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ORIGINAL ARTICLE

Comparative effects of autochthonous single-strain and multi-strain probiotics on the productive performance and disease resistance in *Colossoma macropomum* (Cuvier, 1818)

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Abstract

This study evaluated the effects of autochthonous single-strain and multi-strain (mix) probiotics on the zootechnical performance and sanitary conditions of juvenile neotropical fish. Fingerlings of tambaqui (*Colossoma macropomum*) were fed three diets of single-strain probiotics (two *Enterococcus faecium* strains and one *Bacillus cereus* autochthonous strain) and a multi-strain probiotic diet (a mix of three probiotic strains) for 120 days. After dietary supplementation, 90 tambaquis were intraperitoneally injected with *Aeromonas hydrophila* at a concentration of 1.8×10^8 CFU·g^{-1}. Clinical signs of disease, infectious intensity and accumulated mortality rates were evaluated. The use of diets containing probiotics, regardless of strain, enhanced productive performance from 90 experimental days ($p < 0.05$). The multi-strain probiotics reduced the presence of potentially pathogenic bacteria in the intestine. Fish fed probiotics showed improved resistance to *A. hydrophila* infection, while the diets containing *B. cereus* (an autochthonous probiotic) and multi-strain probiotic promoted the lowest mortality rates and higher leucocyte and thrombocyte counts ($p < 0.05$). The results revealed that the use of probiotics as a single autochthonous or multi-strain probiotic enhanced fish growth, prevented dysbiosis and increased disease resistance.

KEYWORDS
haematology, microbiology, nutrition, pathogen, productive performance

1 | INTRODUCTION

Commercial vaccines and antibiotics are commonly used to prevent and control diseases in fish. Nevertheless, no vaccines are available against all pathogenic bacteria on fish farms (Valladão et al., 2018). Additionally, the indiscriminate use of antibiotics and other chemotherapeutics can potentially lead to resistant microorganisms, causing environmental pollution due to effluent

discharge as well as accumulation of chemicals in the edible tissues of animals, which is harmful to the health of consumers (Liu, Steele, & Meng, 2017; Pilarski et al., 2017; Luiset al., 2019).

In the last 10 years, worldwide federations have been presenting alternative measures for the development of an "antibiotic-free" agriculture, and the use of probiotics has been strongly recommended for several reasons, including their safety, low cost, environmental control, productive performance benefits, pathogen control and disease resistance (Hoseinifar et al., 2018). Nonetheless, most efforts are limited to the application of single strains in the intensive production system, whereas multi-strain probiotics can potentially increase the benefits due to synergism among the bacteria.

The benefits of multi-strain probiotics include improvement in immunomodulation, competitive exclusion of bacterial pathogens, antimicrobial activity, digestive efficacy and confinement stress tolerance (Hoseinifar et al., 2018; Jamal et al., 2019; Mamun et al., 2019).

A consortium of probiotic bacteria allows for the colonization of the gastrointestinal tract and domination of the resident microbiota, which is an improved technique compared to the use of monospecies probiotics (Mohapatra et al., 2012; Kim et al., 2017). However, a recent review states that few studies have compared the use of autochthonous single-strain and multi-strain probiotics (Melo-Bolívar et al., 2021), thereby avoiding accurate multi-strain probiotic recommendations.

Among the organisms mostly used as probiotics in fish farming, *Bacillus* spp. and lactic acid bacteria predominate. Both groups are involved in increasing fish productive performance by secreting proteinases, regulating intestinal pH and improving the absorption of dietary nutrients. Additionally, they release lipopeptides that regulate the intestinal microbiological balance and prevent the growth and virulence of pathogenic bacteria (Yang et al., 2015; Li et al., 2020).

In Latin America, the fish *Colossoma macropomum* is a neotropical species with expressive market value presenting significant growth in exportation annually (4.81%) (Woynárovich & Van Anrooy, 2019; Cavali et al., 2021; Peixe, 2021). However, the intensification of fish production, along with high stocking densities, erroneous productive management, inadequate nutrition and poor water quality, promote the occurrence of diseases, mainly bacterial, which cause an estimated loss of production of approximately $84,000,000 USD (Tavares-Dias & Martins, 2017; Ren et al., 2019; Gallani et al., 2020). Single-strain probiotics have been tested in this species (Dias et al., 2018); however, multi-strain probiotics have not yet been evaluated.

This study aimed to evaluate the dietary use of three probiotic strains: single-strain (two *Enterococcus faecium* strains and an autochthonous *Bacillus cereus* strain) and a multi-strain diet (mix of three probiotic strains) on growth, intestinal microbiological profile and resistance to *Aeromonas hydrophila* infection in juveniles of a neotropical fish species.

2 | MATERIAL AND METHODS

All experiments were approved by the Animal Experimentation Ethics Committee of the Brazilian Agricultural Research Corporation, EMBRAPA (no. 0034/2020).

2.1 | The probiotics

The probiotic strains used in this study included two strains of *Enterococcus faecium* (*E. faecium* 1, Dias et al., 2019; *E. faecium* 2, Sousa et al., 2019) and one autochthonous *Bacillus cereus* strain from tambaqui (Dias et al., 2018), which were acquired from the Laboratory of Health of Aquatic Organisms at the Brazilian Agriculture Research Corporation (EMBRAPA) in Aracaju, Sergipe/Brazil.

Bacterial identification was performed using a Microflex Maldi-TOF mass spectrometer (Bruker Daltonics). Mass spectra were automatically acquired using a Flex Control operating system with a laser frequency of 60 Hz. An HCCA (α-cyano-4-hydroxycinnamic acid) matrix solution was used for the simple direct transfer of parts of a single colony-forming unit (CFU) bacteria method, and the mass spectra profiles were compared with those present in the library provided by Bruker Daltonik GmbH using the Maldi Biotyper 3.0 software. Bacterial identification at the genus and species level was considered when log scores were ≥1.7 and ≥2.3, respectively (Cardoso et al., 2021).

2.2 | In vitro assays

2.2.1 | Antagonism among probiotic strains

An in vitro test was performed to evaluate the antagonism between the probiotic strains following method of Ramírez et al. (2006). Initially, the bacteria were placed in a Man Rogosa & Sharpe (MRS) broth culture medium and incubated at 35°C for 24 h. After microbiological growth, 5 μl of serial dilutions (10^{-2}, 10^{-3}, and 10^{-4}) of each bacterial strain was inoculated onto paper filter discs (250 μm). The disc contents of each probiotic dilution were placed in MRS plates previously inoculated with 10^4 CFU·ml^{-1} of each probiotic (*E. faecium* 1 and 2 or *Bacillus cereus*), incubated at 35°C for 48 h.

Finally, inhibitory halos and antagonism between probiotic strains were observed. The multi-strain probiotic formulation was determined considering the concentrations that cause less interference in bacterial growth.

2.2.2 | Antagonism against Aeromonas hydrophila

The effects of the probiotic strains and the multi-strain probiotic against the pathogen *Aeromonas hydrophila* were also evaluated. The pathogen used had the registration code CPQBA22808 DRM

and was provided by the Shrimp Marine Laboratory of the Federal University of Santa Catarina, Brazil. Species identification of the pathogen was confirmed through matrix-assisted laser desorption/ionization and time-of-flight (MALDI-TOF; Angeletti, 2017).

The in vitro test was performed following the methods described by Vieira et al. (2013) and Paixão et al. (2019). The probiotics were cultivated in MRS broth culture medium and 100 µl ($\times 10^9$ CFU·ml^{-1}) of each treatment was inoculated in MRS agar medium and incubated at 35°C for 48 h. After bacterial growth, 0.8 cm diameter discs were removed from the agar plates, superimposed on plates with Mueller Hinton Agar medium containing *Aeromonas hydrophila*, and incubated at 35°C for 48 h for further analysis of the inhibition halo (mm). The experimental arrangement followed a randomized design with four treatments (*E. faecium* 1, *E. faecium* 2, *Bacillus cereus*, and the probiotic mix) and four replicates for each treatment.

2.3 | Dietary probiotic supplementation - in vivo assay

2.3.1 | Experimental design

A completely randomized design with four treatments in quadruplicate was used, corresponding to two diets with probiotic strains isolated from the continental fish species *Enterococcus faecium* (1) at 2×10^6 CFU·g^{-1} (Dias et al., 2019) and *Enterococcus faecium* (2) at 1×10^8 CFU·g^{-1} of feed (Sousa et al., 2019); one diet with a species-specific probiotic *Bacillus cereus* at 2.8×10^6 CFU.g^{-1} (Dias et al., 2018); one diet with a multi-strain probiotic composed of *E. faecium* (1 and 2) and *B. cereus* strains at 1×10^8 CFU·g^{-1}, which were stipulated in the previous in vitro experiment; and a control group (without the inclusion of the probiotic).

2.3.2 | Preparation of strains and diets

The probiotic strains were grown in MRS liquid medium at 35°C for 24 h, centrifuged at 1800 *g* for 15 min, and resuspended in sterile saline solution (SSE 0.65%) to the desired concentrations, according to the methodology of Jatobá et al. (2008). Subsequently, the saline suspensions were sprinkled on extruded commercial fish feed with guaranteed levels of 32% crude protein, 10% moisture, 7% ether extract and 4.5% crude fibre (Lima et al., 2016; Buzollo et al., 2019).

To monitor the concentration of probiotics in the diet, 1 g of each type of experimental fish feed (w/v) was ground weekly and diluted in sterile saline solution (0.65%; 1:10). An aliquot of 100 µl was plated on MRS agar culture medium to aid in the count of the probiotic bacteria (CFU·g^{-1}), and posterior identification was performed using Gram staining and MALDI-TOF methods (Jatobá & Mouriño, 2015; Angeletti, 2017). The fish feed was renewed every 7 days and stored in a refrigerator at 4°C.

2.3.3 | Experimental conditions

Twenty 150-L tanks were used that were coupled to a water recirculation system with mechanical, biological and UV filters. Juvenile *Colossoma macropomum* individuals (n = 400, weight 1.13 ± 0.01 g, and total length 41.76 ± 0.99 mm) were randomly distributed at a stocking density of 20 individuals per tank (initial biomass of 22.69 ± 0.9 g) and maintained for 120 days.

The animals were fed daily for 120 days at an initial rate of 8% relative to biomass, and the quantity was adjusted according to monthly biometric values (Silva et al., 2007; Dias et al., 2018).

Water parameters of temperature 28.9 ± 2.7 °C, dissolved oxygen 6.2 ± 1.2 mg·L^{-1}, pH 5.8 ± 0.4, and total ammonia 0.69 ± 1.21 mg·L^{-1} were monitored on alternate days during the 120 days (Professional*Plus* YSI multiparameter), to ensure that they remained within range for the species (Silva & Fujimoto, 2015; Dias et al., 2018; Silva et al., 2021).

2.3.4 | Productive parameters

Monthly biometric evaluations were performed on all fish to measure the total length, standard length, weight, feed intake and subsequent determination of weight gain (final weight − initial weight); total length gain (final total length − initial total length); standard length gain (final standard length − initial standard length); specific growth rate (SGR) [ln (final weight in grams) − ln (initial weight in grams) × 100/t (days of experiment)]; Fulton's condition factor (K) (final body weight/total length (cm)^3]; feed conversion ratio (FCR) [feed intake (kg)/weight gain (g)], uniformity (U) [(N ± 20%)/Nt), Nt = total number of fish in each experimental unit; and N ± 20% = number of animals with the parameter weight/length within ±20% around the mean of the experimental unit], and survival rate [(Final number of fish/initial number of fish) × 100] (Furuya et al., 1998; Gonçalves-Junior et al., 2013).

2.3.5 | Haematological analysis

At the end of the dietary supplementation period (120 days), ten fish from each treatment group were randomly selected for blood sampling and evaluation of haematological parameters.

The animals were anaesthetised using a solution of 60 mg·L^{-1} of eugenol, and 1 ml of blood from each fish was drawn from the caudal vessel using syringes moistened with EDTA (10%).

Additionally, an aliquot of 5 µl of the total volume was used to measure blood glucose (AccuCheck Active). Aliquots of the same volume were used to determine triglyceride and cholesterol levels (Accutrend® Plus). Leucocyte and thrombocyte counts were manually performed using the blood smear method after staining with NewProv panoptic haematological dye (Fontes et al., 2014).

To determine the total erythrocyte count (10^6 cell·µl^{-1}), 10 µl of blood was added to 2.5 ml microtubes that were filled with 1 ml

of sterile saline solution (0.65%), and homogenized for subsequent counting in a Neubauer chamber (Tavares-Dias & Moraes, 2004). Haematocrit was determined by applying the microcentrifugation methodology described by Goldenfarb et al. (1971).

Total plasma protein was measured using a refractometer (QuimisRHC-200ATC). Haemoglobin concentrations were analysed in 10 μl of blood added to 2.5 ml of cyanide and measured using a ThermoPlate biochemical analyser (Kamper & Zijlstra, 1961).

Finally, haematimetric indices were calculated for mean corpuscular volume (MCV: [Ht/er]×10), mean corpuscular haemoglobin (MCH: [Hb/er]×10), and mean corpuscular haemoglobin concentrations (MCHC: [Hb/Ht]×100), where Ht is haematocrit value, er is erythrocyte count and Hb is haemoglobin value (Vallada, 1999; Tavares-Dias & Moraes, 2006).

2.3.6 | Somatic indices

The same fish were euthanized by deep anaesthesia followed by medullary sectioning of the sampled liver, spleen and viscera. The organs were weighed to determine the hepatosomatic index (HSI) [(weight of liver of fish/body weight of fish)×100], splenosomatic index (SSI) [(spleen weight/carcass weight)×100], and viscerosomatic index (VI) [(visceral weight/final weight)×100].

2.3.7 | Intestinal microbiota

To assess the intestinal microbiota (probiotic bacteria×potentially pathogenic bacteria), fragments of the foregut and midgut were collected after euthanasia and macerated using a mortar and pestle in the proportion of weight by volume (1:10 w/v %) with 0.65% sterile saline solution for subsequent serial dilution of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} in 15 ml test tubes.

In addition, 100 μl aliquots of each dilution were plated in Petri dishes containing MRS and tryptone soy agar (TSA) to evaluate the growth of probiotic and potentially pathogenic bacteria respectively. These plates were incubated at 35 °C for 48 h, the cells were counted (CFU·g^{-1}) (Jatobá et al., 2008) and bacterial identification was made using the MALDI-TOF technique (Angeletti, 2017).

2.4 | Aeromonas hydrophila challenge infection-in vivo assay

2.4.1 | Pathogen preparation

Aeromonas hydrophila were grown for 24 h in brain heart infusion (BHI) broth enriched with 10% sterile fish blood and incubated at 35°C for 24 h according to Mouriño et al. (2017). After this period, the bacteria were centrifuged for 30 min at 1800 *g*, and the pellet was resuspended in sterile NaCl (0.65%) solution. The lethal infection concentration (1.8×10^8 CFU·g^{-1}) was determined by serial dilution in a 1:10 factor and confirmed by CFU by plating on BHI agar medium (Silva et al., 2012; Angeletti, 2017; Mouriño et al., 2017).

2.4.2 | Infection with aeromonas hydrophila

At the end of 120 days of probiotic supplementation, the fish from the treatments were housed in aquariums with capacities of 60 L in a static system with constant aeration, and five animals from each replicate were injected intraperitoneally with 300 μl of *Aeromonas hydrophila* at a lethal concentration of 1.8×10^8 CFU·g^{-1}. In addition, fish in the non-supplemented group were divided into two control groups: one group with free-probiotic supplementation injected with the pathogen ($n = 5$ per replicate, positive control) and another group with free-probiotic supplementation injected with sterile saline solution (0.65%) ($n = 5$ per replicate, negative control). The design was completely randomized, with six treatments and three replicates.

A fish sentinel, represented by an infection-free specimen, was added to each aquarium to assess water quality and cross-contamination during the acute infection period (Boijink & Brandão, 2004).

The injected animals were monitored for clinical signs, such as skin darkening, erratic swimming, exophthalmos, opercular haemorrhage, lethargy, ocular opacity, epidermal ulcerations and daily mortality for 96 h (Silva et al., 2012). The infection intensity was classified on a scale of zero to five, adapted from the protocol of Fishbein et al. (2005), considering the estimated percentage of observed clinical signs: grade 0, <1% of clinical signs; 1, 1%–5%; 2, 6%–10%; 3, 11%–25%; 4, 26%–50%; and grade 5, >50%.

For this experiment, the water variables were maintained according to Silva and Fujimoto (2015), Dias et al. (2018) and Silva et al. (2021), and monitored daily using the Professional*Plus* YSI multiparameter. The temperature was 28.9 ± 1.2°C, dissolved oxygen was 5.6 ± 0.2 mg·L^{-1}, pH was 5.8 ± 0.8, and total ammonia content was 1.2 ± 0.3 mg·L^{-1}.

Dying fish from the bacterial infection experiment and the survivors ($n = 10$ per treatment) at the end of the observation period had their blood sampled and analysed.

2.4.3 | Koch postulation

For confirmation of pathogen infection after the experimental challenge, blood, intraperitoneal swabs and liver samples were collected for microbiological diagnosis and bacterial re-isolation in BHI agar culture medium at 35°C for 48 h to confirm Koch's postulate. After bacterial growth, colonies were identified using the MALDI-TOF method (Angeletti, 2017).

2.5 | Statistical analysis

All data were evaluated for normality and homoscedasticity using the Shapiro–Wilk and Bartlett tests respectively. In case

of heterogeneity of variance, the data were transformed to log10 (x + 1). Mortality data were transformed into arcsine roots ($x \cdot 100^{-1}$). Microbiological counts were converted into square roots before being subjected to statistical tests. The data with normally distributed residues were subjected to analysis of variance (ANOVA) and means were compared by post hoc Tukey's test at 5% probability using the statistical programs BioEstat 5.0 and Past 4.0.

3 | RESULTS

3.1 | In vitro assays

3.1.1 | Antagonism among probiotic strains

The results for the multi-strain formulation showed concentrations of 1×10^2 CFU·ml^{-1} for *E. faecium* strains and 1×10^4 CFU·ml^{-1} for *B. cereus*, with no inhibition halo (Table 1), and a final concentration of 1×10^8 CFU·ml^{-1} for the multi-strain probiotic. This value was equivalent to CFU·g^{-1} in the fish feed after 48 h of incubation.

3.1.2 | Antagonism against Aeromonas hydrophila

All probiotic bacterial strains and the bacterial mix showed antagonistic effects against *A. hydrophila*. Nevertheless, there was no statistical difference between the single strains and the mixed formulation, which demonstrated mean inhibitory halos of 15.7 ± 0.46 mm (Table 1).

TABLE 1 Formulation of multi-strain treatment (mix) based on inhibition halo of the concentrations probiotic strains among themselves and in vitro inhibition halos of multi- and single-strain probiotics against the pathogen of *Aeromonas hydrophilia*

Antagonism probiotic strains assay	Concentration (CFU·ml^{-1})	BC	EF1	EF2
Bacilus cereus (BC)	10^2	–	–	–
	10^3	–	–	–
	10^4	–	–	–
Enterococcus faecium strain 1 (EF1)	10^2	–	–	–
	10^3	+	–	–
	10^4	+	–	–
Enterococcus faecium strain 2 (EF2)	10^2	–	–	–
	10^3	+	–	–
	10^4	+	–	–
Antagonism against pathogen assay		*Aeromonas hydrophila*		
E. faecium 1		15.6 ± 0.54		
E. faecium 2		15.4 ± 0.50		
B. cereus		16.0 ± 0.44		
Multi strain		16.1 ± 0.37		

Note: (–) without inhibition halo; (+) with inhibition halo.

3.1.3 | Dietary probiotic supplementation – in vivo assay

The inclusion of probiotic bacteria in the diets was confirmed in concentrations of $1.7 \pm 0.09 \times 10^6$, $1.2 \pm 0.13 \times 10^8$, $2.1 \pm 0.45 \times 10^6$ and $1.2 \pm 0.93 \times 10^8$ CFU·g^{-1} for the strains of *E. faecium* (1 and 2), *B. cereus*, and the multi-species mix respectively. Subsequently, the colonies were identified using the MALDI-TOF method with a score greater than 2.0 at the genus and species level, which ensured the stipulated experimental levels up to 7 days of storage under refrigeration at 4°C. No probiotic bacteria were detected in the control group.

There were no differences in productive performance between the treatment and control groups ($p < 0.05$) until 90 days. However, it was noted that the fish fed the *B. cereus* (species-specific) strain exhibited greater length gains, weights and weight gains compared with other treatments after 90 days (Table 2).

At 120 days, the fish fed *E. faecium* (2), *B. cereus*, and the multi-strain probiotics exhibited greater values of total length, weight and weight gain than fish fed *E. faecium* (1) and the control group (Table 2). The weight gain parameter demonstrated a significant increase of 50% compared with that of the control group.

Furthermore, it was possible to confirm the colonization of probiotic bacteria in the intestine, where the concentrations found were $0.9 \pm 1.68 \times 10^4$, $1.0 \pm 0.33 \times 10^4$, $1.2 \pm 0.47 \times 10^4$ and $1.0 \pm 0.78 \times 10^6$ CFU·g^{-1} for *E. faecium* (1 and 2), *B. cereus* and the mix respectively. The intestinal microbiota in the multi-strain treatment group was composed of 70% *B. cereus* and 30% *E. faecium*.

Pathogenic bacteria, such as *Aeromonas hydrophila*, *Staphylococcus aureus* and *Listeria monocytogenes* were found in the intestines of animals, and this microbiological diversity was influenced by the inclusion of probiotics in the feed. In fish fed *E. faecium* (1), *Staphylococcus aureus* ($1.1 \pm 0.13 \times 10^3$) and *Listeria monocytogenes* ($0.9 \pm 0.51 \times 10^3$ CFU·g^{-1}) were identified; in the group fed *E. faecium* (2), *Staphylococcus aureus* ($1.0 \pm 0.64 \times 10^4$ CFU·g^{-1}) was found; and in the control group, *Aeromonas hydrophila* and *Staphylococcus aureus* were observed at concentrations of $0.9 \pm 0.72 \times 10^4$ and $1.0 \pm 0.32 \times 10^4$ CFU·g^{-1} respectively. The growth of any potentially pathogenic bacteria in the intestinal microbiota of animals fed *B. cereus* and multi-strain probiotics was not observed.

Concerning the hepatosomatic index, splenosomatic index and viscerosomatic index, there was no statistical difference between the treatments ($p > 0.05$), and the mean index values registered were 1.56 ± 0.27, 0.058 ± 0.01, and 7.63 ± 0.46, respectively (Table 3).

Furthermore, the haematological analysis at the end of 120 days showed a reduction ($p < 0.05$) in the cholesterol and triglyceride levels in the juvenile tambaquis compared with the control group regardless of the probiotics used. In contrast, the levels of erythrocytes, thrombocytes, lymphocytes and neutrophils were elevated in the probiotic groups compared with the control group (Table 4).

TABLE 2 Productive performance of *Colossoma macropomum* juveniles supplemented with *Enterococcus faecium* (1 and 2), *Bacillus cereus* and multi-strains (mix) probiotics

30 days													
Treatment	TL	TLG	TLU	SL	SLG	SLU	WEIGHT	WG	WU	FCR	SGR	K	S
Control	70.1 ± 2.9	28.3 ± 2.7	96.2 ± 7.5	54.5 ± 3.1	23.1 ± 3.3	95.0 ± 5.7	5.7 ± 0.8	4.5 ± 0.8	78.75 ± 4.78	1.6 ± 0.13	5.3 ± 0.5	1.6 ± 0.14	100.00 ± 0.0
E. faecium 1	73.7 ± 1.2	31.7 ± 1.9	100.0 ± 0.0	54.5 ± 2.6	23.6 ± 1.4	97.5 ± 2.8	6.4 ± 0.4	5.3 ± 0.4	85.00 ± 4.08	1.5 ± 0.04	5.7 ± 0.2	1.6 ± 0.07	100.00 ± 0.0
E. faecium 2	72.3 ± 0.8	30.8 ± 1.3	100.0 ± 0.0	53.4 ± 1.0	22.3 ± 1.0	98.7 ± 2.5	6.2 ± 0.2	5.1 ± 0.2	66.25 ± 14.36	1.5 ± 0.03	5.6 ± 0.1	1.6 ± 0.03	100.00 ± 0.0
B. cereus	73.0 ± 2.3	31.3 ± 2.6	100.0 ± 0.0	55.2 ± 1.8	24.0 ± 2.1	98.7 ± 2.5	6.6 ± 0.5	5.4 ± 0.5	71.25 ± 4.78	1.4 ± 0.05	5.8 ± 0.3	1.6 ± 0.02	100.00 ± 0.0
Mix	73.9 ± 0.9	32.3 ± 2.3	100.0 ± 0.0	55.4 ± 1.5	24.3 ± 1.6	97.5 ± 5.0	6.8 ± 0.3	5.6 ± 0.3	77.50 ± 15.54	1.4 ± 0.02	5.9 ± 0.1	1.6 ± 0.08	100.00 ± 0.0
60 days													
Treatment	TL	TLG	TLU	SL	SLG	SLU	WEIGHT	WG	WU	FCR	SGR	K	S
Control	86.6 ± 6.0	12.5 ± 5.7	98.7 ± 2.5	66.4 ± 4.2	11.8 ± 2.8	98.7 ± 2.5	10.7 ± 1.8	5.0 ± 1.3	72.2 ± 11.6	2.9 ± 0.8	2.0 ± 0.3	2.0 ± 0.19^{b}	98.75 ± 2.5
E. faecium 1	91.8 ± 4.6	18.1 ± 4.7	100.0 ± 0.0	69.9 ± 3.6	14.8 ± 4.3	98.7 ± 2.5	12.2 ± 1.8	5.7 ± 1.8	73.4 ± 7.6	2.4 ± 0.7	2.0 ± 0.5	1.5 ± 0.01^{a}	98.75 ± 2.5
E. faecium 2	92.4 ± 4.0	20.1 ± 3.3	100.0 ± 0.0	70.7 ± 3.3	17.3 ± 3.3	100.0 ± 0.0	13.5 ± 0.9	6.5 ± 1.3	60.0 ± 7.0	2.3 ± 0.4	2.2 ± 0.2	1.5 ± 0.04^{a}	100.00 ± 0.0
B. cereus	93.7 ± 4.2	20.6 ± 3.5	100.0 ± 0.0	71.4 ± 3.3	16.2 ± 2.4	100.0 ± 0.0	13.6 ± 1.9	7.0 ± 1.6	60.0 ± 14.1	2.3 ± 0.4	2.3 ± 0.3	1.6 ± 0.07^{a}	100.00 ± 0.0
Mix	92.6 ± 3.1	18.7 ± 2.9	100.0 ± 0.0	70.2 ± 2.6	14.8 ± 2.0	100.0 ± 0.0	12.5 ± 1.8	5.7 ± 1.4	67.5 ± 11.9	2.4 ± 0.3	1.9 ± 0.3	1.5 ± 0.10^{a}	100.00 ± 0.0
90 days													
Treatment	TL	TLG	TLU	SL	SLG	SLU	WEIGHT	WG	WU	FCR	SGR	K	S
Control	117.5 ± 7.5	33.9 ± 1.0	93.3 ± 2.8	94.3 ± 6.1	27.1 ± 2.1^{b}	90.0 ± 8.6	29.5 ± 2.1^{b}	18.2 ± 1.2^{b}	53.3 ± 7.6	2.1 ± 0.2	3.1 ± 0.1	1.7 ± 0.0	98.75 ± 2.5
E. faecium 1	122.1 ± 6.0	29.3 ± 3.3	93.1 ± 5.9	95.5 ± 6.4	24.7 ± 4.5^{b}	90.0 ± 8.6	31.3 ± 1.0^{b}	17.6 ± 2.4^{b}	62.4 ± 16.4	1.9 ± 0.5	2.8 ± 0.3	1.6 ± 0.0	98.75 ± 2.5
E. faecium 2	124.0 ± 6.5	31.7 ± 2.3	90.0 ± 8.6	99.6 ± 3.4	28.7 ± 0.7^{b}	93.1 ± 5.9	33.6 ± 2.1^{ab}	19.7 ± 1.1^{b}	60.0 ± 21.7	1.8 ± 0.4	2.7 ± 0.4	1.7 ± 0.1	100.00 ± 0.0
B. cereus	130.6 ± 9.5	35.8 ± 5.4	93.3 ± 11.5	109.0 ± 6.3	37.1 ± 2.7^{a}	95.0 ± 8.6	37.0 ± 1.8^{a}	23.9 ± 0.8^{a}	56.0 ± 6.6	1.8 ± 0.4	3.0 ± 0.1	1.6 ± 0.0	100.00 ± 0.0
Mix	126.3 ± 4.9	32.3 ± 3.0	95.0 ± 8.6	99.7 ± 5.8	28.3 ± 4.5^{b}	95.0 ± 8.6	33.4 ± 2.7^{ab}	20.1 ± 1.2^{b}	56.6 ± 5.7	1.9 ± 0.2	2.9 ± 0.1	1.6 ± 0.1	100.00 ± 0.0
120 days													
Treatment	TL	TLG	TLU	SL	SLG	SLU	WEIGHT	WG	WU	FCR	SGR	K	S
Control	14.1 ± 0.8^{b}	23.8 ± 5.7	91.4 ± 10.3	11.0 ± 0.0^{b}	16.0 ± 3.2	91.4 ± 10.3	47.9 ± 3.8^{b}	18.4 ± 1.2^{b}	54.6 ± 4.4	2.0 ± 0.2	1.5 ± 0.3	1.6 ± 0.1	88.33 ± 10.4
E. faecium 1	14.4 ± 0.3^{b}	22.5 ± 2.3	96.6 ± 5.7	11.2 ± 0.0^{b}	16.2 ± 5.8	96.6 ± 5.7	53.3 ± 2.2^{b}	21.8 ± 2.0^{b}	63.3 ± 20.2	1.8 ± 0.1	1.6 ± 0.4	1.7 ± 0.0	100.00 ± 0.0
E. faecium 2	15.3 ± 1.1^{a}	29.6 ± 5.4	95.0 ± 0.0	12.7 ± 0.1^{a}	21.1 ± 2.6	91.4 ± 10.3	61.7 ± 3.0^{a}	28.1 ± 2.5^{a}	63.3 ± 12.5	1.8 ± 0.1	1.8 ± 0.1	1.6 ± 0.0	100.00 ± 0.0
B. cereus	15.5 ± 1.1^{a}	25.1 ± 7.0	91.4 ± 10.3	12.1 ± 0.4^{a}	12.1 ± 4.0	95.0 ± 0.0	63.8 ± 6.6^{a}	26.7 ± 1.2^{a}	56.1 ± 1.5	1.8 ± 0.3	1.9 ± 0.2	1.6 ± 0.0	95.00 ± 5.0
Mix	14.9 ± 1.5^{ab}	25.7 ± 8.0	96.6 ± 2.8	11.8 ± 0.3^{ab}	18.3 ± 3.0	96.6 ± 2.8	61.1 ± 4.3^{ab}	27.7 ± 2.6^{a}	59.4 ± 9.2	1.8 ± 0.2	1.9 ± 0.1	1.7 ± 0.0	98.33 ± 2.8

Note: Distinct letters in each column indicate significant differences among treatments as determined by Tukey's test ($p < 0.05$).

Abbreviations: FCR, Feed Conversion Rate; K, Fulton's condition factor; S, Survival rate (%); SGR, Specific Growth Rate (%·day^{-1}); SL, Standard Length (mm); SLG, Standard Length Gain (mm); SLU, Standard Length Uniformity (%); TL, Total Length (mm); TLG, Total Length Gain (mm); TLU, Total Length Uniformity (%); WG, Weight gain (g); WU, Weight Uniformity (%).

TABLE 4 Biochemical, haematological and haematimetric parameters of *Colossoma macropomum* juveniles supplemented with *Enterococcus faecium* (1 and 2), *Bacillus cereus* and multi-strain probiotics

Treatment	Control	*E. faecium* 1	*E. faecium* 2	*B. cereus*	Mix
Glucose ($mg{\cdot}dl^{-1}$)	48.6 ± 8.4	52.3 ± 14.7	60.6 ± 10.0	60.5 ± 15.2	58.8 ± 6.7
Cholesterol ($mg{\cdot}dl^{-1}$)	176.1 ± 33.8^{a}	163.7 ± 18.7^{ab}	131.1 ± 10.1^{b}	140.1 ± 11.1^{b}	128.1 ± 13.1^{b}
Triglycerides ($mg{\cdot}dl^{-1}$)	299.3 ± 22.1^{a}	256.1 ± 27.3^{a}	230.1 ± 12.0^{ab}	196.1 ± 12.0^{b}	200.1 ± 11.1^{b}
Erythrocytes ($\times10^{6}{\cdot}\mu l^{-1}$)	1.1 ± 0.07^{b}	1.4 ± 0.06^{a}	1.5 ± 0.03^{a}	1.6 ± 0.01^{a}	1.6 ± 0.01^{a}
Haematocrit (%)	38.3 ± 9.8	33.6 ± 6.7	37.8 ± 8.1	35.5 ± 13.6	42.3 ± 12.4
Protein ($g{\cdot}dl^{-1}$)	4.8 ± 0.3	5.0 ± 0.5	5.2 ± 0.3	5.1 ± 0.5	4.6 ± 0.6
Haemoglobin ($g{\cdot}dl^{-1}$)	14.8 ± 4.4	9.6 ± 3.7	12.7 ± 4.6	13.1 ± 4.2	16.3 ± 5.2
MCV (fl)	147.9 ± 8.4	166.0 ± 10.1	167.5 ± 7.2	165.3 ± 9.1	156.9 ± 6.2
MCH ($g{\cdot}dl^{-1}$)	65.2 ± 7.91	62.9 ± 8.2	61.8 ± 9.1	63.4 ± 8.2	63.1 ± 7.4
MCHC ($g{\cdot}dl^{-1}$)	37.2 ± 6.1	37.5 ± 6.3	35.4 ± 3.7	39.4 ± 7.0	39.3 ± 8.1
Thrombocytes ($\times10^{3}{\cdot}\mu l^{-1}$)	39.0 ± 2.2^{b}	44.9 ± 1.5^{b}	47.7 ± 2.4^{b}	67.0 ± 7.4^{a}	66.9 ± 11.0^{a}
Leucocytes ($\times10^{3}{\cdot}\mu l^{-1}$)	38.0 ± 7.6^{b}	52.7 ± 4.3^{a}	56.7 ± 8.4^{a}	60.9 ± 9.8^{a}	58.9 ± 6.8^{a}
Lymphocytes ($\times10^{3}{\cdot}\mu l^{-1}$)	31.7 ± 8.4^{b}	45.0 ± 4.1^{a}	48.4 ± 3.8^{a}	50.2 ± 9.2^{a}	49.7 ± 7.8^{a}
Monocytes ($\times10^{3}{\cdot}\mu l^{-1}$)	2.1 ± 2.7	3.0 ± 4.4	2.2 ± 3.1	4.0 ± 6.2	4.1 ± 4.5
Neutrophils ($\times10^{3}{\cdot}\mu l^{-1}$)	1.5 ± 1.4^{b}	3.5 ± 2.4^{ab}	3.7 ± 2.1^{ab}	5.2 ± 0.9^{a}	5.0 ± 1.0^{a}

Note: Different letters in the same row indicate significant differences after Tukey's test ($p < 0.05$).

Abbreviations: MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume.

3.2 | Aeromonas hydrophila challenge infection – in vivo assay

The negative control group showed 100% survival, as did sentinel fish. These results revealed the safe conditions of the experiment without interference from management or external factors during the investigation period.

Tambaquis fed probiotics demonstrated higher ($p < 0.05$) survival and resistance during acute infection with *A. hydrophila*. Fish fed *E. faecium* (1 and 2), *B. cereus*, and the multi-strain probiotics showed maximum mortality rates of 40%, 33.3%, 26% and 20%, respectively, between 30 and 62 h, and subsequent stabilization until the end of the experiment at 96 h (Figure 1).

The first clinical signs of infection, such as skin darkening, occurred after 28 h of observation. However, animals that received *B. cereus* and the multi-strain mix in the diet recovered normal coloration after 42 h (Figure 2).

In contrast, the fish from the positive control group (no supplemented or pathogen injected) presented intense clinical signs at 31–32 h post injection, such as skin darkening, erratic swimming and exophthalmos. Additionally, other alterations, including haemorrhagic petechiae in the epidermis, lethargy, pale gills, fin erosion and haemorrhages on the mouth were pronounced at 55 h after injection (Figure 1).

E. faecium (1 and 2) treatments and the positive control injected with *A. hydrophila* displayed the highest clinical infection intensities with pathological classifications of 47.05% (4), 41.17% (4) and 82.35% (5) respectively (Table 5).

Regarding the haematological aspects, the animals from the positive control had the highest glucose levels (155.8 ± 61.9 $mg{\cdot}dl^{-1}$) and lowest haemoglobin means (2.2 ± 1.0 $g{\cdot}dl^{-1}$), which highlights the impossibility of determining the concentrations of erythrocytes, haematocrit, total plasma protein and haematimetric parameters (Table 6). In contrast, the values of the haematological parameters of the groups fed probiotics were similar to those of the negative control, except for that of glucose, where the treatments with probiotics promoted intermediate values of glycaemia (Table 6).

At the end of acute *A. hydrophila* infection, lower values of thrombocytes, lymphocytes, monocytes, neutrophils and basophils ($p < 0.05$) were observed in the positive control (Table 6). The treatments that included the inclusion of probiotics in the feed had higher concentrations of leucocytes (treatment mean of $63.0 \pm 3.3 \times 10^{3}{\cdot}\mu l^{-1}$) and lymphocytes ($53.1 \pm 2.2 \times 10^{3}{\cdot}\mu l^{-1}$) compared with the positive and negative controls. In addition, higher mean ($p < 0.05$) thrombocyte concentrations were noted in treatments with probiotics containing *B. cereus* and the multi-strain mix (Table 6).

Koch's postulate was confirmed by re-isolating *A. hydrophila* in the blood, intraperitoneal cavity and liver of recently deceased animals.

4 | DISCUSSION

4.1 | Probiotics in fish diets

The probiotics in the fish diet were established within the experimental concentrations. This result indicates effective diet preparation with no cross-contamination between treatments (Dias et al., 2018).

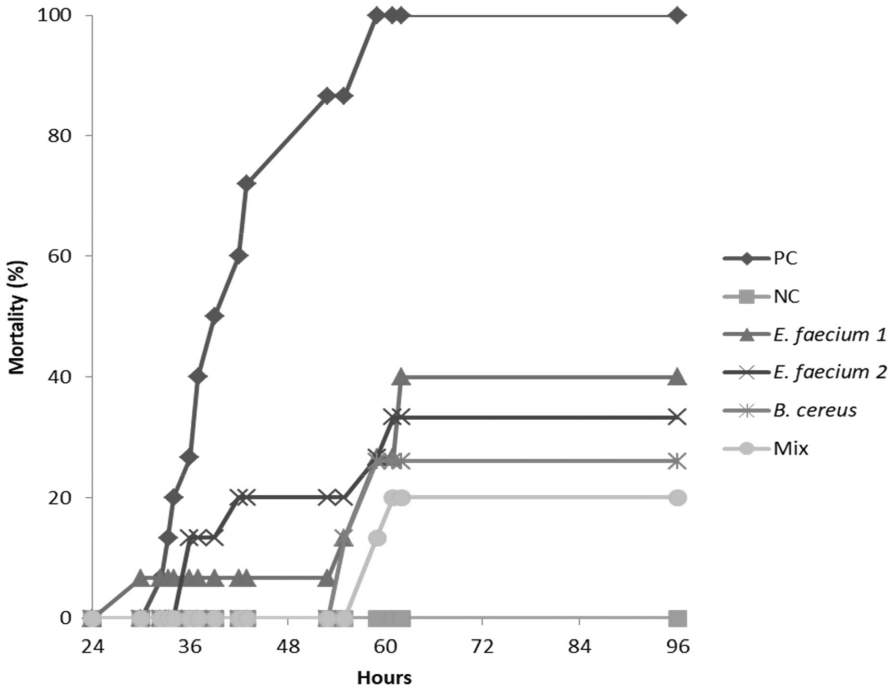

FIGURE 1 Accumulated mortality up to 96 h after infection with *Aeromonas hydrophila* in juveniles of tambaquis (*Colossoma macropomum*) fed for 120 days diets supplemented with *Enterococcus faecium* (1 and 2), *Bacillus cereus* and multi-strain probiotics. PC, Positive Control; NC, Negative Control

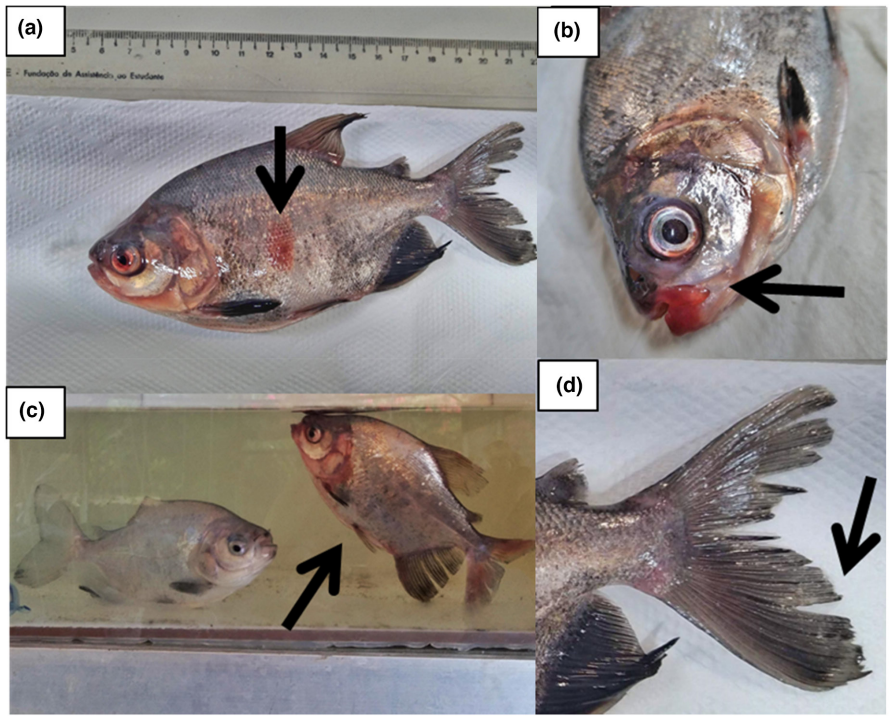

FIGURE 2 Clinical signs of *Colossoma macropomum*, supplemented for 120 days with the probiotics *Enterococcus faecium* (1) 2×10^{6} CFU·g^{-1}, *Enterococcus faecium* (2) 1×10^{8} CFU·g^{-1}, *Bacillus cereus* 2.8×10^{6} CFU·g^{-1} and multi-strain (mix) 1×10^{8} CFU·g^{-1}. (a) Petechial Haemorrhage in the epidermis; (b) Haemorrhage on the mouth; (c) erratic swimming and (d) fin erosion

4.2 | Probiotic intestinal colonization and its effects on microbiota

The probiotics resisted the digestive processes and colonized the intestines of the animals in all probiotic treatments similarly to those of Merrifield et al. (2010) and Dias et al. (2018). The treatments with *B. cereus* and the multi-strain mix demonstrated the lowest logarithmic losses in the intestines of the animals.

The *B. cereus* and multi-strain probiotics acted on the tambaqui microbiota modulation to prevent pathogenic bacterial colonization. This probiotic effect on pathogenic bacteria is a promising feature related to the competitive exclusion of these microorganisms from the production of antimicrobials and quorum sensing (Miller & Bassler, 2001; Gatesoupe, 2008; Mouriño et al., 2017; Jatobá et al., 2018).

In the multi-strain treatment, *E. faecium* and *B. cereus* produced acetic and propionic acids, which have bactericidal action, thus controlling

TABLE 5 Clinical signs of *Colossoma macropomum* challenged with *Aeromonas hydrophila* after supplementation with *Enterococcus faecium* (1 and 2), *Bacillus cereus* and multi-strain probiotics

Treatment	PC	NC	*E. faecium* 1	*E. faecium* 2	*B. cereus*	Mix
Dark coloration of the epidermis	*	-	*	*	*	*
Erratic swimming	*	-	*	*	-	-
Exoftalmia	*	-	*	*	-	-
Haemorrhagic petechiae in the epidermis	*	-	*	*	-	-
Lethargy	*	-	*	*	-	-
Gill pallor	*	-	*	*	-	-
Erosion of the fins	*	-	*	*	-	-
Haemorrhages on mouth	*	-	*	-	-	-
Hyperaemia in the eye region	*	-	-	-	-	-
Inflamed urogenital pore	*	-	-	-	-	-
hyperaemia in the nostril	*	-	-	-	-	-
Abdominal distension	*	-	-	-	-	-
Ocular opacity	*	-	-	-	-	-
Liver change	*	-	-	-	-	-
Gallbladder change	-	-	-	-	-	-
Kidney change	-	-	-	-	-	-
Spleen change	-	-	-	-	-	-
Occurrence of clinical signs (%)	82.35	0.00	47.05	41.17	5.88	5.88
Degree of infection	5	0	4	4	1	1

Note: (*) Degree of infection; (–) Absence of clinical signs. Adapted from the protocol by Fishbein et al. (2005).
Abbreviations: NC, Negative control; PC, Positive control.

intestinal and intracellular pH, increasing epithelial growth, promoting intestinal microbiota balance, preventing dysbiosis and maintaining microbiological stability in the host intestine (Aly et al., 2008; Amiri, & Yousefian, 2009; Standen et al., 2016; Doan et al., 2018; Elsabagh et al., 2018; Ghori et al., 2018; Poolsawat et al., 2020).

4.3 | Growth parameters

In the current study, an improvement ($p < 0.05$) in the productive parameters of total length, weight and weight gain after 90 days of feeding with the *B. cereus* and multi-strain probiotics mix was noted. Growth improvements are related to the production of bacterial enzymes such as proteases and amylases, which improve the use of dietary amino acids, fatty acids, carbohydrates and minerals (Essa et al., 2010; Pirarat et al., 2011).

Furthermore, the higher erythrocyte, glycaemia, thrombocyte, lymphocyte and neutrophil counts in supplemented fish corroborate the improvement in growth performance and health. In previous studies, animals fed probiotics exhibited the highest glycaemic concentrations, reflected by better nutrient absorption (Ranzani-Paiva et al., 2013; Munir et al., 2018). Supplementation with probiotics promotes a response to stressful conditions and increases metabolism and tissue oxygenation. As a result, performance levels and animal health are improved as previously reported by Burgos-Aceves et al. (2019) and Mukherjee et al. (2019).

Regular consumption of probiotics also contributed to a reduction in cholesterol and triglyceride levels. These substances help probiotic bacteria to break down, synthesize, oxidize and absorb medium- and short-chain fatty acids in the intestine of animals. Consequently, this metabolic process not only reduces the enzymatic activities of cholesterol biosynthesis but also reduces lipogenesis, leading to greater catabolism of fatty acids. Therefore, beneficial nutritional effects on the host were observed due to the reduction in these parameters, which corroborates the findings of Holzapfel & Schillinger (2002) and Munir et al. (2018).

4.4 | Bacterial challenge

In the positive control (no supplementation-pathogen injection), the maximum degree of virulence of the infectious agent with haemorrhagic processes in animals was observed, and it was not possible to detect the concentrations of erythrocytes, haematocrit or total plasma protein. The *Aeromonas* genus causes haemolytic anaemia and erythrocyte abnormalities in fish (Clauss et al., 2008). This haemolytic activity results from extracellular products, such as exotoxins, which help the pathogen to adhere to cells and produce proteases that are responsible for anaemia caused by the rupture of erythrocytes (Ribeiro et al., 2016; Munir et al., 2018).

However, the higher values of lymphocytes, monocytes and neutrophils observed in probiotic treatments compared with the positive

TABLE 6 Biochemical and haematological parameters of *Colossoma macropomum* juveniles infected with *Aeromonas hydrophila* after supplementation with *Enterococcus faecium* (1 and 2), *Bacillus cereus*, and multi-strain probiotics

Treatment	PC	NC	*E. faecium* 1	*E. faecium* 2	*B. cereus*	Mix
Glucose ($mg{\cdot}dl^{-1}$)	155.8 ± 61.9^{a}	27.9 ± 3.7^{d}	90.0 ± 27.7^{b}	39.9 ± 6.4^{c}	42.8 ± 8.1^{c}	44.7 ± 11.1^{c}
Erythrocytes ($\times 10^{6}{\cdot}\mu l^{-1}$)	ND	0.892 ± 0.0	1.225 ± 0.0	1.136 ± 0.0	1.342 ± 0.0	1.288 ± 0.0
Haematocrit (%)	ND	22.8 ± 2.2	29.0 ± 2.8	24.3 ± 5.7	26.6 ± 5.1	26.7 ± 3.4
Haemoglobin ($g{\cdot}dl^{-1}$)	2.2 ± 1.0^{b}	15.4 ± 10.2^{a}	8.9 ± 6.0^{a}	8.8 ± 4.6^{ab}	15.1 ± 9.5^{a}	14.7 ± 4.0^{a}
TTP ($g{\cdot}dl^{-1}$)	ND	4.2 ± 0.6	4.5 ± 0.3	4.8 ± 0.2	4.8 ± 0.4	4.8 ± 0.4
MCV (fl)	-	119.3 ± 9.7	132.0 ± 8.0	132.8 ± 5.7	125.5 ± 8.2	132.2 ± 4.9
MCH ($g{\cdot}dl^{-1}$)	-	52.1 ± 6.3	49.4 ± 6.5	51.1 ± 7.2	50.5 ± 6.5	50.7 ± 5.9
MCHC ($g{\cdot}dl^{-1}$)	-	29.7 ± 4.8	28.3 ± 5.0	30.0 ± 2.9	31.4 ± 5.6	31.5 ± 6.4
Thrombocytes ($\times 10^{3}{\cdot}\mu l^{-1}$)	5.4 ± 0.3^{d}	27.3 ± 1.5^{c}	58.3 ± 1.9^{b}	62.0 ± 3.1^{b}	87.1 ± 9.6^{a}	87.0 ± 14.3^{a}
Leucocytes ($\times 10^{3}{\cdot}\mu l^{-1}$)	8.3 ± 1.6^{c}	41.8 ± 8.3^{b}	58.0 ± 4.7^{a}	62.4 ± 9.2^{a}	67.0 ± 10.7^{a}	64.8 ± 7.4^{a}
Lymphocytes ($\times 10^{3}{\cdot}\mu l^{-1}$)	6.9 ± 1.8^{c}	34.9 ± 9.2^{b}	49.5 ± 4.5^{a}	53.2 ± 4.1^{a}	55.2 ± 10.1^{a}	54.7 ± 8.5^{a}
Monocytes ($\times 10^{3}{\cdot}\mu l^{-1}$)	0.427 ± 0.5^{b}	2.1 ± 2.9^{a}	3.0 ± 4.8^{a}	2.27 ± 3.4^{a}	4.0 ± 6.8^{a}	4.1 ± 4.9^{a}
Neutrophils ($\times 10^{3}{\cdot}\mu l^{-1}$)	0.378 ± 0.3^{c}	1.8 ± 1.6^{b}	4.2 ± 2.8^{ab}	4.4 ± 2.5^{a}	6.2 ± 1.0^{a}	6.0 ± 1.2^{a}
Basophils ($\times 10^{3}{\cdot}\mu l^{-1}$)	ND	0.131 ± 0.0^{b}	0.118 ± 0.0^{b}	1.414 ± 0.0^{a}	1.249 ± 0.0^{a}	1.145 ± 0.0^{a}

Note: Different letters in the same row indicate significant differences after Tukey's test ($p < 0.05$).

Abbreviations: MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; NC, Negative control; ND, Not detected; PC, Positive control; TTP-, total plasma protein.

control directly interfered with the health and survival of animals during the bacterial infection process. These statements are corroborated by Farias et al. (2016), who supplemented *Piaractus mesopotamicus* diets with a mix of *B. cereus* and *B. subtilis* (1×10^{8} CFU·g^{-1}) and Tanekhy et al., (2016), who used a mix of *Lactobacillus sp.*, *Pediococcus sp.*, *Gluconacetobacter sp.* and *Saccharomyces sp.* (1.5×10^{10} CFU·g^{-1}) to control *A. hydrophila* infection.

This improvement in the cellular immune system resulted in the lowest mortality rates during the bacterial challenge assay, and was responsible for colour restoration and enhanced animal behaviour at 42 h post infection with *A. hydrophila* in fish fed *B. cereus* and the mix. These results indicated the positive effects of probiotic supplementation.

E. faecium and *B. cereus* stimulated the lymphoid tissue, and consequently prevented the attachment of pathogenic bacteria to the epithelial cells of the gut-associated lymphoid tissue (GALT), leading to the recruitment and activation of neutrophils and monocytes (Nayak, 2010; Bloes et al., 2012; Feria et al., 2017).

Similar findings were observed in fish that received a combination of *Pediococcus pentosaceus* and *Bacillus subtilis*, *B. subitilis* and *B. licheniformis*, and *Bacillus subtilis and L. acidophilus* during 60 days of supplementation, which presented survival rates of 25%, 56% and 52%, respectively, after infection with *A. hydrophila* and *A. salmonicida* (Aly, Ahmed, Ghareeb and Mohamed, 2008; Kaew-on, Areechon, and Wanchaitanawong, 2016; Park et al., 2016). Nevertheless, these survival rates were lower than observed in the present study, which may be related to the origin of the strains (autochthonous or allochthonous), dosage, time of use, production system, pathogen virulence and synergistic interactions between the probiotic bacteria applied (Ng et al., 2014).

Therefore, the use of probiotic strains in the diet of tambaquis improved haematological and biochemical responses, and increased leucocyte and thrombocyte concentrations, favouring animal resistance during the acute infection process with *A. hydrophila*. These findings corroborate those of Dias et al. (2018) and Paixão et al. (2020), who used a single-strain probiotic diet with *Bacillus* spp. or lactic acid bacteria. Nonetheless, the mechanisms of molecular and cell-to-cell synergistic actions in the use of multispecies probiotics need to be better understood in terms of the health and performance aspects of confined animals.

4.5 | Remarking

The use of multi-strain probiotics in tambaqui production was revealed to be an efficient alternative similar to the species-specific probiotics used in high doses as reported by Dias et al. (2018) with autochthonous monostrains for *Colossoma macropomum*.

This beneficial interaction of the bacterial mix demonstrates relevant prophylactic advantages as described in the literature. The action of multi-strain probiotics may be more effective than that of single-strain probiotics, regardless of whether the strains are suitable for each organism, even at lower concentrations, because of a positive synergism between the microorganisms (Timmerman et al., 2004; Douillard et al., 2018).

5 | CONCLUSION

Enterococcus faecium (1 and 2), *B. cereus* and the multi-strain probiotic favoured productive and haematological parameters, as well as the survival and rehabilitation after *Aeromonas hydrophila* infection in juvenile tambaquis.

Moreover, the application of species-specific strains and the use of the multi-species treatment inhibited the growth of potentially pathogenic bacteria in the animal intestine after 120 days of supplementation.

Finally, probiotics demonstrated significant effectiveness as an alternative method to avoid dysbiosis in juvenile tambaquis, which suggests their application as a prophylactic measure in intensive production systems of the respective species.

AUTHOR CONTRIBUTION

All authors listed executed substantial contributions to the conception or design of thework; or the acquisition, analysis, or interpretation of data for the work; and drafting the work or revising it critically for important intellectual content; and final approval of the version to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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ETHICS STATEMENT

The study was conducted in accordance with the guidelines on the care and use of animals for scientific purposes set up by Ethics Committee in Animal experimentation of the Brazilian Agricultural Research Corporation (EMBRAPA), no. 0034/2020, Sergipe, Brazil.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the author upon reasonable request.

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