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Research Article

Immunostimulatory effects of multispecies probiotics against *Aeromonas hydrophila* in *Colossoma macropomum* reared in biofloc and recirculating aquaculture systems

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ABSTRACT. This study evaluated the immunostimulatory and protective effects of a multispecies probiotic formulation -comprising *Bacillus subtilis*, *Lactobacillus plantarum*, and *Pediococcus acidilactici*- on juvenile *Colossoma macropomum* reared in biofloc and recirculating aquaculture systems over 70 days. The probiotic was administered via feed, water, or both, and its effects on growth performance, hematological and biochemical profiles, innate immune responses, and resistance to *Aeromonas hydrophila* infection were assessed. The combined probiotic strategy (feed + water) led to enhanced weight gain, improved serum lysozyme and protease activity, and higher reactive oxygen species production. Fish reared in biofloc systems exhibited higher hematocrit, hemoglobin, and innate immune markers than those reared in recirculating systems. However, recirculating aquaculture systems yielded superior growth and survivability. Following bacterial challenge, the combined probiotic treatment achieved 100% survival in the biofloc system, while the control group showed 50% survival. These findings highlight the potential of multispecies probiotics to improve immune competence and disease resistance in tambaqui, supporting their use as sustainable biotechnological tools in aquaculture. The choice of probiotic administration route and farming system significantly influenced host physiology and response to bacterial infection. Importantly, probiotic supplementation may serve as a viable alternative to antibiotics, reducing the risk of antimicrobial resistance and promoting more sustainable fish farming practices.

Keywords: *Colossoma macropomum*; aquaculture systems; innate immunity; multispecies probiotics; prophylaxis

INTRODUCTION

Aquaculture is a globally expanding economic sector, driven by the growing demand for aquatic protein and the need for sustainable alternatives to extractive fisheries (Lutz 2024). For the first time, aquaculture has surpassed extractive fishing, now accounting for 51% of total global aquatic production (FAO 2024). In Brazil, tambaqui (*Colossoma macropomum*) is the second most farmed fish species, representing approxi-

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mately 113.6 thousand tons, about 17,3% of the country's total aquaculture output (IBGE 2024). It is widely cultivated across the Amazon basin and in other regions of Brazil, as well as in several Central American and Asian countries (Woynárovich & Van Anrooy 2019, Araújo et al. 2024). Beyond Brazil, *C. macropomum*, commonly known as tambaqui, is among the most culturally and economically significant freshwater fish species farmed across tropical South America. It is widely cultivated in Peru, where it is known as gamitana and ranks among the leading species in inland aquaculture (PRODUCE 2023). In neighboring countries, such as Colombia, Ecuador, Bolivia, and Venezuela, it is commonly referred to as cachama negra, cachama amazónica, or again gamitana, reflecting its broad geographical and cultural relevance (Woynárovich & Van Anrooy 2019). The species is also known in English-language literature as tambaqui or black pacu (Hilsdorf et al. 2022). Its high adaptability to environmental fluctuations, rapid growth, and excellent meat quality make it well-suited to intensive farming systems (Val & De Oliveira 2021). However, the intensification of production has raised concerns about disease outbreaks and environmental sustainability, highlighting the need for integrated health management strategies.

Probiotics represent a promising, sustainable approach to enhancing fish performance and disease resistance. These live microorganisms, when administered in adequate amounts, promote host health by improving gut microbiota balance, stimulating immune responses, and potentially suppressing pathogenic bacteria (Khanjani et al. 2024, Mathan-Muthu et al. 2024). Multispecies probiotics are gaining attention due to their greater microbial diversity and synergistic effects, which may outperform single-strain formulations in terms of immunomodulatory capacity and environmental resilience (Azevedo et al. 2016, Wang et al. 2019, Pardosi et al. 2024).

Multispecies probiotics can be administered via feed or directly into the water. When provided through feed, they modulate the intestinal microbiota, enhancing digestion and nutrient absorption. Water-based applications help balance the environmental microbiota and improve water quality by reducing pathogen loads and organic waste (Tachibana et al. 2019, Fonseca et al. 2020). However, the efficacy of probiotic supplementation is context-dependent, varying with rearing system, fish species, and the probiotic strains used (Mello et al. 2013, Costa et al. 2021).

Among the most widely used aquaculture systems are biofloc technology (BFT) and recirculating aquaculture systems (RAS). BFT relies on microbial flocs for nutrient recycling and pathogen control, while RAS is based on water recirculation and filtration, providing stable environmental conditions and reduced effluent discharge (Ogello et al. 2021, Lal et al. 2024). The interaction between probiotics and these contrasting environments remains understudied, especially concerning their effects on immune function and disease resistance. *Aeromonas hydrophila* is an opportunistic pathogen of economic relevance in tropical aquaculture and a major cause of hemorrhagic septicemia in tambaqui (Claudiano et al. 2019, Gallani et al. 2020). It represents an effective model for evaluating host resistance and immunomodulation strategies under intensive farming conditions.

In this study, we evaluated the effects of a commercial multispecies probiotic on growth performance, immune and biochemical parameters, and survival of juvenile *C. macropomum* challenged with *A. hydrophila*, under both biofloc and recirculating aquaculture systems. The goal was to assess the immunostimulatory and protective potential of different probiotic administration routes and their interaction with farming systems, contributing to the development of sustainable health strategies in aquaculture.

MATERIALS AND METHODS

Fish sampling and experimental design

A total of 192 tambaquis (111.56 ± 3.31 g) were distributed across 24 polyethylene tanks (300 L) at a density of 27 fish m^{-3} (n = 8). The study was approved by the UFOPA Ethics Committee on Animal Use (No. 008577/12) and carried out in accordance with Brazilian animal welfare standards and the ISO (2006) standard. A 2×4 factorial design was applied, considering BFT and water recirculation system (WRS) farming systems and employing four probiotic use strategies (added to feed, PF; added to water, PW; added to both, PFW; and a control group not exposed to the probiotic, CTL), each in triplicate, for 70 days.

Fish were fed an extruded commercial feed containing 28% crude protein and 6% lipid, with a granulometry ranging from 4 to 6 mm, at a daily rate of 3% of the total fish biomass, divided into three feedings (9:00, 15:00, and 18:00 h). Throughout the entire experimental period, biweekly biometric assessments were conducted to monitor the fish and allow for potential adjustments to the feeding rate (Fig. 1). The

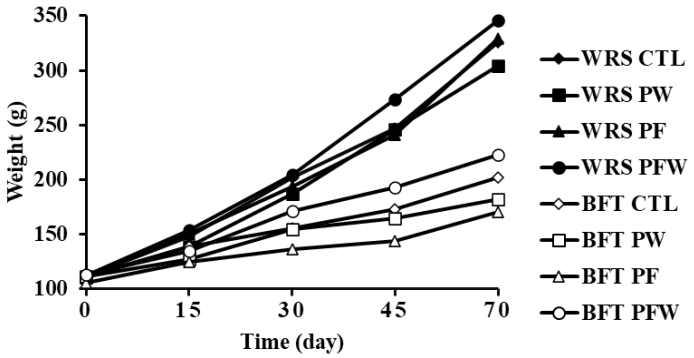

Figure 1. Average growth of *Colossoma macropomum* juveniles reared under different multispecies probiotic supplementation strategies in recirculating aquaculture (WRS) and biofloc technology (BFT) systems over 70 days. WRS PF: aquaculture recirculation system with added probiotic in the feed, WRS PW: aquaculture recirculation systems with added probiotic in the water, WRS PFW: aquaculture recirculation systems with probiotic in both, and WRS CTL: control group without probiotic, BFT PF: biofloc technology with added probiotic in the feed, BFT PW: biofloc technology with added probiotic in the water, BFT PFW: biofloc technology with probiotic in both, and BFT CTL: control group without probiotic.

multispecies probiotic was composed of *Bacillus subtilis* (3.4×10^9 CFU g^{-1}), *Lactobacillus plantarum* (1.2×10^9 CFU g^{-1}), and *Pediococcus acidilactici* (1.2×10^9 CFU g^{-1}), applied according to the manufacturer's recommendations (Table 1; Kera Nutrição Animal®, São Paulo, Brazil).

The PF treatment consisted of supplementing the diet with 2 kg of multispecies probiotic per ton of commercial feed. For the PW treatment, 1 kg of probiotic was added per 10 m^3 of water, corresponding to 0.03 g per experimental unit. In this case, the probiotic was first diluted in 10 mL of water and then homogeneously distributed into each experimental tank, approximately 30 min before the final feeding of the day. For the PFW treatment, both methods were applied simultaneously, with the same probiotic doses and preparation procedures described above. All treatments were administered daily throughout the 70-day experimental period. At the end of the experimental period, seven fish from each treatment were separated for the analyses described below.

Water quality variables

Water quality parameters, including dissolved oxygen (mg L^{-1}), temperature (ºC), and hydrogen potential (pH), were determined daily in all experimental units using a multiparameter probe (Model YSY PRO1020).

Total ammonia (mg L^{-1}) (Verdouw et al. 1978), nitrite (mg L^{-1}) (Boyd & Tucker 1993), and alkalinity (Clesceri 1998) determinations, as well as turbidity assessments using a digital turbidimeter (Model TU430), were carried out three times a week. Sugarcane molasses were added to establish a 6:1 carbon:nitrogen (C/N) ratio when total ammonia concentrations were ≥1 mg L^{-1} in the BFT system (Ebeling et al. 2006).

Settleable solids (SS) (mL L^{-1}) in the BFT system treatments were monitored twice a week, using graduated Imhoff cones (Avnimelech 2007). When water pH was below 7.0, sodium bicarbonate ($NaHCO_3$) was added to maintain pH between 7 and 7.5 in all experimental units and to maintain alkalinity above 120 mg L^{-1} of CaCO3 for the maintenance of heterotrophic and chemoautotrophic bacteria (Jiménez-Ojeda et al. 2018).

Zootechnical performance and bromatological analyses

All tambaqui individuals from each group were evaluated concerning zootechnical performance at the beginning and after 70 days. The following indices were determined: weight gain (g) = final weight (g) - initial weight (g); final biomass (g) = final average weight (g) × number of fish at the end of the production process; productivity (kg m^{-3}) = final biomass (kg) / volume used (m^3); apparent feed conversion = amount of feed provided (kg) / total biomass (kg); and survival (%) = (final number of animals / initial number of animals) × 100.

At the end of the experimental period, seven animals from each group were euthanized with benzocaine (200 mg L^{-1}) and dissected (Ross & Ross 2008). Muscle samples were stored at -18°C for bromatological analyses, which were performed in triplicate following

Table 1. Application of a multispecies probiotic during the rearing phase of tambaqui *Colossoma macropomum* in a water recirculation system (WRS) and a biofloc technology (BFT) system for 70 days.

Treatment	Probiotic amount	Inclusion frequency
CTL - No probiotic	None	None
PW - Probiotic added to water	1 kg 10.000 m^{-3} of water	Weekly
PF - Probiotic added to feed	2 kg 1.000 kg^{-1} of feed	Daily
PFW - Probiotic addes ot both feed and water	2 kg 1.000 kg^{-1} of feed +1 kg 10.000 m^{-3} of water	Daily and weekly

AOAC (1997) protocols, except for lipid content, which was determined using the method of Folch et al. (1957). Moisture was determined by gravimetric drying, ash by calcination, protein by the Kjeldahl method (using a nitrogen-to-protein conversion factor of 6.25), and lipids by cold extraction (n = 10 per treatment).

Biochemical blood and serum assessments

Blood (1 mL) from seven animals per treatment was obtained by caudal vasopuncture following anesthesia with benzocaine (0.1 g L^{-1}) at the end of the experimental period (n = 10 per treatment). The samples were stored in Eppendorf tubes containing 20 µL of EDTA (Sousa et al. 2021).

Total red blood cell (RBC) counts (10^6 cells μL^{-1}) were performed in a Neubauer chamber using Natt and Herrik's diluent (1:200) (Sousa et al. 2021). Aliquots were used for haematocrit (Htc, %) (Goldenfarb et al. 1971) and haemoglobin concentration (Drabkin & Austin 1935) determinations. Haematimetric indices, comprising mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC), were also assessed (Acerete et al. 2004).

Blood smears were stained by rapid panoptic staining for total thrombocyte, total leukocyte, and differential leukocyte (neutrophils, monocytes, and lymphocytes) counts by light microscopy employing an indirect method (Sousa et al. 2021). Briefly, 2,000 erythrocytes were counted in blood extensions for total leukocyte and thrombocyte determinations as follows: total leukocytes (µL) = number of leukocytes in the sample × number of erythrocytes counts in the Neubauer chamber / 2,000 erythrocytes in the sample; total thrombocytes (µL) = number of thrombocytes in the sample × number erythrocytes count in the Neubauer chamber (µL) / 2,000 erythrocytes in the sample (Sousa et al. 2021).

Biochemical variables were determined using a semiautomatic biochemical analyzer as follows: total protein by the biuret method; albumin by the bromocresol green method; globulin by subtraction of albumin from total protein values; albumin/globulin ratio (A/G) by direct calculation; lactate by the enzymatic–colorimetric method; alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by the kinetic UV-IFCC method (Brito et al. 2015, Claudiano et al. 2020). Blood glucose was determined according to the method of Castro et al. (2014).

Innate immunity

The respiratory burst was determined in caudal vein blood samples (n = 7 per treatment) using a turbidometric assay with nitroblue tetrazolium (NBT) (Biller-Takahashi et al. 2013) to quantify blood leukocyte reactive oxygen species (ROS) (Blázquez-Castro et al. 2014, Claudiano et al. 2019).

For lytic serum activity determinations, the bacterial strain's growth standard was initially standardized. First, a test was carried out to determine the dilution factor for colony counts between 30 and 300 CFU $plate^{-1}$, according to Claudiano et al. (2019). Then, for the definitive assays, *A. hydrophila* strains (10^6 CFU/1: 100,000) and final sample volume (50 µL *A. hydrophila* + 50 µL of serum from the samples) were established according to Biller-Takahashi et al. (2013). The resulting suspensions (100 µL) were plated and incubated as mentioned previously, followed by colony counting. A turbidimetric assay determined serum lysozyme concentrations according to Marzocchi-Machado et al. (1999) and Blázquez-Castro et al. (2014).

The equivalence point between the amounts of CFU and serum in U-bottom microtitre plates in the dose-response relationship was previously established for bacterial agglutination activity determinations (Claudiano et al. 2019). A dose-response of 1.8×10^9 CFU was deter-

mined. Serum bacterial agglutination activity was determined in samples from all fish groups using 50 µL of serially diluted fish serum. The total antibody titre was defined as the highest serum dilution that exhibited positive agglutination. The results were expressed as $\log_2$ of the reciprocal of the serum titres (Fernandes et al. 2015).

Clinical signs and survival analysis

To determine the inoculum dose, 20 fish (179.44 ± 27.15 g) inoculated with increasing *A. hydrophila* concentrations of 1.5×10^8; 6.0×10^8; 1.2×10^9; 2.4×10^9 CFU mL^{-1}, and a control group, distributed in five tanks (n = 5/100 L) (Claudiano et al. 2019), were used. The DL 50-96 h value was calculated using the trimmed Spearman-Karber method as 9×10^8, with lower and upper limits of 1.78×10^9 and 4.65×10^9, respectively.

To evaluate clinical signs and survival, 500 µL of an *A. hydrophila* suspension (corresponding to LD50% = 9.0×10^8 CFU mL^{-1}) was inoculated in the coelomic cavity of 56 juvenile tambaqui (n = 7 per treatment). Survival was determined by visual observation for up to 10 days. Fish were subjected to cranial kidney bacterial reisolation at the end of the experimental period or at the time of death, confirming sepsis in all exposed animals.

Statistical analysis

The results were first analyzed for normality (Shapiro-Wilks) and homoscedasticity (Levene). A two-way analysis of variance (ANOVA) was used for normally distributed data, followed by Tukey's *post-hoc* test to identify differences between means. Non-normally distributed data were subjected to the Kruskal-Wallis and Dunn's *post-hoc* tests ($P < 0.05$).

RESULTS

Water quality, zootechnical performance, and bromatology analyses

Significantly higher levels of pH, dissolved oxygen, turbidity, and nitrite were observed in the BFT system compared to the WRS, while total ammonia concentrations were lower ($P < 0.05$; Table 2). Regarding the probiotic supplementation treatments, no significant differences were observed for most evaluated parameters ($P > 0.05$), except in the PW treatment, which showed reduced nitrite levels in both WRS and BFT systems compared with PF and PFW ($P < 0.05$, Table 2).

The zootechnical parameters evaluated -final weight, weight gain, apparent feed conversion, productivity, and biomass- are presented in Table 3. All parameters were significantly higher in fish reared in the WRS compared to the BFT system ($P < 0.05$), although no differences in survival rate were observed ($P > 0.05$). Additionally, no significant differences in zootechnical parameters were noted ($P > 0.05$) among the multispecies probiotic supplementation treatments. However, the highest final weight and weight gain values were observed in the PFW group of WRS fish, whereas the lowest values were found in the PF group of BFT fish ($P < 0.05$, Table 3).

The production system had no significant effect on fish muscle protein content (Table 4) ($P > 0.05$). However, moisture content was significantly higher in BFT fish ($P < 0.05$), while ash and lipid contents were significantly higher in WRS fish ($P < 0.05$). None of the probiotic supplementation forms significantly affected tambaqui moisture, ash, or protein contents ($P > 0.05$, Table 4), although lipid content was significantly reduced in the PF treatment ($P < 0.05$). A significant interaction between the farming system and the forms of probiotic supplementation was also observed ($P < 0.05$). Ash content was higher in the PF group of WRS fish and lower in BFT fish supplemented by the same route. Similarly, higher lipid content was recorded in the PW group of WRS fish, while the lowest values were observed in the PF group of BFT fish.

Blood, biochemical profiles, and innate immunity

Erythrocyte counts, MCV, and MCH did not differ significantly between the two farming systems or among the multispecies probiotic supplementation treatments ($P > 0.05$, Table 5), although hemoglobin levels and hematocrit values were significantly higher in BFT fish ($P < 0.05$). Regarding leukograms, no differences in total leukocyte, granulocyte, or thrombocyte counts were observed between systems. However, WRS fish exhibited higher lymphocyte counts compared to BFT fish, with an inverse pattern observed for monocytes ($P < 0.05$, Table 5). Probiotic supplementation alone did not significantly alter most hematological parameters, whereas the PFW treatment resulted in significant reductions in total leukocyte and thrombocyte counts ($P < 0.05$, Table 5).

Regarding serum biochemistry, significantly elevated AST and glucose levels were observed in WRS fish, while total protein levels were higher in BFT fish ($P < 0.05$, Table 5). No significant differences were found between systems for ALT, albumin, globulins, or

Table 2. Water quality parameters (mean ± standard deviation) observed during *Colossoma macropomum* rearing under different multispecies probiotic supplementation in a water recirculation system (WRS) and biofloc technology (BFT) system for 70 days. CTL: control; PW: probiotic added to water; PF: probiotic added to feed; PFW: probiotic added to both feed and water; DO: dissolved oxygen; SS: settleable solids. Different capital letters between columns indicate statistically significant differences between production systems, and different lowercase letters between columns indicate statistically significant differences between probiotic use by the Tukey or Kruskal-Wallis test (*) ($P < 0.05$).

Parameter	Multispecies probiotic supplementation							
	Water recirculation system				Biofloc system			
	CTL	PW	PF	PFW	CTL	PW	PF	PFW
Temperature (ºC)	27.71 ± 0.19A^{ab}	27.68 ± 0.22A^{b}	27.70 ± 0.29A^{b}	27.95 ± 0.18A^{a}	27.58 ± 0.19A^{ab}	27.40 ± 0.22A^{b}	27.55 ± 0.18A^{b}	27.63 ± 0.29A^{a}
*pH	6.86 ± 0.00B^{a}	6.91 ± 0.00B^{a}	6.91 ± 0.00B^{a}	6.92 ± 0.00B^{a}	7.16 ± 0.02A^{a}	7.16 ± 0.01A^{a}	7.22 ± 0.03A^{a}	7.21 ± 0.03A^{a}
*DO (mg L^{-1})	6.82 ± 0.00B^{a}	6.75 ± 0.00B^{a}	6.75 ± 0.00B^{a}	6.79 ± 0.00B^{a}	7.22 ± 0.04A^{a}	7.32 ± 0.09A^{a}	7.34 ± 0.07A^{a}	7.26 ± 0.19A^{a}
*Turbidity (NTU)	1.16 ± 0.00B^{a}	1.01 ± 0.00B^{a}	0.36 ± 0.00B^{a}	0.36 ± 0.00B^{a}	55.88 ± 2.64A^{a}	51.29 ± 4.58A^{a}	39.28 ± 8.31A^{a}	51.36 ± 11.55A^{a}
Alkalinity (mg L^{-1} $CaCO_3$)	29.02 ± 0.00A^{a}	28.37 ± 0.00A^{a}	25.22 ± 0.00A^{a}	28.35 ± 0.00A^{a}	30.94 ± 0.37A^{a}	26.47 ± 0.54A^{a}	30.24 ± 2.01A^{a}	28.22 ± 4.09A^{a}
*Total ammonia (mg L^{-1})	0.10 ± 0.00A^{a}	0.09 ± 0.00A^{a}	0.10 ± 0.00A^{a}	0.11 ± 0.00A^{a}	0.05 ± 0.01B^{a}	0.05 ± 0.01B^{a}	0.07 ± 0.02B^{a}	0.07 ± 0.03B^{a}
Nitrite (mg L^{-1})	0.77 ± 0.00B^{a}	0.73 ± 0.00B^{b}	0.81 ± 0.00B^{a}	1.02 ± 0.00B^{a}	1.01 ± 0.04A^{a}	0.68 ± 0.10A^{b}	1.05 ± 0.18A^{a}	0.85 ± 0.02A^{a}
SS (mL L^{-1})	-	-	-	-	5.85 ± 0.72^{a}	5.52 ± 1.24^{a}	3.01 ± 1.57^{a}	6.06 ± 0.57^{a}
Factor	*P*-value							
	Temperature	pH	DO	Turbidity	Alkalinity	Total ammonia	Nitrite	SS
System	0.1386	0.0001	0.0001	0.0001	0.0832	0.0003	0.0481	-
Probiotic	0.0260	0.5633	0.9595	0.3472	0.0641	0.2253	0.0002	-
Interaction	0.0142	0.0026	0.0038	0.0033	0.0124	0.0143	0.0003	0.1349

Table 3. Zootechnical *Colossoma macropomum* performance parameters (mean ± standard deviation) under different multispecies probiotic supplementation in a water recirculation system (WRS) and a biofloc technology (BFT) system for 70 days. CTL: control; PW: probiotic added to water; PF: probiotic added to feed; PFW: probiotic added to both feed and water; AFC: apparent feed conversion. Capital letters between columns indicate statistically significant difference between production systems, and different lowercase letters between columns indicate statistically significant difference between probiotic use by the Tukey or Kruskal-Wallis test (*) ($P < 0.05$).

Parameter	Multispecies probiotic supplementation							
	Water recirculation system				Biofloc system			
	CTL	PW	PF	PFW	CTL	PW	PF	PFW
Initial weight (g)	111.75 ± 1.72A^{a}	110.87 ± 1.72A^{a}	111.71 ± 1.72A^{a}	112.58 ± 1.72A^{a}	111.25 ± 1.72A^{a}	111.04 ± 1.72A^{a}	105.79 ± 1.72A^{a}	113.46 ± 1.72A^{a}
*Final weight (g)	325.54 ± 10.07A^{a}	304.33 ± 10.07A^{a}	329.41 ± 10.07A^{a}	345.79 ± 10.07A^{a}	202.21 ± 10.07B^{a}	182.21 ± 10.07B^{a}	170.46 ± 10.07B^{a}	222.70 ± 10.07B^{a}
*Weight gain (g)	213.67 ± 9.31A^{a}	193.67 ± 9.31A^{a}	218.00 ± 9.31A^{a}	233.33 ± 9.31A^{a}	91.00 ± 9.31B^{a}	71.00 ± 9.31B^{a}	64.67 ± 9.31B^{a}	109.33 ± 9.31B^{a}
AFC	1.38 ± 0.12B^{a}	1.49 ± 0.12B^{a}	1.39 ± 0.12B^{a}	1.42 ± 0.11B^{a}	1.74 ± 0.12A^{a}	1.87 ± 0.12A^{a}	1.80 ± 0.12A^{a}	1.71 ± 0.12A^{a}
*Yield (kg m^{-3})	9.000 ± 550.41A^{a}	8.057 ± 1170.46A^{a}	8.887 ± 396.81^{a}	9.221 ± 249.97A^{a}	5.392 ± 85.55B^{a}	4.858 ± 758.57B^{a}	4.545 ± 311.04B^{a}	5.938 ± 717.41B^{a}
Biomass (kg m^{-3})	2.604 ± 1.14A^{a}	2.334 ± 1.14A^{a}	2.635 ± 1.14A^{a}	2.766 ± 1.14A^{a}	1.419 ± 1.14B^{a}	1.457 ± 1.14B^{a}	1.363 ± 1.14B^{a}	1.781 ± 1.14B^{a}
*Survival (%)	100.00 ± 1.41A^{a}	96.00 ± 1.41A^{a}	100.00 ± 1.41A^{a}	100.00 ± 1.41A^{a}	100.00 ± 1.41A^{a}	100.00 ± 1.41A^{a}	100.00 ± 1.41A^{a}	100.00 ± 1.41A^{a}

Factor	*P*-value						
	Initial weight	Final weight	Weight gain	AFC	Yield	Biomass	Survival
System	0.2855	0.0001	0.0001	0.0008	0.0001	0.0001	0.3173
Probiotic	0.1384	0.5074	0.4443	0.9502	0.6305	0.5425	0.3916
Interaction	0.2228	0.0046	0.0044	0.1040	0.0068	0.0054	0.4289

Table 4. Proximate muscle composition values (means ± standard deviation) of juvenile *Colossoma macropomum* reared under different multispecies probiotic supplementation strategies in a water recirculation system (WRS) and a biofloc technology (BFT) system for 70 days. CTL: control; PW: probiotic added to water; PF: probiotic added to feed; PFW: probiotic added to both feed and water. Capital letters between columns indicate statistically significant differences between production systems, and lowercase letters indicate statistically significant differences between probiotic treatments, as determined by Tukey's ($P < 0.05$).

Parameter	Multispecies probiotic supplementation							
	Water recirculation system				Biofloc system			
	CTL	PW	PF	PFW	CTL	PW	PF	PFW
Moisture (%)	75.94 ± 0.90B^{a}	76.41 ± 2.30B^{a}	76.03 ± 0.65B^{a}	76.90 ± 0.28B^{a}	76.87 ± 0.52A^{a}	77.44 ± 0.77A^{a}	77.90 ± 0.30A^{a}	76.95 ± 0.53A^{a}
Ash (%)	1.24 ± 0.02A^{a}	1.19 ± 0.02A^{a}	1.26 ± 0.02A^{a}	1.19 ± 0.02A^{a}	1.18 ± 0.02B^{a}	1.19 ± 0.02B^{a}	1.13 ± 0.02B^{a}	1.22 ± 0.02B^{a}
Lipids (%)	2.89 ± 0.15A^{a}	3.52 ± 0.15A^{a}	2.90 ± 0.15A^{b}	2.93 ± 0.15A^{ab}	2.86 ± 0.15B^{a}	2.13 ± 0.15B^{a}	1.60 ± 0.15B^{b}	2.09 ± 0.15B^{ab}
Protein (%)	18.91 ± 0.44A^{a}	17.29 ± 0.44A^{a}	19.37 ± 0.44A^{a}	18.10 ± 0.44A^{a}	18.97 ± 0.44A^{a}	18.74 ± 0.44A^{a}	18.22 ± 0.44A^{a}	18.72 ± 0.44A^{a}

Factor	*P*-value			
	Moisture	Ash	Lipids	Protein
System	0.0290	0.0212	0.0001	0.4458
Probiotic	0.7322	0.8743	0.0024	0.1920
Interaction	0.4888	0.0090	0.0013	0.0592

Table 5. Hematological and biochemical parameters (mean ± standard deviation) of the blood of juvenile *Colossoma macropomum* reared under different multispecies probiotic supplementation in a water recirculation system (WRS) and a biofloc technology (BFT) system for 70 days. CTL: control; PW: probiotic added to water; PF: probiotic added to feed; PFW: probiotic added to both feed and water; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; TGP/ALT: alanine aminotransferase; TGO/AST: aspartate aminotransferase; TGP: TGO: capital letters between columns indicate statistically significant difference between production systems, and different lowercase letters between columns indicate statistically significant difference between probiotic use by the Tukey or Kruskall-Wallis test (*) ($P < 0.05$).

Parameter	Multispecies probiotic supplementation							
	Water recirculation system				Water recirculation system			
	CTL	PW	PF	PFW	CTL	PW	PF	PFW
Total erythrocytes ($\times 10^6$ mm^{-3})*	1.68 ± 0.64A^a	2.03 ± 1.16A^a	1.71 ± 0.90A^a	1.26 ± 0.33A^a	1.69 ± 0.45A^a	1.69 ± 0.64A^a	1.56 ± 0.38A^a	1.55 ± 0.39A^a
Hemoglobin (g dL^{-1})	7.06 ± 0.36B^a	6.6 ± 0.36B^a	7.08 ± 0.36B^a	6.52 ± 0.36B^a	6.60 ± 0.36A^a	7.89 ± 0.36A^a	7.47 ± 0.36A^a	8.18 ± 0.36A^a
Hematocrit (%)	31.80 ± 1.42B^a	31.14 ± 1.42B^a	30.43 ± 1.42B^a	29.57 ± 1.42B^a	35.57 ± 1.42A^a	34.43 ± 1.42A^a	35.86 ± 1.42A^a	34.14 ± 1.42A^a
MCV (fL)	216.32 ± 28.28A^a	189.73 ± 28.28A^a	203.82 ± 28.28A^a	256.71 ± 28.28A^a	224.05 ± 28.28A^a	226.02 ± 28.28A^a	243.76 ± 28.28A^a	231.25 ± 28.28A^a
MCH (pg)	48.28 ± 6.88A^a	41.62 ± 6.88A^a	48.82 ± 6.88A^a	56.41 ± 6.88A^a	43.01 ± 6.88A^a	51.96 ± 6.88A^a	50.48 ± 6.88A^a	54.59 ± 6.88A^a
MCHC (g dL^{-1})	22.05 ± 1.12A^a	21.68 ± 1.12A^a	23.86 ± 1.12A^a	22.10 ± 1.12A^a	18.66 ± 1.12A^a	22.96 ± 1.12A^a	20.95 ± 1.12A^a	23.87 ± 1.12A^a
Total leukocytes ($\times 10^5$ µL^{-1})*	1.47 ± 1.81A^a	1.78 ± 2.22A^a	1.50 ± 1.86A^a	0.02 ± 0.01A^b	1.48 ± 1.80A^a	1.48 ± 1.81A^a	1.36 ± 1.66A^a	1.36 ± 1.66A^b
Total lymphocytes ($\times 10^5$ µL^{-1})*	2.29 ± 3.96A^a	5.90 ± 12.0A^a	4.69 ± 4.03A^a	0.06 ± 0.10A^a	3.45 ± 8.19B^a	0.25 ± 7.21B^a	0.23 ± 0.44B^a	0.77 ± 0.75B^a
Total granulocytes ($\times 10^3$ µL^{-1})*	0.79 ± 0.80A^a	1.33 ± 1.38A^a	1.27 ± 1.22A^a	0.05 ± 0.03A^a	1.17 ± 1.01A^a	0.73 ± 1.16A^a	0.59 ± 0.90A^a	0.91 ± 1.07A^a
Total monocytes* ($\times 10^3$ µL^{-1})	0.00 ± 0.0B^a	0.24 ± 0.49B^a	1.76 ± 4.14B^a	0.19 ± 0.28B^a	3.87 ± 3.66A^a	1.65 ± 2.13A^a	0.73 ± 0.82A^a	3.66 ± 3.50A^a
Total thrombocytes (*10^5µL^{-1})*	1.36 ± 1.71A^a	1.58 ± 1.99A^a	1.30 ± 1.67A^a	0.01 ± 0.01A^b	1.29 ± 1.72A^a	1.38 ± 1.69A^a	1.29 ± 1.58A^a	1.22 ± 1.54A^b
Glycemia (g dL^{-1})	139.00 ± 10.27A^a	138.86 ± 10.27A^a	166.00 ± 10.27A^a	141.29 ± 10.27A^a	139.14 ± 10.27B^a	90.43 ± 10.27B^a	83.86 ± 10.27B^a	83.86 ± 10.27B^a
TGP/ALT (U d^{-1} L^{-1})	9.65 ± 2.91A^a	7.72 ± 1.70A^a	9.82 ± 1.01A^a	11.65 ± 7.29A^a	5.42 ± 1.06B^a	4.90 ± 0.21B^a	5.72 ± 0.19B^a	5.66 ± 1.39B^a
TGO/AST (U d^{-1} L^{-1})	17.25 ± 11.15A^a	9.60 ± 6.71A^a	8.87 ± 1.94A^a	8.62 ± 4.58A^a	5.66 ± 1.48A^a	5.07 ± 1.03A^a	6.08 ± 1.13A^a	8.62 ± 4.58A^a
Lactate (mmol L^{-1})	0.30 ± 0.03A^a	0.21 ± 0.03A^b	0.36 ± 0.03A^a	0.42 ± 0.03A^a	0.34 ± 0.03A^a	0.24 ± 0.03A^b	0.31 ± 0.03A^a	0.33 ± 0.03A^a
Albumin (A; g dL^{-1})	0.74 ± 0.03A^a	0.76 ± 0.03A^a	0.76 ± 0.03A^a	0.70 ± 0.03A^a	0.74 ± 0.03A^a	0.74 ± 0.03A^a	0.75 ± 0.03A^a	0.74 ± 0.03A^a
Total protein (g dL^{-1})	3.07 ± 0.32B^a	2.24 ± 0.32B^a	2.75 ± 0.30B^a	3.19 ± 0.35B^a	3.17 ± 0.32A^a	3.63 ± 0.32A^a	3.20 ± 0.32A^a	3.19 ± 0.32A^a
Globulin (G; g dL^{-1})	2.33 ± 0.33A^a	1.49 ± 0.33A^a	2.00 ± 0.33A^a	2.42 ± 0.33A^a	2.43 ± 0.33A^a	2.81 ± 0.33A^a	2.45 ± 0.33A^a	2.45 ± 0.33A^a
*A:G ratio (g dL^{-1})	0.37 ± 0.12A^a	0.58 ± 0.22A^a	0.38 ± 0.45A^a	0.30 ± 0.08A^a	0.33 ± 0.09A^a	0.28 ± 0.08A^a	0.32 ± 0.07A^a	0.31 ± 0.05A^a

P-value

Factor	Total erythrocytes	Hemoglobin	Hematocrit	MCV	MCH	MCHC	Total leukocytes
System	0.5718	0.0089	0.0001	0.4703	0.8021	0.3090	0.1086
Probiotic	0.5301	0.4597	0.5351	0.6435	0.4896	0.1109	0.0117
Interaction	0.8552	0.0258	0.8430	0.6329	0.7011	0.0420	0.0035
Factor	Total lymphocytes	Total granulocytes	Total monocytes	Total thrombocytes	Glycemia	TGP/AST	TGO/ALT
System	0.0008	0.9091	0.0003	0.0857	0.0001	0.0001	0.2239
Probiotic	0.8488	0.0799	0.1663	0.0127	0.0509	0.7149	0.2883
Interaction	0.0021	0.0088	0.0007	0.0033	0.0022	0.0220	0.3290
Factor	Lactate	Albumin	Total protein	Globulin	A:G ratio		
System	0.6536	0.6620	0.0341	0.0546	0.1894		
Probiotic	0.0012	0.8183	0.8341	0.7612	0.2976		
Interaction	0.0085	0.6998	0.1408	0.1408	0.0908		

albumin:globulin ratios. Additionally, lactate levels were significantly reduced in the PW groups of both the WRS and BFT systems ($P < 0.05$). These findings indicate that both the farming system and probiotic supplementation treatment influenced the hematological, leukocyte, and biochemical profiles of juvenile tambaqui (Table 5).

Variations in innate immunity parameters were observed according to probiotic supplementation treatment and farming system (Fig. 2). Blood leukocyte granule ROS concentrations were higher in BFT fish than in WRS fish ($P < 0.05$) and lower in the PFW groups ($P < 0.05$, Fig. 2a) compared to other treatments. Serum protease lytic activity against bacterial colonies was greater in BFT fish than in WRS fish, with higher activity observed in the PF and PFW groups ($P < 0.05$, Fig. 2b). Serum lysozyme levels were elevated in fish from the PFW group in both systems (Fig. 2d). The agglutination capacity of tambaqui antibodies against *A. hydrophila* did not differ significantly between systems or probiotic supplementation treatments ($P > 0.05$, Fig. 2c).

Clinical signs and survival

Infected fish presented petechiae and suffusions in the dorsal region near the inoculation site at two days post-inoculation, with clinical signs worsening throughout the experimental period. Significantly higher survival rates were observed for WRS fish compared to BFT fish ($P < 0.05$, Fig. 3a) during the 10-day monitoring period following *A. hydrophila* infection. No significant differences were observed between any of the probiotic supplementation treatments (Fig. 3b) in WRS fish. Significant differences, however ($P < 0.05$), were observed for BFT fish, in which the control group presented a 50% survival rate. In contrast, the PW and PF groups exhibited 57.1 and 71.4% survival rates, respectively, increasing to 100% in the PFW group (Fig. 3c). Consequently, mortality rates were zero for WRS fish, except for the PFW group (14.2%). In contrast, in the BFT system, these values increased to 52.7% in the control group, 42.8% in the PW group, 28.57% in the PF group, and zero in the PFW group (Fig. 3d).

DISCUSSION

Both tambaqui rearing systems and probiotic supplementation treatments significantly influenced several physio-immunological variables and, consequently, fish production. Higher pH, turbidity, and nitrite values were observed in the BFT system, although within the recommended ranges for the species (Wood et al. 2018). Lower total ammonia concentrations were, however, observed, suggesting that the BFT environment favors ammonia oxidation (Dos Santos et al. 2023) due to higher solids load and distinct microbial nitrifying and heterotrophic bacteria dynamics that efficiently mediate ammonia assimilation and reduction (Ebeling et al. 2006). The addition of mature biofloc inocula to the experimental units at the beginning of the study may also have contributed to these results, as observed in other BFT assessments (Chen et al. 2006, Santos et al. 2019). In contrast, more stable conditions were observed in the WRS, with lower turbidity and nitrite values. However, higher total ammonia levels were likely associated with the appropriate WRS management established during the study and with an efficient bacterial nitrification process. These parameters did not compromise the well-being of tambaqui juveniles.

Regarding zootechnical performance, WRS fish demonstrated superior weight gains, productivity, and biomass compared to BFT fish. The PFW treatment enhanced these gains in the WRS, suggesting that simultaneous probiotic application via both water and feed creates a synergistic, growth-favoring effect, possibly due to improved nutrient digestion and utilization, which corroborates the association between *Bacillus subtilis*, *Lactobacillus plantarum*, and *Pediococcus acidilactici*, all widely employed as probiotics in aquaculture, and higher tambaqui weight gains and final weight (Sáenz De Rodrigáñez et al. 2009, Kim et al. 2010, Nayak 2010). Several studies on other teleost species have demonstrated that probiotic bacteria with exoenzymatic activity can enhance digestive efficiency, thereby improving zootechnical performance, such as increased weight gain and superior feed conversion ratios (Han et al. 2015). For instance, Mello et al. (2013) observed significant improvements in growth performance of juvenile *Oreochromis niloticus* (100 ± 8.2 g) after 80 days of dietary supplementation with *Bacillus cereus* and *B. subtilis* at concentrations of 4.0×10^8 CFU g^{-1} each.

In addition to improving production performance, the applied probiotic species significantly improved tambaqui body composition, although the effects varied across production systems. In this sense, WRS fish exhibited higher ash and lipid contents, while BFT fish exhibited higher moisture levels. The PF treatment significantly reduced lipid muscle content, which is desirable to improve fish nutritional quality, as lower lipid deposition can contribute to a more balanced body profile (NRC 2011). A significant interaction was also

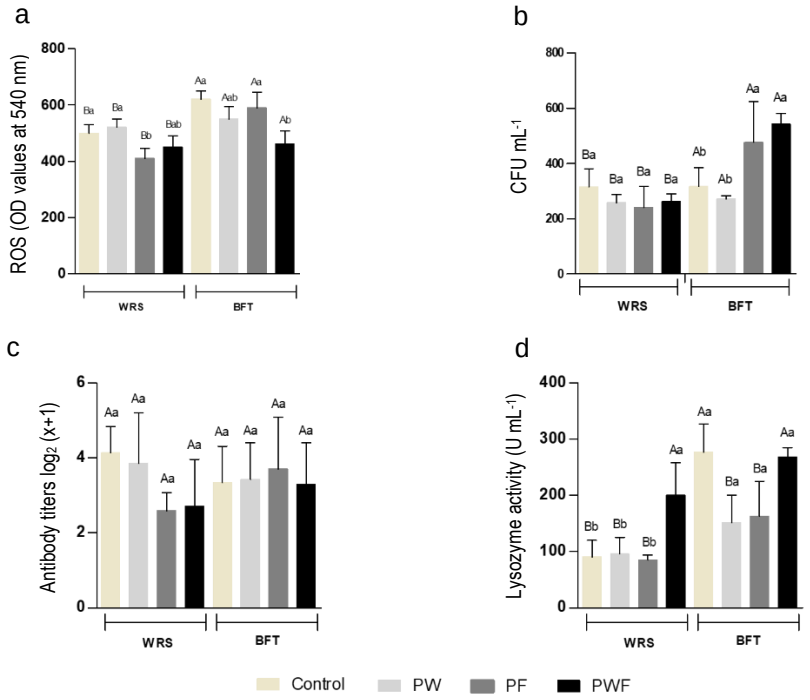

Figure 2. Analysis of innate immunity in juvenile *Colossoma macropomum* reared under different multispecies probiotic supplementation strategies in recirculating aquaculture (WRS) and biofloc technology (BFT) systems. a) Reactive oxygen species (ROS) production in blood leukocytes (OD values at 540 nm); b) serum proteolytic (lytic) activity (CFU mL^{-1}); c) natural antibody titers (log_2 (x + 1)); d) serum lysozyme activity (U mL^{-1}). Vertical bars represent mean values, and error bars indicate standard deviation. Columns sharing the same letter do not differ significantly at the 5% level according to Tukey's test (a) and Kruskal-Wallis test (b-d). ROS: reactive oxygen species; OD: optical density; WRS: water recirculation system; BFT: biofloc technology; Control: no probiotic supplementation; PW: probiotic in water; PF: probiotic in feed; PWF: probiotic in water and feed.

observed in the BFT system, where the PF treatment was associated with decreases in lipid and ash content.

These findings diverge from previous studies on Nile tilapia (*O. niloticus*), which reported no significant differences in ash, lipid, or protein content between fish fed probiotics and those in the control group (Albuquerque et al. 2015, Da Silva 2020). Dos Santos et al. (2021), working with *C. macropomum* juveniles (23.24 ± 0.34 g) for 60 days in a BFT system supplemented with *B. subtilis* in the water (4×10^8 cells mL^{-1}), observed that probiotic supplementation resulted only in an increase in crude protein content in the fish body composition. In contrast, Azevedo et al. (2016) demonstrated that tambaqui (2.4 ± 0.2 g) supplemented with *B. subtilis* for eight weeks exhibited increased lipid deposition and decreased mineral matter content as body weight increased. These findings complement the present study, particularly in the WRS, where higher final weights were associated with elevated lipid content and reduced ash levels, suggesting that the effects of probiotics on body composition may vary across rearing conditions and fish growth stages.

Furthermore, this study's results indicate that the effects of multispecies probiotic supplementation on the centesimal composition of juvenile tambaqui are influenced by the rearing system. Although bioflocs are recognized as a supplementary nutrient source - providing microbial biomass that can enhance protein and lipid intake (Rajkumar 2024)-, their presence and potential ingestion by fish in the BFT system did not translate into improvements in body composition in the present study, which may be attributed to environmental or physiological trade-offs that limit nutrient assimilation efficiency under biofloc conditions.

Overall, these findings underscore the importance of considering the rearing environment when assessing the nutritional impact of probiotics on tambaqui. Responses to supplementation may differ substantially

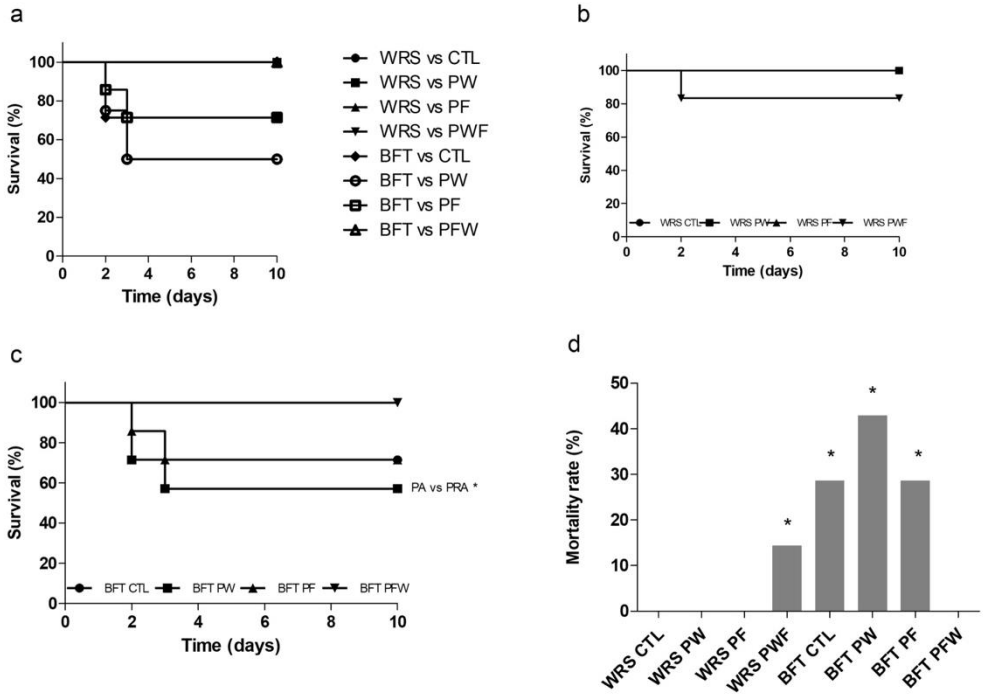

Figure 3. Survival and mortality of *Colossoma macropomum* after sepsis induction by *Aeromonas hydrophila* (n = 7). a) Overall survival percentage comparing the biofloc technology (BFT) and the water recirculation system (WRS), b) survival percentage in the WRS among probiotic-treated groups and the control, c) survival percentage in the BFT among probiotic-treated groups and the control, d) mortality rate comparing both systems (BFT *vs*. WRS) and the probiotic-treated groups vs. the control. Vertical bars represent the mean values for each group. Survival data were analyzed using the log-rank test ($P < 0.05$), and mortality rates were compared using Tukey's test ($P < 0.05$). WRS PF: aquaculture recirculation system with added probiotic in the feed, WRS PW: aquaculture recirculation systems with added probiotic in the water, WRS PFW: aquaculture recirculation systems with probiotic in both, and WRS CTL: control group without probiotic, BFT PF: biofloc technology with added probiotic in the feed, BFT PW: biofloc technology with added probiotic in the water, BFT PFW: biofloc technology with probiotic in both, and BFT CTL: control group without probiotic.

between WRS and BFT systems due to factors such as microbial community interactions, nutrient availability, water quality, and energy expenditure related to environmental adaptation (Austin & Sharifuzzaman 2022).

Both the production system and probiotic supplementation influenced the physio-immunological responses of juvenile tambaqui. Although erythrocyte counts, MCV, and MCH did not differ significantly between fish reared in the WRS and those in the BFT system, nor among the different probiotic treatments, hemoglobin concentration and hematocrit values were notably higher in BFT-reared fish. These alterations suggest an enhanced oxygen transport capacity to peripheral tissues, likely as an adaptive response to the more variable and demanding environmental conditions typical of the biofloc system (Tavares-Dias & Moraes 2004, Mokhtar et al. 2023).

The BFT environment is characterized by increased turbidity, higher organic load, and frequent physico-chemical fluctuations, including dissolved oxygen levels, which can impose physiological stress on aquatic organisms. In such settings, fish may activate compensatory mechanisms to preserve homeostasis and ensure adequate tissue oxygenation. One such mechanism involves erythropoietin stimulation, leading to elevated red blood cell production and increased hemoglobin synthesis. These changes enhance the oxygen-carrying capacity of the blood, enabling fish to meet the elevated metabolic demands imposed by environmental stressors (Evans et al. 2005). This hematological adjustment is not only indicative of physiological resilience but also reflects an evolved plasticity of tambaqui in coping with unstable environments, such as those found in BFT systems. Similar hematological profiles were observed by Dos

Santos et al. (2024), who reported elevated hematocrit and hemoglobin values in tambaqui juveniles reared in BFT at varying stocking densities. Their findings reinforce the notion that this system induces physiological shifts favoring increased oxygen transport efficiency as a survival strategy.

By contrast, fish maintained in WRS are subjected to more stable environmental conditions, with controlled water quality and reduced organic matter accumulation, thus requiring less physiological adjustment. The consistently high water quality in WRS reduces the necessity for hematological compensation, which explains the lower hemoglobin and hematocrit levels observed in this group (Tavares Dias & Moraes 2004, Baldisserotto 2025). Taken together, these results suggest that the hematological profiles observed in BFT fish are part of a broader metabolic and physiological adaptation aimed at maintaining functional equilibrium in fluctuating and microbially enriched environments. Such insights are crucial for understanding the biological trade-offs associated with different aquaculture systems and for designing management strategies that align with species-specific physiological capacities.

Multispecies probiotic supplementation in tambaqui juveniles improved blood cell profiles, increasing lymphocyte counts in WRS fish and monocyte counts in BFT fish. These cells play essential immune system roles in fish, including direct defense against infections and maintenance of homeostasis (Claudiano et al. 2013, 2019, Yunis-Aguinaga et al. 2016). The combined probiotic strategy resulted in a significant decrease in total leukocytes and thrombocytes, suggesting modulation of the immune response and reduced stress levels (Liu et al. 2022). Regarding biochemical parameters, WRS fish exhibited significantly higher AST and glycemia levels, while BFT fish presented higher serum total protein levels. Furthermore, the PW treatment reduced lactate levels in both systems, suggesting improved liver integrity and reduced metabolic stress (Lee et al. 2023). These results are consistent with those of Naiel et al. (2022), who reported that supplementation with probiotic strains (*Bacillus toyonensis* and *Geobacillus stearothermophilus*) at concentrations of 1 to 2×10^5 CFU mL^{-1} in the rearing water of *O. niloticus* juveniles (20 ± 0.03 g), over 70 days, led to enhanced antioxidant and immune responses. Similarly, Adorian et al. (2019) demonstrated that *Lates calcarifer* (1.5 ± 0.2 g) fed diets containing *Bacillus licheniformis* and *B. subtilis* at concentrations of 1×10^3, 1×10^6, and 1×10^9 CFU g^{-1} for eight weeks exhibited significant improvements in physiological responses, reinforcing the beneficial impact of probiotics on fish metabolism and health.

Higher ROS levels in leukocyte granules were observed in BFT fish, reflecting an intensified oxidative response in the more complex biofloc system environment (Gómez & Balcázar 2008). These molecules are produced by activated neutrophils and macrophages during the respiratory burst and play a crucial role in microbial killing (Claudiano et al. 2019). Lytic serum protease activities were also higher in BFT fish, while serum lysozyme levels increased considerably in the PWF groups of both systems. These enzymes are key effectors of innate immunity. They are secreted in response to microbial components recognized by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), that activate signaling cascades and trigger inflammatory and antimicrobial responses (Vallejos-Vidal et al. 2016, Mokhtar et al. 2023). The combined delivery of probiotics in water and feed likely promoted mucosal and systemic immune activation via gut-associated lymphoid tissue (GALT) and skin/gill epithelia, thereby enhancing immune competence (Zhou et al. 2020, Wang et al. 2021).

On the other hand, antibody agglutination against *A. hydrophila* was not significantly different between treatments, suggesting immune modulation mainly through innate mechanisms (Fernandes et al. 2015, Claudiano et al. 2019). It is consistent with the study's duration, as adaptive responses, such as antibody titers, may require longer periods or booster exposure to become evident (Fernandes et al. 2015).

Higher survival rates over 10 days were observed for WRS fish compared to BFT fish during aeromonosis, evidencing the influence of a more stable environment on tambaqui resistance. While the different probiotic supplementation strategies did not significantly alter WRS survival rates, the combined PFW treatment was noteworthy in the BFT system, resulting in 100% survival, compared with 50% in the control group and intermediate rates for each isolated probiotic supplementation treatment. These almost non-existent variations in WRS fish and progressively reduced variations in BFT fish suggest that the less stable BFT environment requires an integrated supplementation approach to optimize immune responses and mitigate stress in tambaqui, reinforcing the need to consider both the rearing system and the probiotic supplementation strategy to maximize fish resistance to infections.

Importantly, the observed protection against *A. hydrophila*-induced septicemia without antibiotic use underscores the relevance of multispecies probiotics as viable alternatives to antimicrobials in aquaculture. It is

especially relevant in the context of antimicrobial resistance, as such strategies contribute to sustainable production and reduce selective pressure on resistant bacterial strains (Austin & Sharifuzzaman 2022, Khanjani et al. 2024).

Taken together, these findings indicate that the fish production environment and the probiotic application method not only modulate hematological and biochemical parameters in tambaqui but also influence immune response capacity and resistance to infections (Austin & Sharifuzzaman 2022). While the WRS appears to favor a metabolic and hematological response that contributes to greater resilience, the BFT environment, although stimulating a more intense innate immune response, may require more effective supplementation strategies to mitigate stress and improve survival rates, as noted for the PFW treatment. Therefore, adequate environmental management, in combination with optimized probiotic application strategies, is paramount for maximizing juvenile tambaqui performance and health.

CONCLUSION

This study demonstrates that aquaculture systems distinctly influence the physiology and immunity of juvenile *C. macropomum*. While RAS enhanced growth performance and survival, the biofloc system promoted stronger innate immune responses, including increased levels of hemoglobin, hematocrit, lysozyme, protease, and ROS. Among the tested strategies, combined probiotic supplementation (PFW) provided the most consistent benefits, notably under biofloc conditions, where it ensured complete protection against *A. hydrophila*. These findings support the use of multispecies probiotics as effective immunostimulants and reinforce their potential as sustainable alternatives to antibiotics when integrated with optimized aquaculture systems.

Credits author contribution

G.T.J. Reis: contributed to the experimental design, data collection, statistical data analysis, data interpretation and manuscript preparation; L.A.B. Pereira: data collection, data interpretation and manuscript preparation; L.P.C. Santos: data collection, data interpretation and manuscript preparation; A.L. Leão: data collection, data interpretation and manuscript preparation; A.S. Oliveira: data collection, data interpretation and manuscript preparation; D.M.K.T. Moreira: statistical data analysis, data interpretation and manuscript preparation; L. Jensen: experimental design and manuscript preparation; G.S. Claudiano: data interpretation and manuscript preparation; M.M.S. Fugimura: experimental design, data analysis, data interpretation, manuscript preparation and study coordination.

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

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