± 0.16 A	2.51 ± 0.14 A	2.66 ± 0.24 A	2.64 ± 0.22 A
PROD (Kg/m³)	6.80 ± 0.47 A	6.59 ± 1.22 A	5.63 ± 0.70 A	6.67 ± 1.58 A	6.37 ± 1.34 A
SUR (%)	100 ± 0.00 A	100 ± 0.00 A	100 ± 0.00 A	100 ± 0.00 A	100 ± 0.00 A

Histological analysis of the intestinal villi showed that the lamina propria was composed of loose connective tissue containing lymphocytes, eosinophilic granular cells, rodlet cells, and macrophages, in addition to smooth muscle fibers, lymphatic vessels, and blood capillaries. In all groups, no signs of inflammatory process were observed, such as marked inflammatory infiltrate or cellular and circulatory alterations, indicating preservation of the intestinal architecture (Fig. 1A and B). Regarding histomorphometry, the villus height in the control group did not differ significantly from that observed in the supplemented groups ($p > 0.05$) (Fig. 1C). Likewise, villus width in probiotic-supplemented tambaqui did not differ significantly from the control group (Fig. 1D).

No significant differences were observed among treatments for moisture, ash, or lipid contents ($p > 0.05$; Table 2). Regarding dry protein content, fish supplemented with strain I10, provided once or three times daily, showed the highest values, which differed significantly from those of the control, M14-3F and M14-1F groups (Table 2). As for wet protein content, the I10-1F group showed a significantly higher value than the M14-3F group, did not differ from the control group. In contrast, carbohydrate content was significantly reduced in the groups supplemented with strain I10 compared with the control group and those supplemented with strain M14 (Table 2).

Table 2

Mean ± standard deviation of the proximate composition of juvenile *Colossoma macropomum* supplemented with diets containing probiotic *Weissella cibaria* strains for 90 days.

Parameter	Treatment				
	CTL	**M14-3F**	**M14-1F**	**I10-3F**	**I10-1F**
Moisture (%)	77.58 ± 1.18 A	78.09 ± 1.09 A	77.82 ± 2.29 A	77.83 ± 1.80 A	78.03 ± 1.49 A
Ash (%)	1.31 ± 0.13 A	1.23 ± 0.26 A	1.24 ± 0.28 A	1.25 ± 0.21 A	1.20 ± 0.47 A
Dry-basis lipid (%)	2.47 ± 0.80 A	2.97 ± 1.46 A	3.09 ± 1.06 A	2.75 ± 0.76 A	2.67 ± 0.75 A
Wet-basis lipid (%)	0.56 ± 0.20 A	0.70 ± 0.41 A	0.70 ± 0.28 A	0.61 ± 0.20 A	0.59 ± 0.19 A
Dry-basis protein(%)	77.68 ± 6.54 C	72.06 ± 4.24 C	75.56 ± 5.14 C	81.31 ± 5.01 A	82.89 ± 3.58 A
Wet-basis protein(%)	17.35 ± 1.48 AB	15.99 ± 1.12 B	16.70 ± 1.59 AB	18.07 ± 1.91 AB	18.20 ± 1.39 A
Carbohydrates (%)	3.21 ± 1.54 AB	3.99 ± 0.79 A	3.54 ± 1.04 AB	2.26 ± 1.08 C	1.98 ± 0.64 C

CTL, control; M14-3F, diet supplemented with strain M14 and provided three times daily; M14-1F, diet supplemented with strain M14 and provided once daily; I10-3F, diet supplemented with strain I10 and provided three times daily; and I10-1F, diet supplemented with strain I10 and provided once daily. Different letters within columns indicate significant differences among groups according to Tukey's and Dunn's tests (*) ($p < 0.05$).

Regarding red blood cell indices, fish from the M14-3F group showed higher hemoglobin concentration than those from the control, I10-3F, and I10-1F groups ($p < 0.05$; Table 3), did not differ from the M14-1F group. Mean corpuscular hemoglobin (MCH) was higher in the M14-3F group than in the I10-1F group, with no significant differences from the other groups (Table 3). In the evaluation of blood smears, lymphocytes, monocytes, granulocytes, and thrombocytes were identified, whereas basophils and eosinophils were not observed. Leukocytosis was observed in the I10-1F group compared with the control group ($p < 0.05$; Table 3). The number of monocytes was higher in the I10-3F group than in the control and M14-3F groups ($p < 0.05$). Lymphocytosis was also observed in the I10-1F group compared with the control and M14-3F groups ($p < 0.05$). Regarding granulocytes, the I10-1F group showed a higher cell count, differing significantly from the control, M14-3F, and I10-3F groups ($p < 0.05$), but did not differ from the M14-1F group (Table 3).

Table 3

Means ± standard deviation of hematological and biochemical parameters of juvenile *Colossoma macropomum* supplemented with feed containing probiotic strains of *Weissella cibaria* for 90 days.

Parameter	Treatment				
	CTL	**M14-3F**	**M14-1F**	**I10-3F**	**I10-1F**
Hematocrit (%)	30.60 ± 2.88 A	34.10 ± 2.47 A	34.20 ± 4.94 A	32.30 ± 2.75 A	31.50 ± 3.37 A
Hemoglobin (g/dL)	8.38 ± 0.57 B	9.01 ± 0.47 A	8.90 ± 0.66 AB	8.32 ± 0.44 BC	7.93 ± 0.41 B
MCH (pg)	45.91 ± 21.43 AB	54.98 ± 16.10 A	54.74 ± 13.02 AB	46.60 ± 16.56 AB	45.42 ± 10.91 B
MCHC (g/dL)	27.53 ± 2.33 A	26.49 ± 1.53 A	26.49 ± 4.00 A	25.89 ± 2.20 A	25.12 ± 2.84 A
MCV (fL)	169.30 ± 81.81 A	208.23 ± 59.17 A	212.24 ± 66.54 A	180.84 ± 59.90 A	181.54 ± 21.88 A
Erythrocytes ($x10^6/mm^3$)	1.43 ± 0.425 A	1.44 ± 0.301 A	1.70 ± 0.358 A	1.61 ± 0.419 A	1.81 ± 0.350 A
WBC ($x10^4\ \mu L^{-1}$)	7.14 ± 2.13 B	7.20 ± 1.50 AB	8.49 ± 1.79 AB	8.05 ± 2.09 AB	9.06 ± 1.75 A
Lymphocytes ($x10^4\ \mu L^{-1}$)	0.87 ± 0.80 B	0.47 ± 0.40 B	1.55 ± 1.05 AB	1.42 ± 0.86 AB	2.07 ± 1.25 A
Thrombocytes ($x10^4\ \mu L^{-1}$)	5.85 ± 2.80 A	6.11 ± 2.51 A	6.77 ± 1.42 A	6.34 ± 1.98 A	6.70 ± 1.93 A
Monocytes ($x10^3\ \mu L^{-1}$)	0.09 ± 0.27 B	0.04 ± 0.12 B	0.35 ± 0.46 AB	1.61 ± 1.70 A	0.37 ± 0.51 AB
Granulocytes ($x10^3\ \mu L^{-1}$)	0.29 ± 0.40 C	0.17 ± 0.35 C	1.36 ± 0.96 AB	1.34 ± 1.69 BC	2.51 ± 1.93 A
GLIC (mg/dL)	103.24 ± 22.42 A	115.10 ± 35.18 A	90.78 ± 14.49 A	99.73 ± 25.01 A	104.03 ± 49.69 A
COL (mg/dL)	111.74 ± 14.94 A	95.68 ± 10.19 A	126.70 ± 18.25 A	121.22 ± 42.29 A	109.08 ± 15.23 A

MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; WBC: White Blood Cells; GLIC, glucose; COL, cholesterol; TRIG, triglycerides; ALB, albumin; TP, total proteins; A:G, albumin:globulin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. CTL, control; M14-3F, diet supplemented with strain M14 and provided three times daily; M14-1F, diet supplemented with strain M14 and provided once daily; I10-3F, diet supplemented with strain I10 and provided three times daily; and I10-1F, diet supplemented with strain I10 and provided once daily. Different letters within columns indicate significant differences among groups according to Tukey's and Dunn's tests ($p < 0.05$).

Parameter	Treatment				
	CTL	M14-3F	M14-1F	I10-3F	I10-1F
TRIG (mg/dL)	339.36 ± 75.26 A	295.08 ± 68.75 A	422.28 ± 114.39 A	366.18 ± 153.63 A	371.65 ± 102.60 A
ALB (g/dL)	1.14 ± 0.48 A	0.87 ± 0.08 A	0.93 ± 0.07 A	0.93 ± 0.12 A	0.85 ± 0.14 A
TP (g/dL)	3.46 ± 0.93 A	2.87 ± 0.38 A	3.49 ± 0.32 A	3.15 ± 0.49 A	3.03 ± 0.50 A
Globulins (g/dL)	2.32 ± 0.47 A	2.00 ± 0.31 A	2.56 ± 0.29 A	2.22 ± 0.39 A	2.18 ± 0.44 A
A:G Ratio (g/dL)	0.48 ± 0.10 A	0.44 ± 0.05 A	0.37 ± 0.05 A	0.43 ± 0.05 A	0.40 ± 0.08 A
AST (U/dL)	123.38 ± 24.61 A	119.95 ± 18.08 A	162.88 ± 27.27 A	157.54 ± 45.80 A	138.23 ± 37.83 A
ALT (U/dL)	6.96 ± 2.37 A	6.70 ± 1.35 A	10.60 ± 3.44 A	7.24 ± 3.43 A	8.08 ± 3.25 A
ALP (U/dL)	17.96 ± 3.56 A	16.53 ± 5.46 A	22.96 ± 9.33 A	14.18 ± 5.75 A	13.38 ± 3.78 A

MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; WBC: White Blood Cells; GLIC, glucose; COL, cholesterol; TRIG, triglycerides; ALB, albumin; TP, total proteins; A:G, albumin:globulin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. CTL, control; M14-3F, diet supplemented with strain M14 and provided three times daily; M14-1F, diet supplemented with strain M14 and provided once daily; I10-3F, diet supplemented with strain I10 and provided three times daily; and I10-1F, diet supplemented with strain I10 and provided once daily. Different letters within columns indicate significant differences among groups according to Tukey's and Dunn's tests ($p < 0.05$).

No significant differences were observed among treatments for the evaluated serum biochemical parameters, including glucose (GLIC), cholesterol (COL), triglycerides (TRIG), albumin (ALB), total protein (TP), globulins, albumin:globulin ratio (A:G), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) ($p > 0.05$; Table 3).

A significant reduction ($p < 0.05$) in total hepatic protein levels was observed in the groups supplemented with Weissella cibaria strains compared with the control group (Fig. 2A). Hepatic catalase activity was significantly higher in fish supplemented with probiotics than in the control group ($p < 0.05$; Fig. 2B). No significant differences were observed among groups in hepatic superoxide dismutase (SOD) activity ($p > 0.05$; Fig. 2C).

No significant differences were observed in natural antibody titers between probiotic-supplemented groups and the control group ($p > 0.05$; Fig. 2D). Regarding serum lysozyme concentration, all supplemented groups showed lower values than the control group ($p < 0.05$), with the lowest concentrations observed in the groups supplemented with strain I10, especially I10-1F (Fig. 2E). For

serum myeloperoxidase activity, a significant reduction was observed in the groups supplemented with strain I10 compared with the control group ($p < 0.05$; Fig. 2F).

Aeromonose: hemogram, blood biochemistry, innate immunity and evaluation of clinical signs and survival

Infection was confirmed by positive blood culture in all fish inoculated with Aeromonas hydrophila. The hematological and serum biochemical changes resulting from infection are presented in Table 4. In the erythrogram, a significant reduction in erythrocyte count and hematocrit was observed in the control group compared with the supplemented and challenged groups ($p < 0.05$). Among the hematological indices, a reduction in MCV was observed in the groups supplemented with strain I10 compared with the control and M14-1F groups, whereas MCH was lower in the M14-3F, I10-3F, and I10-1F groups than in the control group. No significant differences were observed for total hemoglobin or MCHC ($p > 0.05$; Table 4). In the leukogram, leukocytosis was observed in the supplemented groups compared with the control group ($p < 0.05$), with no significant differences among the treated groups. No significant differences were detected for lymphocytes or monocytes. Regarding granulocytes, the M14-1F group showed a lower cell count than the M14-3F and I10-3F groups, did not differ from the control and I10-1F groups. For thrombocytes, thrombocytopenia was observed in the control group compared with the supplemented groups ($p < 0.05$), whereas the M14-1F group showed lower values than those observed in the I10-3F group (Table 4).

Table 4

Means ± standard deviation of hematological and biochemical parameters of juvenile *Colossoma macropomum* supplemented with feed containing probiotic strains of *Weissella cibaria* for 90 days and challenged with *Aeromonas hydrophila*.

Parameter	Treatment				
	CTL	**M14-3F**	**M14-1F**	**I10-3F**	**I10-1F**
Hemoglobin (g/dL)	8.00 ± 0.81 A	8.18 ± 0.96 A	8.32 ± 1.38 A	8.14 ± 0.84 A	8.29 ± 0.99 A
Hematocrit (%)	37.80 ± 8.95 B	46.20 ± 8.51 A	44.40 ± 8.86 A	41.70 ± 6.53 A	39.40 ± 2.99 A
Erythrocytes ($x10^6/mm^3$)	1.18 ± 0.13 B	1.81 ± 0.34 A	1.50 ± 0.30 A	1.77 ± 0.43 A	2.12 ± 0.42 A
MCH (μm^3)	324.20 ± 84.92 A	266.05 ± 76.83 AB	310.55 ± 94.05 A	251.36 ± 77.42 B	191.07 ± 33.96 B
MCH (pg)	68.86 ± 11.52 A	46.99 ± 11.84 B	58.47 ± 18.63 AB	50.63 ± 22.41 B	40.92 ± 11.14 B
MCHC (%)	21.85 ± 3.98 A	18.40 ± 4.58 A	19.47 ± 5.46 A	20.03 ± 3.91 A	21.22 ± 3.49 A
WBC ($x10^3\ \mu L^{-1}$)	6.80 ± 13.4 B	14.10 ± 4.9 A	9.90 ± 4.8 A	11.80 ± 6.7 A	10.80 ± 7.0 A
Lymphocytes ($x10^3\ \mu L^{-1}$)	5.06 ± 9.31 A	6.82 ± 3.30 A	4.74 ± 5.26 A	5.66 ± 4.89 A	7.71 ± 9.66 A
Monocytes ($x10^3\ \mu L^{-1}$)	3.44 ± 4.62 A	1.42 ± 1.68 A	1.58 ± 1.92 A	2.48 ± 2.83 A	1.08 ± 1.66 A
Granulocytes ($x10^3\ \mu L^{-1}$)	2.92 ± 2.05 AB	4.16 ± 2.50 A	1.31 ± 1.37 B	2.28 ± 1.97 A	3.00 ± 3.01 AB
Thrombocytes ($x10^4\ \mu L^{-1}$)	4.15 ± 0.66 C	7.80 ± 1.74 AB	6.71 ± 1.25 B	7.81 ± 2.02 AB	9.45 ± 1.90 A
GLIC (mg/dL)	88.42 ± 20.01 A	85.58 ± 22.61 A	85.03 ± 21.02 A	89.80 ± 10.34 A	104.79 ± 12.83 A

MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC: White Blood Cells; GLIC, glucose; COL, cholesterol; TRIG, triglycerides; ALB, albumin; TP, total proteins; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. CTL, control; M14-3F, diet supplemented with strain M14 and provided three times daily; M14-1F, diet supplemented with strain M14 and provided once daily; I10-3F, diet supplemented with strain I10 and provided three times daily; and I10-1F, diet supplemented with strain I10 and provided once daily. Different letters within columns indicate significant differences among groups according to Tukey's and Dunn's tests ($p < 0.05$).

Parameter	Treatment				
	CTL	M14-3F	M14-1F	I10-3F	I10-1F
COL (mg/dL)	124.77 ± 11.61 A	132.76 ± 25.39 A	119.95 ± 23.27 A	124.52 ± 35.63 A	123.29 ± 23.05 A
TRIG (mg/dL)	153.39 ± 12.22 B	209.90 ± 20.55 A	181.11 ± 35.73 A	187.47 ± 33.50 A	199.81 ± 38.48 A
ALB (g/dL)	0.94 ± 0.09 A	0.93 ± 0.05 A	0.93 ± 0.09 A	0.92 ± 0.13 A	0.84 ± 0.08 A
TP (g/dL)	3.13 ± 0.39 A	3.09 ± 0.23 A	3.21 ± 0.33 A	2.99 ± 0.42 A	3.06 ± 0.49 A
Globulins (g/dL)	2.19 ± 0.37 A	2.15 ± 0.26 A	2.27 ± 0.29 A	2.02 ± 0.28 A	2.08 ± 0.35 A
AST (U/dL)	181.53 ± 77.24 A	174.06 ± 50.02 AB	123.04 ± 55.71 B	179.98 ± 67.89 AB	123.31 ± 30.07 B
ALT (U/dL)	9.18 ± 4.39 A	9.75 ± 2.41 A	8.29 ± 1.36 AB	8.05 ± 3.44 AB	6.53 ± 1.50 B
ALP (U/dL)	37.50 ± 10.89 A	26.84 ± 9.00 A	25.29 ± 18.24 A	27.10 ± 10.63 A	40.60 ± 21.73 A

MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC: White Blood Cells; GLIC, glucose; COL, cholesterol; TRIG, triglycerides; ALB, albumin; TP, total proteins; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. CTL, control; M14-3F, diet supplemented with strain M14 and provided three times daily; M14-1F, diet supplemented with strain M14 and provided once daily; I10-3F, diet supplemented with strain I10 and provided three times daily; and I10-1F, diet supplemented with strain I10 and provided once daily. Different letters within columns indicate significant differences among groups according to Tukey's and Dunn's tests ($p < 0.05$).

Regarding serum biochemical parameters, the supplemented groups showed significantly higher triglyceride concentrations than the control group ($p < 0.05$). Reduced serum concentrations of AST and ALT were also observed in supplemented fish compared with the control, with statistically significant differences for AST in the M14-1F and I10-1F groups, and for ALT in the I10-1F group. No significant differences were detected for glucose, cholesterol, albumin, total protein, globulins, or alkaline phosphatase between the supplemented groups and the control (Table 4).

The analysis of innate immune parameters in *A. hydrophila*-induced sepsis is presented in Fig. 3. Quantification of reactive oxygen species (ROS) produced by blood leukocytes showed no significant differences among treatments and the control group ($p > 0.05$; Fig. 3A). The agglutination capacity of natural antibodies against *A. hydrophila* showed significantly lower serum titers in the control group than in supplemented fish ($p < 0.05$; Fig. 3B). A similar pattern was observed for serum lysozyme concentration and myeloperoxidase activity, which were significantly higher in the supplemented groups compared with the control group ($p < 0.05$; Fig. 3C and D)

After the experimental challenge with *A. hydrophila*, mortality was observed in all groups. The control and M14-1F groups showed the lowest survival rates (71.4%) at the end of the observation period, whereas the M14-3F, I10-1F, and I10-3F groups showed a final survival of 85.7% (Fig. 4). However, Kaplan–Meier survival analysis, with curves compared by the log-rank (Mantel–Cox) test, showed no significant differences among treatments ($p = 0.9025$). Clinical signs of infection included external hemorrhages, mainly in the gills. Petechiae, fin erosion, and erythrodermatitis were observed in all groups. Fish from the control group showed a higher frequency of ocular ulceration, exophthalmia, and abdominal distension than the supplemented groups. Macroscopic evaluation of internal organs revealed that non-supplemented fish exhibited more flaccid organs, less intact kidneys, and greenish livers, whereas supplemented fish showed livers with a color closer to dark red.

Intestinal microbiota profile before and after *Aeromonas hydrophila* **challenge**

No significant differences were observed in alpha diversity indices among treatments, either before or after the experimental challenge, at both the species and phylum levels, indicating that supplementation with Weissella cibaria strains did not promote detectable changes in the overall diversity of the intestinal microbiota (Supplementary Tables S1 and S2; Supplementary Figures S1–S9).

In contrast, beta diversity analysis based on Bray–Curtis distance revealed a significant effect of the experimental challenge on the structure of the intestinal microbial community. At the species level, the challenge explained 56.47% of the variation observed among samples (PERMANOVA, $F = 35.43$; $R^2 = 0.5647$; $p = 0.001$), with no significant effect of probiotic supplementation ($R^2 = 0.0566$; $p = 0.511$) or of the probiotic-by-challenge interaction ($R^2 = 0.0600$; $p = 0.480$). At the phylum level, the challenge explained 84.02% of the total variation (PERMANOVA, $F = 126.53$; $R^2 = 0.8402$; $p = 0.001$), also with no significant effect of treatment ($R^2 = 0.0142$; $p = 0.679$) or of the treatment-by-challenge interaction ($R^2 = 0.0127$; $p = 0.724$) (Supplementary Tables S3 and S4). PCoA ordination supported these results, showing a clear separation between pre- and post-challenge samples along the primary axis (Fig. 5A).

Analysis of average relative abundance showed that, before the challenge, the intestinal microbiota displayed a relatively homogeneous profile among groups, with a predominance of *Cetobacterium somerae* at the species level and Fusobacteria at the phylum level. After challenge with *A. hydrophila*, a marked reduction in the relative abundance of *C. somerae* and Fusobacteria was observed, accompanied by an increase in taxa mainly associated with the family Enterobacteriaceae, including *Enterobacter, Citrobacter, and Cronobacter*, as well as an increase in Proteobacteria at the phylum level (Fig. 5B and C). Despite this compositional shift after challenge, no consistent structural differences were observed among treatments within each experimental period. Taken together, these results indicate that the infectious challenge was the main factor modulating the intestinal microbiota, whereas probiotic supplementation did not promote detectable global restructuring of the microbial community.

This study demonstrated that supplementation with autochthonous *W. cibaria* strains generated measurable benefits in juvenile tambaqui, including improved protein deposition, reduced muscle carbohydrate content, modulation of leukocyte and thrombocyte profiles, enhancement of innate

immune and antioxidant responses, and mitigation of septic-associated hematological, biochemical, and organ alterations after *A. hydrophila* challenge. Although no broad taxonomic restructuring of the intestinal microbiota was detected, probiotic supplementation clearly improved host functional responses and physiological stability. By contrast, infection was the dominant factor driving intestinal dysbiosis, reducing taxa associated with microbial homeostasis and reshaping community composition. These results show that probiotic efficacy in teleosts extends beyond microbiota composition alone and should also be defined by its capacity to improve host immune competence and resilience under infectious challenge. Therefore, autochthonous *W. cibaria* represents a strong prophylactic candidate for sustainable health management and reduced antimicrobial use in tropical aquaculture.

Discussion

In the present study, dietary supplementation with autochthonous probiotic strains primarily promoted host immunophysiological modulation in juvenile tambaqui (*Colossoma macropomum*), with limited effects on the overall taxonomic structure of the intestinal microbiota under basal conditions. These findings reinforce the potential of *W. cibaria* to support intestinal health through functional modulation of host physiology rather than broad restructuring of microbial communities. Furthermore, supplementation was associated with enhanced immune responsiveness and positive shifts in muscle proximate composition, indicating relevant metabolic and physiological effects.

Weissella spp. have previously demonstrated probiotic potential in in vitro screening assays and in vivo experiments with tambaqui larvae, where improved growth performance and immune responses against *A. hydrophila* challenge were observed [8]. However, in the present study with juveniles, no significant changes were detected in growth performance or intestinal histomorphometry. These findings suggest that, although the strains exert clear immunomodulatory effects, their inclusion does not compromise growth, productive efficiency, or intestinal integrity, characterizing a physiological maintenance effect. Similar outcomes have been reported for multistrain synbiotics [16], isolated probiotics [17] and commercial multistrain probiotics [18], reinforcing that these responses are strain-specific and dependent on host, dosage, and supplementation conditions.

Despite the absence of changes in growth performance and intestinal histomorphometry, *W. cibaria* supplementation exerted measurable physiological effects dependent on strain and feeding frequency. The intestinal microbiota remained globally stable across treatments prior to the *Aeromonas* challenge, with no significant differences in alpha diversity or overall community structure. This indicates that supplementation did not promote broad taxonomic restructuring of the intestinal microbiota in juvenile *C. macropomum*. The maintenance of a relatively homogeneous microbial profile, together with improvements in host physiological parameters, supports the hypothesis that probiotic benefits arise primarily from host-mediated functional modulation rather than global microbial remodeling. This observation is consistent with previous reports highlighting the context-dependent and strain-specific nature of probiotic effects in fish [19,20].

Fish supplemented with strain I10, particularly in the I10-1F treatment, exhibited higher protein content, suggesting improved nutrient utilization and protein deposition. Similar findings have been reported in *Mugil capito* [21]. *Pagrus major* [22] and *Oreochromis niloticus* [7]. Additionally, the reduction in muscle carbohydrate content suggests alterations in energy metabolism, possibly reflecting increased mobilization or utilization of glycogen reserves. This effect may be associated with the metabolic versatility described for *Weissella* spp. [23,24] Collectively, these results suggest that probiotic supplementation can influence nutrient partitioning and metabolic efficiency, even in the absence of detectable changes in growth performance.

Hematological modulation was primarily observed in cellular components, with increased leukocyte counts, particularly lymphocytes, monocytes, and granulocytes. This effect was more pronounced in fish supplemented with strain I10, suggesting enhanced immune readiness. This response may be associated with interactions between probiotic bacteria and the intestinal epithelium and gut-associated lymphoid tissue (GALT), promoting activation of immune cells and enhancing phagocytic capacity [25]. The observed leukocyte increase likely reflects enhanced immune surveillance rather than an active inflammatory process. Similar responses have been reported in tambaqui and other species receiving probiotic supplementation [26,27].

The absence of significant changes in serum biochemical parameters indicates that *W. cibaria* supplementation did not induce systemic metabolic imbalance under the evaluated conditions, supporting the clinical safety of the tested strains. This is particularly relevant given that *Weissella* species may exhibit opportunistic pathogenicity under certain conditions [11] and is consistent with findings from previous studies using multistrain probiotics in tambaqui [18].

Lower basal lysozyme and myeloperoxidase activities, together with reduced hepatic protein levels, suggest attenuation of immune activation in the absence of bacterial infection, reflecting a homeostatic physiological state. This profile is consistent with a homeostatic state, characterized by controlled immune activity and reduced energetic expenditure associated with inflammatory processes [3,28,29]. This supports the hypothesis that probiotic supplementation promotes immunometabolic stability, allowing energy to be preferentially allocated to anabolic processes such as protein deposition [11,30–32].

The increase in catalase activity observed in supplemented fish further supports a beneficial modulation of antioxidant defenses. Catalase plays a key role in mitigating oxidative stress by decomposing reactive oxygen species [33], and its upregulation suggests enhanced protection against oxidative damage. Similar effects have been reported in probiotic-supplemented fish, where increased antioxidant enzyme activity contributes to improved cellular stability [11,34,35].

Following intracoelomic challenge with *A. hydrophila*, fish developed clinical and physiological alterations consistent with aeromonosis [3,4]. However, probiotic-supplemented fish exhibited reduced organ impairment and milder clinical alterations, suggesting attenuation of disease severity. Although no

significant differences in mortality were observed, the improved physiological condition of supplemented fish indicates enhanced disease resilience.

Fish in the control group showed reduced hematocrit and erythrocyte counts, consistent with typical hematological alterations observed during systemic bacterial infections [36–39]. In contrast, supplemented fish maintained more stable hematological profiles, indicating improved physiological resistance to infection. This is in agreement with previous studies demonstrating that probiotic supplementation can mitigate hematological disturbances associated with bacterial infections [26,40–43].

Supplementation with *W. cibaria* also modulated leukocyte and thrombocyte responses during infection, indicating controlled immune activation. While the control group exhibited leukocytosis indicative of exacerbated inflammatory response, supplemented fish showed a more regulated profile, supporting the immunomodulatory capacity of these strains [11,30]. The preservation of thrombocyte levels in supplemented fish further suggests improved hemostatic balance, reducing the risk of hemorrhagic complications commonly associated with systemic infections [3,44].

Lower AST and ALT levels in supplemented fish indicate reduced hepatic damage, suggesting a hepatoprotective effect of the probiotic strains. This, together with increased triglyceride levels, supports the hypothesis of reduced oxidative stress and improved metabolic stability during infection [45].

The absence of increased leukocyte respiratory activity suggests that probiotic supplementation did not induce excessive reactive oxygen species production, reinforcing the concept of controlled immune activation. At the same time, increased lysozyme activity, myeloperoxidase activity, and bacterial agglutination capacity indicate enhanced humoral and cellular defense mechanisms, contributing to improved pathogen neutralization [46–49].

Microbiota analyses revealed that the infectious challenge was the primary driver of intestinal community restructuring, overriding the effects of probiotic supplementation. Before infection, the microbiota was dominated by *Cetobacterium somerae*, a characteristic taxon in freshwater fish [50]. Following infection, a shift toward increased Proteobacteria and Enterobacteriaceae-related taxa was observed, consistent with dysbiosis associated with pathogenic invasion [51,52]. The limited detection of *A. hydrophila* in intestinal samples, despite positive blood culture in all challenged animals, supports the invasive behavior of this pathogen and its rapid dissemination to internal organs, consistent with the systemic septic condition observed in the present study [3,53].

The absence of persistent *W. cibaria* detection and the stability of alpha diversity suggest transient colonization, a common feature in probiotic studies [54,55]. Importantly, the observed physiological and immunological benefits, even in the absence of stable colonization, indicate that probiotic effects are mediated primarily through functional interactions with the host rather than permanent microbiota integration [56]. These findings highlight that probiotic efficacy in tambaqui should be evaluated based on host functional responses rather than solely on taxonomic changes in the microbiota.

Methods

Probiotic bacteria

In a previous study conducted by the Aquatic Animal Health Research Group (GESAQUI) at the Federal University of Western Pará (UFOPA), bacterial strains were isolated from the gastrointestinal tract of juvenile tambaqui captured in the Amazon River in Santarém, Pará, Brazil (2°26'35.02" S, 54°42'29.99" W). 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. 0620180030). Four strains were initially screened *in vitro* for viability and probiotic potential through auto-aggregation, co-aggregation, biofilm formation, hydrophobicity, antibiotic susceptibility, antagonism against pathogens, and tolerance to varying pH levels and bile salts.

Based on these assays, strains I10 (isolated from the intestine) and M14 (isolated from a mixture of intestine and feces) were selected for *in vivo* experiments due to their superior resistance, adhesion capacity, and antagonistic activity against *A. hydrophila*, as well as their ability to significantly improve survival, performance, water quality parameters, and innate immune responses in tambaqui larvae. For taxonomic characterization, genomic DNA was extracted and subjected to PCR amplification with *Lactobacillus*- and *Bacillus*-specific primers, followed by agarose gel electrophoresis and 16S rRNA gene sequencing, allowing the initial identification of the isolates as *Weissella* spp. [8].

The taxonomic identification of strains I10 and M14 was subsequently refined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using the MALDI Biotyper system, which confirmed the identity of both isolates as *Weissella cibaria* with log (score) values higher than 2.3.

Fish, experimental design, and diets

A total of 300 juvenile tambaqui (*C. macropomum*) (13.1 ± 0.8 g) were used in the experiment. The fish were acclimatized for 15 days in a 3,000 L tank and fed twice daily with a commercial diet containing 32% crude protein, at 5% of biomass. Thereafter, the fish were randomly distributed into 300 L polyethylene boxes at a stocking density of 50 fish m^{-3} (n = 15). All experimental procedures involving the fish were approved by the Ethics Committee on Animal Use of UFOPA (CEUA No. 0620180030) and were conducted in accordance with the principles established by the Brazilian College of Animal Experimentation.

Fish were assigned to a completely randomized design (CRD) and fed for 90 days, three times daily, with either a control or a probiotic-supplemented diet (32% crude protein; 4–6 mm pellet size) at a feeding rate of 3–6% of biomass. The treatments consisted of: control (CT), receiving only the unsupplemented diet; M14-3F and I10-3F, receiving diets supplemented with the respective strains at all three daily

feedings; and M14-1F and I10-1F, receiving the supplemented diet at only one of the three daily feedings and the control diet at the others. Each treatment was performed in quadruplicate (N = 20 experimental units). Feed allowance was adjusted every 15 days based on biometric measurements (n = 8 fish per unit) [18].

The strains were cultured in MRS broth (24 h; 37°C), centrifuged, and resuspended in a 0.65% saline solution to obtain a standardized suspension ($3x10^9$ CFU mL^{-1}). Supplementation was performed by adding 15 mL of the bacterial suspension per kg of feed, with diets prepared every three days. The suspensions were sprayed onto the feed, manually homogenized, and coated with vegetable oil at 3% (v/w) to ensure adhesion and uniform distribution. After application, the feed rested for 30 min for drying and was then stored at 4°C until use [8].

Water temperature (27.52 ± 0.44°C), pH (7.07 ± 0.38), and dissolved oxygen (6.41 ± 0.61 mg) were monitored daily using a multiparameter probe (YSI® 63). The experimental units were siphoned daily for waste removal, followed by a 20% water exchange with pre-treated water. Throughout the experiment, these parameters remained stable and within the recommended comfort range for *C. macropomum* [57,58].

Growth performance and survival

Growth performance was evaluated using the following indices: (a) weight gain (g) = final weight − initial weight; (b) biomass gain (kg) = final biomass − initial biomass; (c) apparent feed conversion ratio (FCR) = total feed provided (kg) / total biomass gain (kg); (d) specific growth rate (SGR, % day^{-1}) = [(ln final weight − ln initial weight) / experimental period (days)] x 100; (e) productivity (kg m^{-3}) = final biomass (kg) / volume (m^3); and (f) survival (%) = (final number of fish / initial number of fish) x 100.

Intestinal villus histomorphometry and proximate composition

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 [7].

Simultaneously, muscle samples (n = 10) were collected for proximate composition analysis according to the [59] except for either extract, which was determined according to [60]. Moisture (AOAC 920.151), ash (AOAC 940.26), and protein (Kjeldahl; AOAC 920.87) contents were quantified. Carbohydrates were calculated by subtracting the sum of protein, lipids, ash, and moisture from 100. All analyses were performed in triplicate and expressed as a percentage [7].

Immunophysiological analyses

At the end of the experiment, blood samples were collected by caudal vein puncture. Following deep anesthesia, liver tissue samples were collected (n = 10 per group) [4]. Blood was transferred to 1.5 mL microcentrifuge tubes containing 2 µL of EDTA for hematological analyses, whereas samples without anticoagulant were centrifuged (1,200 × g, 10 min, 4°C) to obtain serum for biochemical and immunological assays. To determine antioxidant enzyme activity, liver tissue was pooled by treatment, homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 0.15 M KCl and 100 µM PMSF, and centrifuged at 9,000 × g for 30 min at 4°C. The resulting supernatant was collected and transferred to cryovials. Serum samples and liver homogenates were stored at − 80°C until analysis.

Blood counts and serum biochemical assessments

A whole-blood aliquot was used to determine hematocrit (Ht), hemoglobin concentration (Hb), mean corpuscular volume (MCV = Ht x 10 / RBC), mean corpuscular hemoglobin (MCH = Hb x 10 / RBC), and mean corpuscular hemoglobin concentration (MCHC = Hb x 100 / Ht) [61]. Erythrocyte counts (RBC) were determined using the indirect method [61]. Leukocyte and thrombocyte counts were determined indirectly from blood smears stained with a rapid panoptic kit [3,61].

Serum biochemical analyses (glucose, cholesterol, triglycerides, total protein, albumin, globulin, AST, ALT, and alkaline phosphatase) were performed using an automated biochemical analyzer with commercial kits (Labtest®); globulin levels were calculated by the difference between total protein and albumin (TP − ALB) [62].

Innate immune analyses

Serum lysozyme concentration was determined by a turbidimetric assay [63; 64]. For the bacterial agglutination assay, the equivalence point in the dose–response relationship between bacterial CFU and serum volume was previously established in U-bottom microtiter plates and a dose of 1.8 x 10^9 CFU was defined for the analysis [3]. Serum bacterial agglutination activity was determined in all groups using 50 µL of serially diluted serum samples. The total antibody titer was defined as the highest serum dilution showing positive agglutination, and the results were expressed as the $\log_2$ of the reciprocal of the serum titer [65].

Serum myeloperoxidase (MPO) activity was evaluated in 96-well microplates using 25 µL serum aliquots diluted in 125 µL of HBSS without Ca^{2+} and Mg^{2+}, with 20 µL of 3,3',5,5'-tetramethylbenzidine (TMB, 20 mM; Sigma®) and 50 µL of 30% H_2O_2 (5 mM) as substrates. After 2 min of incubation at room temperature, the reaction was stopped by adding 50 µL of H_2SO_4 (4 M), and the absorbance was measured at 450 nm. Activity was expressed as µmol of H_2O_2 reduced per µL of serum [66].

Antioxidant analyses

For the antioxidant enzymatic activity analysis, pooled liver tissue from each treatment (n = 10) was used. Total protein concentration was determined by the Bradford method using a commercial reagent (Scienco Biotech®) and bovine serum albumin (BSA) as the standard. The homogenate was diluted 1:3 in 10x PBS, and 50 µL aliquots were added to 2.5 mL of Bradford reagent. After homogenization and a 5-min incubation in the dark, absorbance was measured at 595 nm.

Catalase (CAT) activity was determined according to [67], based on the decrease in absorbance at 240 nm during H_2O_2 decomposition. Aliquots of 20 µL of liver homogenate were added to 980 µL of H_2O_2 solution, and the reaction was monitored kinetically for 60 s. Activity was expressed as U mg^{-1} of protein, where one unit (U) was defined as the amount of enzyme required to decompose 1 µmol of H_2O_2 per minute.

Superoxide dismutase (SOD) activity was measured in liver homogenates using a commercial colorimetric kit (Thermo Fisher Scientific®, Cat. No. EIASODC), following the manufacturer's instructions. Samples were diluted 1:10, and activity was quantified based on the inhibition of the formation of a chromogenic compound, with absorbance read at 450 nm after a 20-min incubation at room temperature. Activity was expressed as U mL^{-1}, based on a standard curve (0–92 U mL^{-1}) and corrected by the dilution factor.

Gut microbiota metagenomic analysis

For intestinal microbiota analysis, fish were fasted for 24 h, and nine individuals per treatment were euthanized by deep anesthesia with benzocaine (1:20,000). After surface disinfection with 70% ethanol, the distal portion of the intestine, including the associated digesta, was collected under aseptic conditions and stored in sterile buffer until processing.

The microbial community profile was determined by sequencing the V3–V4 hypervariable region of the bacterial 16S rRNA gene using primers 341F and 806R, following a two-step PCR protocol with dual indexing. Amplicon libraries were purified, quantified, normalized, and sequenced on an Illumina MiSeq platform using 2 × 300 bp paired-end chemistry, with a minimum depth of 100,000 reads per sample.

Bioinformatic analyses were performed using the Sentinel pipeline. Sequence quality was assessed, and primers and low-quality reads (Phred < 20) were removed. Paired-end reads were merged, and low-abundance OTUs/ASVs were excluded according to the filtering criteria implemented in the Sentinel pipeline. Taxonomic assignment was performed by BLAST-based alignment against reference 16S rRNA databases [68,69], considering sequence similarity, e-value, and taxonomic consistency.

Aeromonose

At the end of the experimental period, 85 tambaqui (125.73 ± 9.39 g; n = 17 per treatment) were used to evaluate the effects of *Weissella* strain supplementation during *Aeromonas hydrophila*-induced sepsis (CEUA No. 0620180030). For this purpose, infection was induced by intracelomic inoculation of the LD_{50} in previously anesthetized fish assigned to the experimental treatments described previously. At 24 h

post-challenge (HPC), the animals were euthanized by deepening the anesthetic plane with benzocaine [70]. Peripheral blood samples were collected by caudal vessel puncture (N = 10), and serum was obtained by centrifugation (1,200 x g, 5 min, 4°C) and stored at − 80°C until analysis.

Aeromonas hydrophila and median lethal dose (LD_{50}–$_{96\ h}$)

The *A. hydrophila* strain used in the infectious challenge was previously isolated from fish showing clinical signs of aeromonosis [4]. Identification was performed by culture on tryptic soy agar (TSA; Difco®), followed by morphological, staining, and biochemical characterization (Bactray 3 – Laborclin®). Molecular confirmation was achieved via PCR and genomic sequencing [3]. The isolate was confirmed as *A. hydrophila* with 99% similarity (criterion ≥ 98%).

The median lethal dose (LD_{50}–$_{96\ h}$) of *A. hydrophila* was determined in 36 juvenile tambaqui distributed into six groups (n = 6). Fish received an intracoelomic injection (0.5 mL) of increasing bacterial concentrations (0.15 x 10^9, 0.6 x 10^9, 1.2 x 10^9, 2.4 x 10^9, and 3.0 x 10^9 CFU mL^{-1}). Mortality was recorded every 24 hours over a 96-h period. The control group received the same volume of sterile saline solution (0.65% NaCl). The LD_{50} was estimated using the Trimmed Spearman–Karber method, resulting in 1.78 x 10^9 CFU mL^{-1} (95% confidence interval: 1.64–2.30 x 10^9) [3].

Clinical, immunological, and metagenomic analyses

At 24 HPC, ten fish per treatment were used for the analyses described in this section. Confirmation of *A. hydrophila* infection was performed by blood culture; briefly, 100 µL of blood was inoculated onto TSA medium (Difco®) and incubated at 30°C for 24 h. Bacterial growth was assessed visually [3]. Hematological and serum biochemical analyses were performed according to the protocols described previously.

Respiratory burst activity was determined in blood collected from the caudal vein using a turbidimetric assay with nitroblue tetrazolium (NBT) to quantify reactive oxygen species (ROS) produced by leukocytes [3,64]. Serum lysozyme concentration, myeloperoxidase (MPO) activity, and bacterial agglutination activity were determined according to the protocols described previously. Metagenomic analysis of the intestinal microbiota after sepsis induction followed the protocol described previously.

Evaluation of clinical signs and survival analysis

Thirty-five tambaqui were used to evaluate clinical signs and survival. The fish were divided into two groups: one inoculated with 0.5 mL of *A. hydrophila* suspension (1.78 x 10^9 CFU mL^{-1}) and a control group injected with the same volume of sterile saline (0.65% NaCl). Clinical signs and mortality were monitored by direct observation for 5 days following the induction of sepsis [3].

Statistical analysis

Statistical analyses were performed using R software (version 4.4.2; R Core Team, 2024). Data were first subjected to the Shapiro–Wilk normality test and Cochran's test for homogeneity of variances. Normally distributed data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test ($p < 0.05$). Data that did not meet normality assumptions were analyzed using the non-parametric Kruskal–Wallis test, followed by Dunn's post hoc test. Survival after the infectious challenge was analyzed using the Kaplan–Meier method, and survival curves were compared using the log-rank (Mantel–Cox) test. For intestinal microbiota analysis, alpha diversity was estimated using Shannon and Simpson indices at the species and phylum levels. Beta diversity was calculated based on Bray–Curtis dissimilarity and visualized via principal coordinates analysis (PCoA). Differences in community structure were tested by permutational multivariate analysis of variance (PERMANOVA; 999 permutations), and homogeneity of dispersion was assessed by PERMDISP. In all analyses, differences were considered significant at $p < 0.05$.

Declarations

Additional information

The authors declare that they have no competing interests.

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Author Contribution

L. A. F. S.: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review and editing. L. A. B. P.: Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review and editing. L. P. C. S.: Investigation. A. L. L.: Investigation. J. B. T.: Investigation. J. N. F.: Investigation, Visualization. I. B. A. B.: Investigation, Visualization. G. T. J. R.: Investigation. M. M. S. F.: Project administration. L. J. V.: Investigation. P. F. M.: Investigation, Writing – review and editing. C. M. M.: Investigation. Débora K. T. Moreira: Investigation. G. C. T.: Investigation. H. C. P. F.: Investigation. G. S. C.: Supervision, Project administration, Funding acquisition, Conceptualization, Methodology, Formal analysis, Data curation, Validation, Resources, Writing – review and editing.

CTL, control; M14-3F, diet supplemented with strain M14 and provided three times daily; M14-1F, diet supplemented with strain M14 and provided once daily; I10-3F, diet supplemented with strain I10 and provided three times daily; and I10-1F, diet supplemented with strain I10 and provided once daily. Different letters within columns indicate significant differences among groups according to Tukey's and Dunn's tests (*) ($p < 0.05$).

MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; WBC: White Blood Cells; GLIC, glucose; COL, cholesterol; TRIG, triglycerides; ALB, albumin; TP, total proteins; A:G, albumin:globulin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. CTL, control; M14-3F, diet supplemented with strain M14 and provided three times daily; M14-1F, diet supplemented with strain M14 and provided once daily; I10-3F, diet supplemented with strain I10 and provided three times daily; and I10-1F, diet supplemented with strain I10 and provided once daily. Different letters within columns indicate significant differences among groups according to Tukey's and Dunn's tests ($p < 0.05$).

MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBC: White Blood Cells; GLIC, glucose; COL, cholesterol; TRIG, triglycerides; ALB, albumin; TP, total proteins; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. CTL, control; M14-3F, diet supplemented with strain M14 and provided three times daily; M14-1F, diet supplemented with strain M14 and provided once daily; I10-3F, diet supplemented with strain I10 and provided three times daily; and I10-1F, diet supplemented with strain I10 and provided once daily. Different letters within columns indicate significant differences among groups according to Tukey's and Dunn's tests ($p < 0.05$).

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Data Availability

The metagenomic sequencing data generated during the current study are available as supplementary information files. All other experimental datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

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Figures

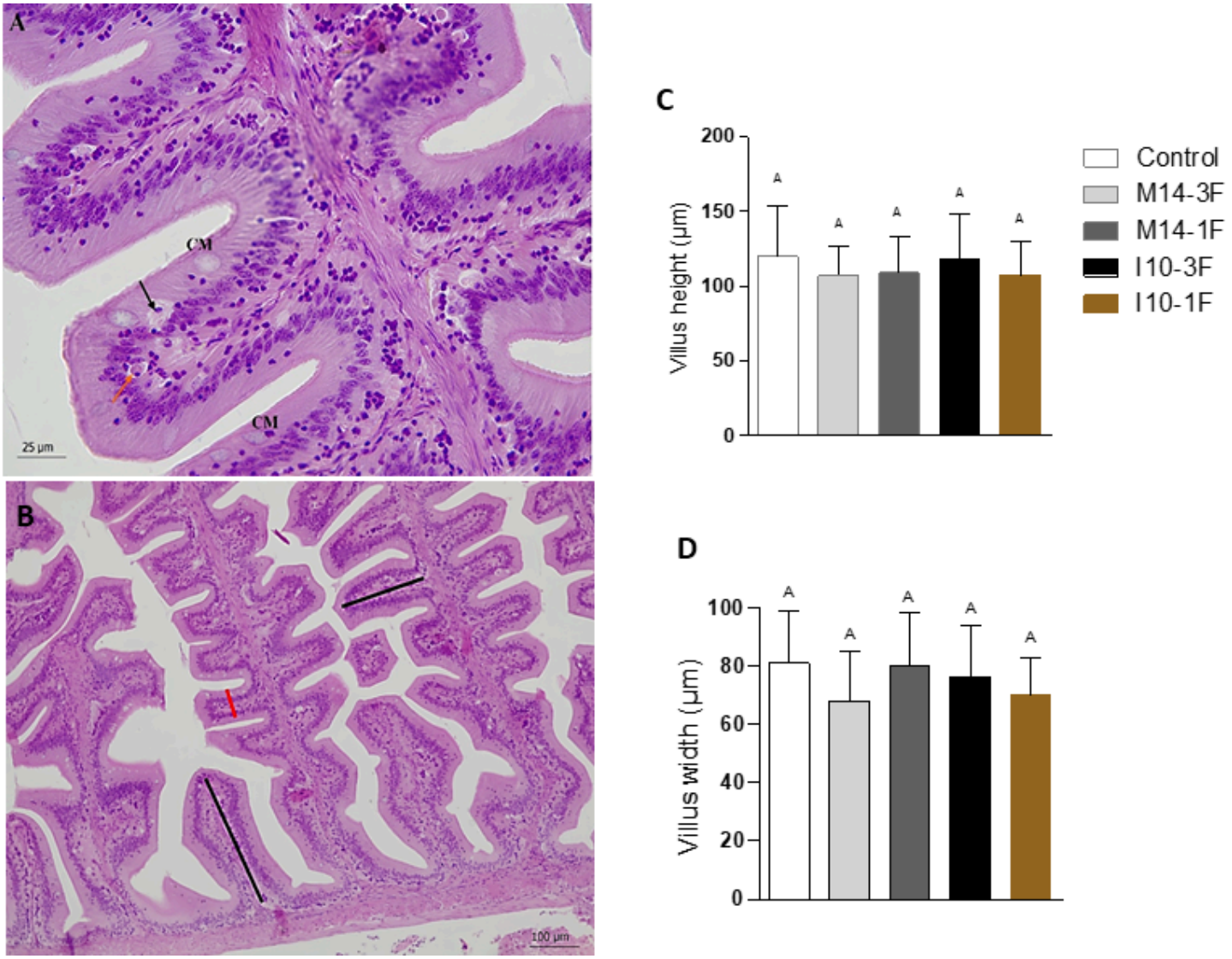


Figure 1

Histological organization and histomorphometry of intestinal villi in juvenile *Colossoma macropomum* supplemented with diets containing probiotic *Weissella cibaria* strains.

(A) Intestinal epithelium showing mucous cells (CM), rodlet cells (black arrow), and enterocytes.

(B) Intestinal villi showing the lacteal vessel. Red arrow: width measurement; Black arrow: height measurement; Orange arrow: macrophage.

(C) Mean ± standard deviation of villus height (µm).

(D) Mean ± standard deviation of villus width (µm).

CM, mucous cells; black arrow, rodlet cell. Scale bars: 25 µm in (A) and 100 µm in (B). Bars represent the mean of each treatment

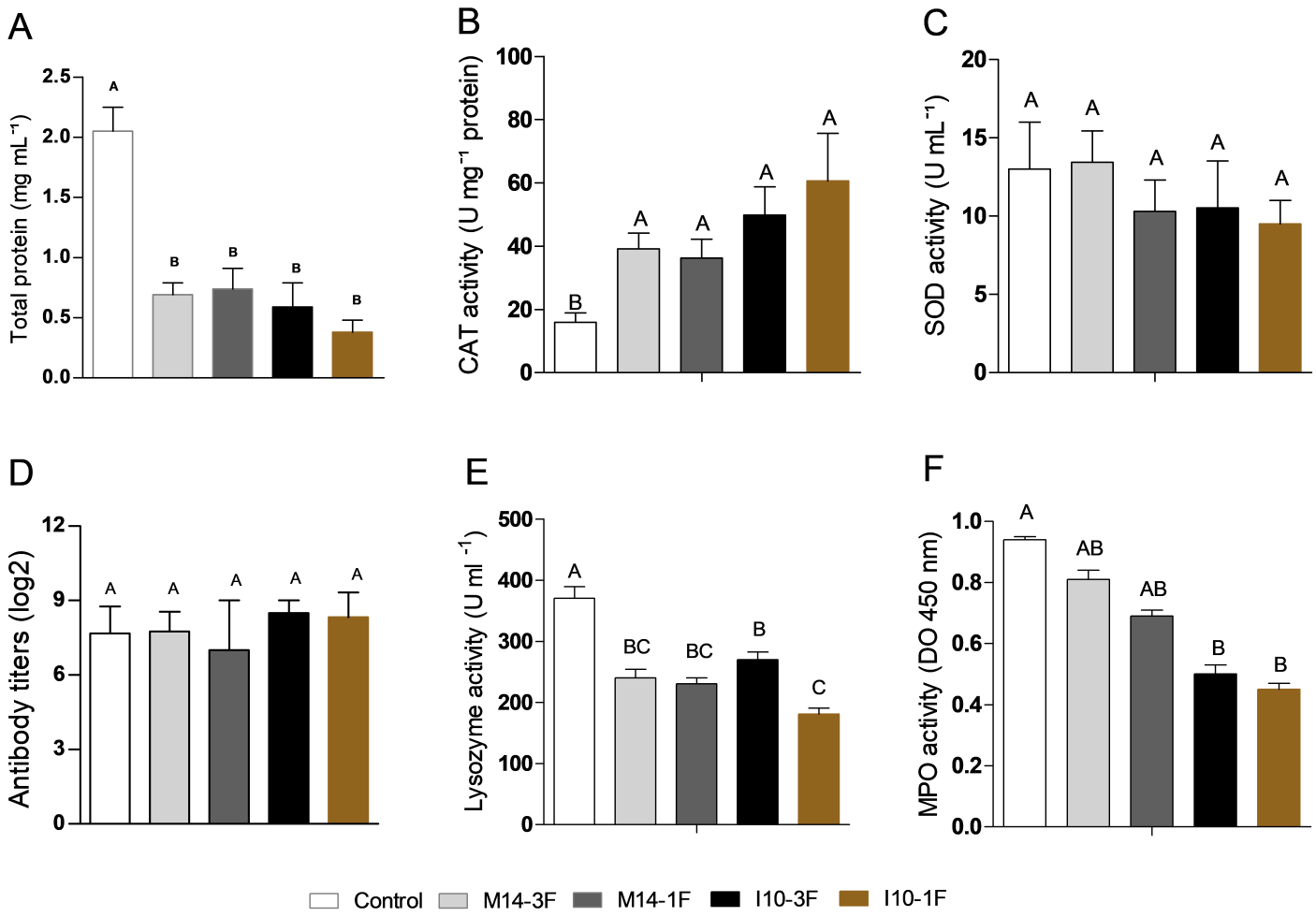


Figure 2

Histological organization and histomorphometry of intestinal villi in juvenile *Colossoma macropomum* supplemented with diets containing probiotic *Weissella cibaria* strains.

(A) Intestinal epithelium showing mucous cells (CM), rodlet cells (black arrow), and enterocytes.

(B) Intestinal villi showing the lacteal vessel. Red arrow: width measurement; Black arrow: height measurement; Orange arrow: macrophage.

(C) Mean ± standard deviation of villus height (µm).

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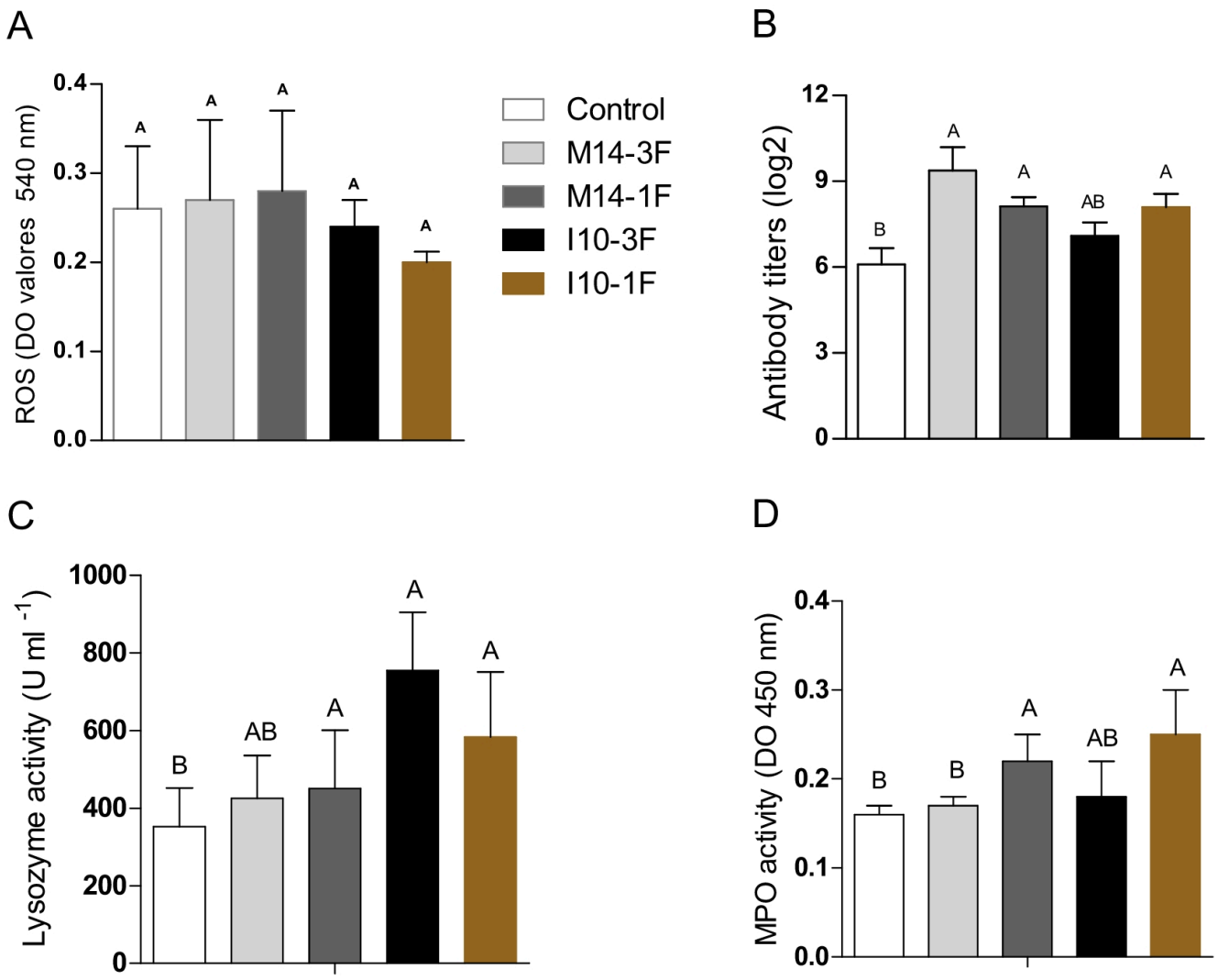


Figure 3

Innate immune parameters in the serum and peripheral blood of tambaqui challenged with *Aeromonas hydrophila* at 24 h post-challenge (n=10).

(A) Respiratory burst activity, expressed as optical density of reactive oxygen species (ROS) at 540 nm in blood leukocytes.

(B) Natural antibody titers (log_2).

(C) Serum lysozyme concentration (U mL^{-1}).

(D) Serum myeloperoxidase (MPO) activity (OD 450 nm).

* Vertical columns represent means of each group in the sampling times. Vertical bars represent the standard error of the mean. Columns with letters in common do not differ by the level of 5% by Tukey test (A, C and D) and Dunn (B). M14-3F, diet supplemented with strain M14 in the three daily feedings; M14-1F, diet supplemented with strain M14 in one daily feeding; I10-3F, diet supplemented with strain I10 in the three daily feedings; and I10-1F, diet supplemented with strain I10 in one daily feeding.

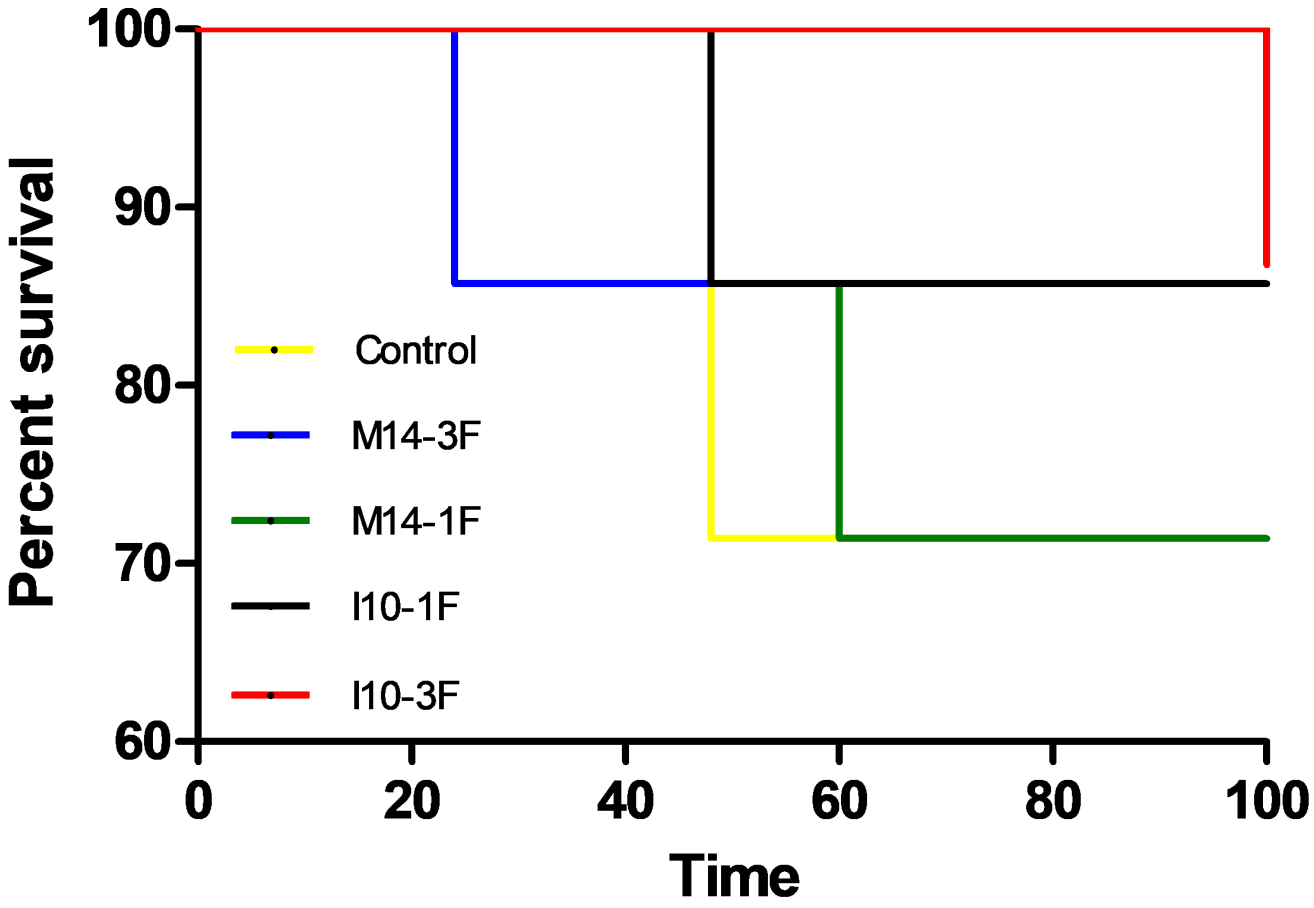


Figure 4

Survival curves of *Colossoma macropomum* after experimental challenge with *Aeromonas hydrophila*. Kaplan–Meier survival analysis of fish from the control group and probiotic-supplemented treatments over the post-challenge period. CTL, control; M14-3F, diet supplemented with strain M14 in the three daily feedings; M14-1F, diet supplemented with strain M14 in one daily feeding; I10-3F, diet supplemented with strain I10 in the three daily feedings; and I10-1F, diet supplemented with strain I10 in one daily feeding.

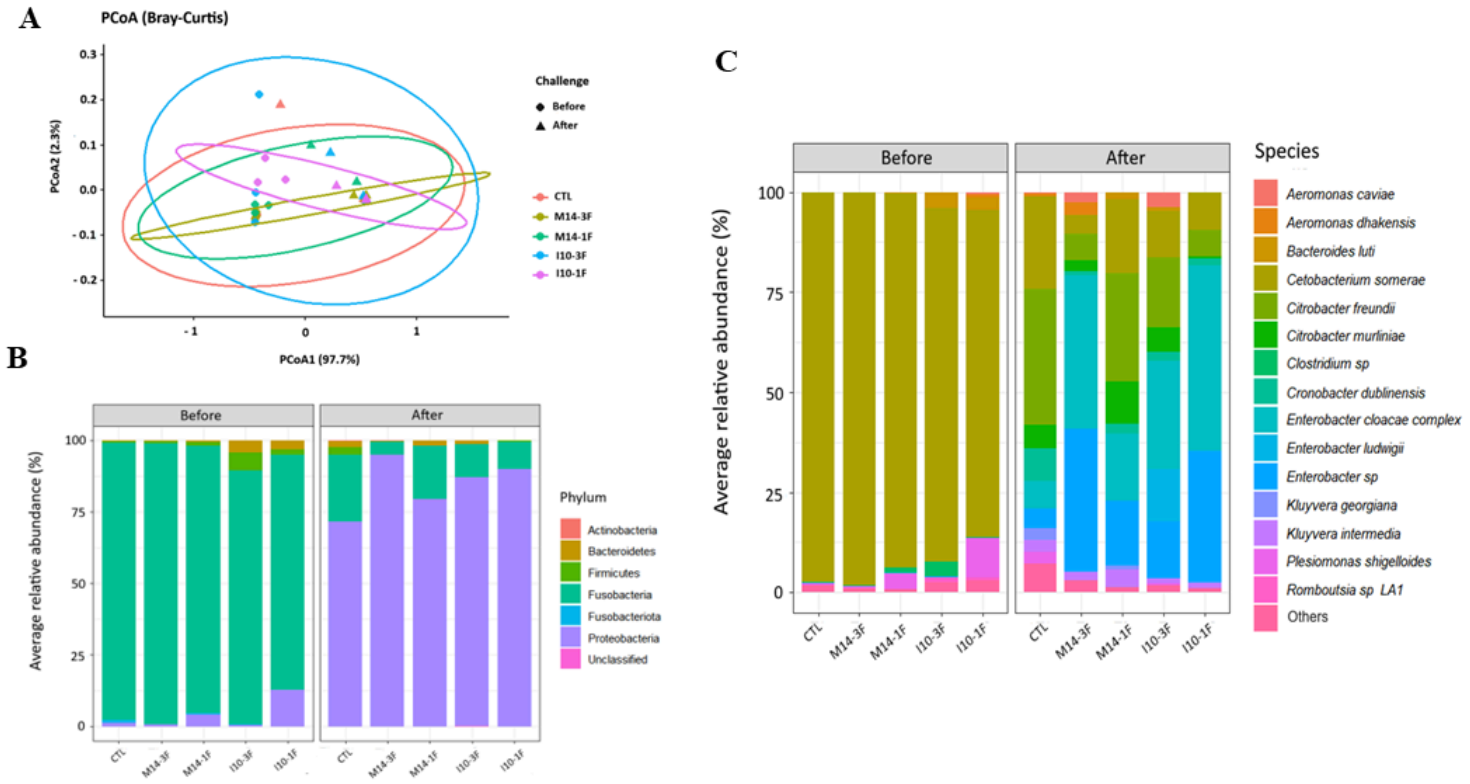


Figure 5

Gut microbiota structure and taxonomic composition of *Colossoma macropomum* before and after experimental challenge with *Aeromonas hydrophila*.

(A) Principal coordinate analysis (PCoA) based on Bray–Curtis distances showing the distribution of samples according to treatment and challenge status (before and after challenge).
(B) Average relative abundance (%) of the main bacterial phyla identified in the intestinal microbiota before and after challenge.

(C) Average relative abundance (%) of the main bacterial species identified in the intestinal microbiota before and after challenge.

CTL, control; M14-3F, diet supplemented with strain M14 in the three daily feedings; M14-1F, diet supplemented with strain M14 in one daily feeding; I10-3F, diet supplemented with strain I10 in the three daily feedings; and I10-1F, diet supplemented with strain I10 in one daily feeding.

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