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ORIGINAL ARTICLE

Dietary supplementation of Probiotic *Enterococcus faecium* improve resistance in *Arapaima gigas* against *Aeromonas hydrophila*

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Abstract

This study evaluated the mortality rate, histopathology and haematological parameters of *Arapaima gigas* against *Aeromonas hydrophila* after 68 days of dietary probiotic supplementation with autochthonous bacterium *Enterococcus faecium*. Three experiments were carried out: the first assay evaluated the lethality of *A. hydrophila*, the second and the third assay evaluated the fish supplemented subjected to *A. hydrophila* lethal dose 30% (10^6 CFU ml^{-1}) and lethal dose 100% (10^8CFU ml^{-1}) respectively. The clinical signs, blood changes, histopathological alterations and mortalities were evaluated. At the first experiment, the concentrations of 10^6 and 10^8CFU.mL^{-1} with *A. hydrophila* caused 33% and 100% of mortality. The *A. hydrophila* infection provoked clinical signs such as dark skin, ulceration, haemorrhage, pale gills and liver, hepatic alterations, hyperaemia, hepatic cord breakdown, cellular deforming, lipid degeneration and necrosis. In the second experiment, no mortality occurred onto fish fed with probiotic. Furthermore, in the third experiment, fish submitted to probiotic supplementation showed reduction in mortality of 75% compared with the control and fish fed with probiotic diets at 10^8CFU g^{-1} not presented any clinical signs. For these reasons, *Arapaima gigas* juveniles previously submitted to probiotic supplementation with *E. faecium* (10^8CFU g^{-1}) shows better physiological and immunological response, improving resistance against *A. hydrophila* infection.

KEYWORDS
challenge, experimental infection, haematological, pirarucu

1 | INTRODUCTION

Diseases have become a serious problem in worldwide fish farming, causing outbreaks resulting in mass mortalities and economic losses of USD 1.05 billion (Hoai et al., 2019; Shinn et al., 2015). In Brazil, the fish farming production in 2017 reached 485 thousand tonnes; however, there was a reduction compared with the previous year (IBGE, 2017; Fao, 2018), partially which occurred due to mortality outbreaks.

Among the most important pathogenic bacteria for fish farming, *Aeromonas hydrophila* has been highlighted as an opportunistic bacterium with zoonotic potential, which can cause rapid infection, reaching 73% mortality in fish farms (Plumb et al., 2011; Silva et al., 2012; El-Bahar et al., 2019; Zhang et al., 2016). However, its

infection depends on the bacteria's virulence, pathogenicity and the host resistance (Dias et al., 2016; El-Bahar et al., 2019).

Fish farmers commonly use antibiotics as the main treatment to control the *Aeromonas* infection (Quesada et al., 2013; Ramesh & Souissi, 2018). However, the eco-friendly strategies using probiotics to prevent fish diseases has been reported improving the immunological system and the resistance against pathogens (Jatobá et al., 2011; Giri et al., 2013; Mouriño et al., 2015; Rihda and Azad, 2016; Shahid et al., 2017; Dias, Santos, et al., 2018; Dias, Abe, et al., 2018; Sousa et al., 2019).

The pirarucu *Arapaima gigas* stands out in South American fish farming because of its rapid growth (10 kg in one year), rusticity and easy adaptation to various rearing systems (Cavero et al., 2003; Oliveira et al., 2012; Scorvo filho et al., 2004). However, in early phases, this species is susceptible to infectious diseases (bacterial infection), causing economic loss to the fish farmers.

Prior report presented improvements on the growth performance and immunological system in *A. gigas* supplemented with autochthonous probiotics (Sousa et al., 2019); however, there is no information regarding the resistance of the *A. gigas* to sanitary challenges. For these reasons, this study evaluated the mortality rate, histopathology and haematological parameters of *Arapaima gigas* challenged against *Aeromonas hydrophila* after dietary probiotic supplementation with autochthonous bacterium *Enterococcus faecium*.

2 | MATERIALS AND METHODS

The ethic committee of experiment with animals approved this study according to protocol CEUA/172008FAPESPA01.

2.1 | Culture of pathogen

The pathogenic bacterium *A. hydrophila* (CPQBA22808) used in this study was provided by Federal University of Santa Catarina. The pathogen was cultured in assay tubes containing Brain Heart Infusion broth (BHI broth) for 24 h at 30°C. After bacterial growth, the bacterial suspension was serially diluted (1:10) and then plated in tryptone soya agar (TSA) for bacterial counting (colony forming unit, CFU ml^{-1}).

For the sanitary challenge, the pathogen was centrifuged for 30 min at 1800 g, the supernatant was removed, and saline solution (NaCl 0.65%) was added to prepare the experimental concentrations.

2.2 | Experiment 1. Lethality of *A. hydrophila* in *A. gigas*

Firstly, 12 pirarucu juveniles (11.23 ± 1.48 g and 12.50 ± 1.35 cm) fed with free-probiotic diet were used to evaluate the lethal concentrations of *A. hydrophila*. The fish received microchips (animal tags) for identification. This experiment was performed using a completely randomized design with four treatments (ISSS—intraperitoneal injection of sterile saline solution NaCl 0.65%; $IAH10^4$–*A. hydrophila* at concentration 1.2×10^4 CFU. ml^{-1}; $IAH10^6$–*A. hydrophila* at concentration 1.2×10^6 CFU ml^{-1}; $IAH10^8$–*A. hydrophila* at a concentration of 1.2×10^8 CFU ml^{-1}) and three replicates (fish itself was a replicate). The fish were maintained in polyethylene tanks (300 L capacity) in a static system for 96 h, and the clinical signs and mortalities were monitored every four hours. The clinical signs evaluated were erratic swimming, lethargic behaviour, individual withdrawal behaviour, spasm and operculum beat alteration (Andrade-Porto et al., 2018; Dias et al., 2016; Dias, Santos, et al., 2018; Dias, Abe, et al., 2018). Each treatment also received a fish, named as *'Sentinel'*, to observe any possible effect of water quality parameter alterations or cross infection, according to Lima Boijink and Brandão (2001).

The concentrations that provoked 30% (LC_{30}) and 100% (LC_{100}) of mortality were used in experiment 2 and 3 respectively.

In the second and third experiment, the fish used were from experiment of Sousa et al. (2019). This previous supplemented experiment used fish that received diets containing *Enterococcus faecium* (CD—control diet using only commercial ration, MRSD—commercial diet containing Man Rugosa Sharped Agar, $D10^6$- Diet containing *E. faecium* in 106 CFU g^{-1} and $D10^8$- Diet containing *E. faecium* in 108 CFU g^{-1}). The feeding procedure occurred three times a day at feeding rate 10% of live weight for 68 days. The commercial diet had crude protein 45%, fat 8%, moisture 12%, fibre 4%, mineral 14%, phosphorus 0.60% and calcium 2.5% (Sousa et al., 2019). The probiotic strains were grown into MRS liquid medium, at 35°C for 24 hours, centrifuged at 1.800 g for 15 min and resuspended in sterile saline solution (SSE 0.65%) (Jatobá et al., 2011) and then sprinkled on commercial ration. The fish food was renewed every seven days and stored in a refrigerator at 4°C.

2.3 | Experiment 2: Bacterial challenge using lethal concentration 30% (LC_{30}) in *A gigas* supplemented with probiotic

A total of 16 juvenile *A. gigas* (128.85 ± 16.2g and 23.17 ± 2.64 cm) from previous probiotic supplementation were used for this experiment.

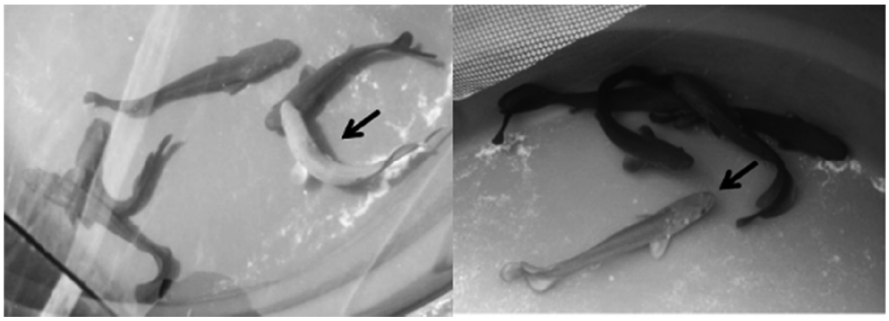
The bacterial challenge assay was carried out using intraperitoneal injection *A. hydrophila* at 1.0×10^6 CFU ml^{-1} equivalent to LC_{30} ($IAH10^6$). All fish were individually tagged with microchips (animaltags) for identification, and the fish itself was a replicate. Furthermore, a *'Sentinel'* fish also included to observe any possible effect of water quality parameter alterations or cross infection. After infection, the fish were maintained in four polyethylene tanks (300 L) in static system. The clinical signs and mortalities were monitored for 192 h continuously every 4 h.

TABLE 1 Water quality parameters (Mean values ± *SD*) for all experiments with fish juveniles *Arapaima gigas* challenged by *Aeromonas hydrophila*

	T (°C)	DO (mg L^{-1})	pH	EC (μS cm^{-1})	TA (mg L^{-1})
Exp 1	27.0 ± 0.7	6.8 ± 0.4	7.0 ± 0.2	225.0 ± 5.0	0.4 ± 0.1
Exp 2	29.2 ± 0.3	7.0 ± 0.2	7.3 ± 0.3	256.0 ± 15.0	0.5 ± 0.1
Exp 3	30.0 ± 0.5	6.5 ± 0.6	7.2 ± 0.1	310.1 ± 25.0	0.8 ± 0.1

Note: Abbreviation: DO, dissolved oxygen; EC, electric conductivity; T, temperature; TA, total ammonia.

FIGURE 1 Clinical signs (darkest skin) of juveniles fish *Arapaima gigas* challenged by *Aeromonas hydrophila* (1.2×10^6 and 1.2×10^8 CFU ml^{-1}). Arrow indicates the sentinel fish without clinical sign *darkest skin*



2.4 | Experiment 3: Bacterial challenge using lethal concentration 100% (LC_{100}) in *A. gigas* supplemented with probiotic

This assay used the same experimental design from previous experiment; however, the *A. hydrophila* dose used was equivalent to LC_{100} (IAH10^8 1.2×10^8 CFU ml^{-1}).

2.5 | Water quality parameters

The water quality parameters, such as temperature (YSI app550A), dissolved oxygen (YSI app550A), pH (YSI60), electric conductivity (AKSO-AK51) and total ammonia (Hanna HI93715), were measured daily in all experiments.

2.6 | Haematological analysis

During the trial period (dying fish) and after 192h of observation (survival fish), the fish underwent an anaesthetic procedure (60 mg L^{-1} eugenol sprinkled on gills), and blood withdrawn by caudal puncture with syringes containing 3% EDTA (Honczaryk & Inoue, 2009). Blood samples were used to perform blood smears dyed with panotic method for leucocytes differential counting and total thrombocytes. In addition, total erythrocytes (Er), haematocrit (Goldenfarb et al., 1971), total plasma protein (refractometer Quimis®) and haemoglobin (Hb) (cianemtahaemoglobin method, Collier 1944) were measured. Finally, hematimetric indices were determined: mean corpuscular volume (MCV: Ht ×10/Er), corpuscular haemoglobin concentration (CHC: Hb ×10/Er) and mean corpuscular haemoglobin concentration (MCHC: Hb ×100/Ht) (Vallada, 1999).

2.7 | Microbiological analysis

To confirm infection, kidney fragments were collected to isolate the pathogen. These fragments were macerated at a proportion of 1 g of kidney for 1 ml of 0.65% NaCl (sterile saline solution). Subsequently, samples were inoculated on Petri dishes containing TSA and cultured for 24 h at 30°C, to confirm Koch's postulates. Furthermore, the colonies were adequately characterized with regard to morphology and Gram coloration and were finally identified by MALDI-TOF method (Seuylemezian et al., 2018; Sousa et al., 2019).

2.8 | Histological analysis

All fish underwent surgery to remove fragments of the liver, which were fixed in formalin (10% formalin +4% tetraborate) for 24 h and then into alcohol 70% until histological analysis. Subsequently, all fragments were dehydrated, diaphanized, and then embedded in paraffin to perform histological cuts at 5 μm, posteriorly stained with haematoxylin-eosin (HE) (Behmer & Tolosa, 1976; Honorato et al., 2014). The severity degree of infection in the liver was determined using the symbol (+) according Van Dyk et al. (2007).

2.9 | Statistical analysis

In the lethal doses experiment, a regression curve of Pearson was applied to determine the lethal dose. Other data were subjected to normality (Shapiro Wilk) and homoscedasticity tests (Levene's), and then subjected to analysis of variance with the post hoc Tukey test ($p < 0.05$) (Zar, 2009).

3 | RESULTS

For all experiments, the water quality parameters did not differ significantly ($p < 0.05$) (Table 1). None of the *sentinel* fish died or showed any behavioural alterations during the experimental period.

3.1 | Experiment 1. Lethality of *Aeromonas hydrophila* in *Arapaima gigas*

In the lethal doses experiment, the highest mortality rate was observed in the first 25 h of the experiment, ranging from 33% to 100% at concentrations of 10^6 and 10^8 CFU.mL^{-1} respectively. A positive correlation ($r^2 = 0.82$, $p = 0.002$, $Y = 6 \times 10^7 X + 8.3778$) allowed determined the lethal dose of 30% of *A. hydrophila* for *A. gigas* at 1.0×10^6 CFU ml^{-1}.

Throughout the trial period (for all experiments), susceptible fish to infection demonstrated lethargy, erratic swimming, quick operculum beating, spasms, long periods on the water surface and dark skin. All symptoms were first registered at 24 h after experimental infection with *A. hydrophila* (Figure 1).

The fish showed ulceration, localized inflammation, haemorrhagic petechiae, pale gills and liver, darkest skin, local depigmentation and internal haemorrhage (Figure 2). These clinical signs have different degrees related to the concentration of infection. All surviving fish, including *sentinel* fish, demonstrated normal behaviour and absence of clinical signs until the end of the experiment.

Higher concentrations of *A. hydrophila* (10^6 and 10^8 CFU ml^{-1}) caused reduced erythrocyte values and increased hematimetric indexes. Other blood parameters did not differ statistically among the treatments (Table 2).

Thrombocytes and neutrophils were also reduced at all pathogen concentrations. Only monocytes showed a reduction at higher concentrations (10^6 and 10^8 CFU ml^{-1}) (Table 3).

3.2 | Experiment 2: Bacterial challenge using lethal concentration 30% (LC_{30}) in *A gigas* supplemented with probiotic

In this challenge, the fish without probiotic supplementation showed 25% mortality rate after subjected to *A. hydrophila* (IAH10^6) injection; however, no mortalities were observed in probiotic-supplemented fish. Furthermore, the fish supplemented (D10^6 and D10^8) showed increased values of erythrocytes (2.32 ± 0.30 and 2.31 ± 0.08 cell × 10^6 µl^{-1}), haematocrit (44.92 ± 2.50 and 45.30 ± 2.16%), haemoglobin (13.80 ± 0.30 e 14.10 ± 0.30 g dl^{-1}), MCV (365.00 ± 32.21 and 380.76 ± 35.86 fl), and MCH (89.40 ± 11.03 and 96.50 ± 5.39 pg), respectively, compared with fish without probiotic supplementation (Table 4).

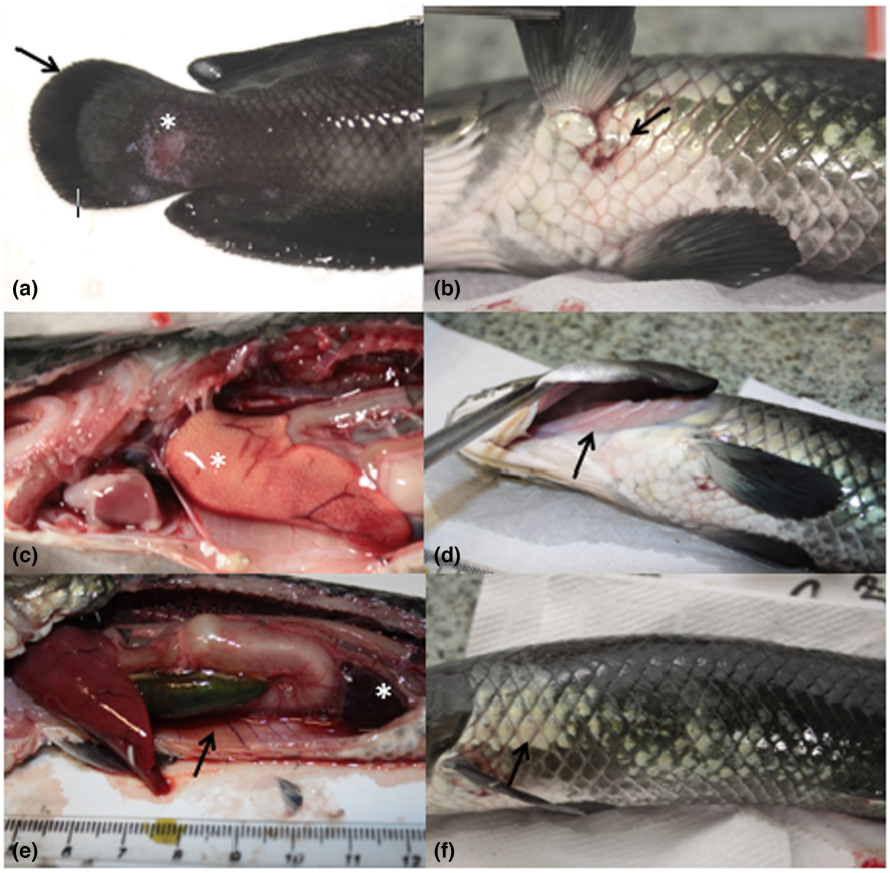


FIGURE 2 Clinical signs of juvenile fish of *Arapaima gigas* challenged by *Aeromonas hydrophila*. (a) Arrow indicates darkest skin and asterisk indicates ulceration close to the caudal fin, (b) local haemorrhage near of local of injection, (c) asterisk indicates pale liver, (d) asterisk indicates pale gills, (e) arrow indicates haemorrhage and asterisk increased spleen, (f) arrow indicates local depigmentation

TABLE 2 Red blood cell (Mean values ± *SD*) of juvenile fish *Arapaima gigas* challenged with *Aeromonas hydrophila* (first experiment)

	ISSS	IAH10^4	IAH10^6	IAH10^8
Er (×10^6 µl^{-1})	2.55 ± 0.31 a	2.65 ± 0.30 a	1.56 ± 0.34 b	1.2 ± 0.09 b
Ht (%)	44.38 ± 12.39 a	41.98 ± 4.60 a	49.00 ± 6.56 a	43.16 ± 1.76 a
Hg (g dl^{-1})	14.55 ± 7.00 a	14.00 ± 2.50 a	13.70 ± 9.60 a	13.50 ± 0.50 a
TPP (g dl^{-1})	3.96 ± 0.80 a	3.72 ± 0.54 a	3.70 ± 0.30 a	3.50 ± 0.50 a
MCV (fl)	166.37 ± 12.73 b	158.42 ± 17.38 b	314.10 ± 42.03 a	359.72 ± 14.63 a
MCH (pg)	47.64 ± 1.10 b	38.15 ± 12.91 b	98.27 ± 15.00 a	126.44 ± 11.76 a
MCHC (g dl^{-1})	26.94 ± 5.88 b	23.81 ± 5.93 b	31.82 ± 7.13 a	35.22 ± 4.08 a

Note: Different letters in the row means statistical difference by Tukey test ($p < 0.05$).

Abbreviatons: Er, erythrocyte; Hg, haemoglobin; Ht, haematocrit; IAH10^4, injection with *A hydrophila* at concentration 10^4; IAH10^6, injection with *A. hydrophila* at concentration 10^6; IAH10^8, injection with *A. hydrophila* at concentration 10^8 CFU ml^{-1}; ISSS, injection with sterile saline solution; MCHC, mean corpuscular haemoglobin concentration; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; TPP, total plasmatic protein.

TABLE 3 White blood cell (Mean values ± *SD*) of juvenile fish *Arapaima gigas* challenged with *Aeromonas hydrophila* (first experiment)

	ISSS	IAH10^4	IAH10^6	IAH10^8
Thrombocyte	11.52 ± 2.9 a	5.86 ± 1.1 b	4.54 ± 0.77 b	5.4 ± 1.4 b
Leucocytes	36.11 ± 2.26 a	34.70 ± 2.04 a	26.01 ± 3.2 b	27.85 ± 1.9 b
Lymphocyte	13.42 ± 1.2 a	16.26 ± 2.09 a	16.25 ± 1.07 a	16.11 ± 1.04 a
Monocyte	14.10 ± 0.3 a	15.9 ± 1.08 a	7.38 ± 0.47 b	8.75 ± 0.48 b
Neutrophil	8.55 ± 1.38 a	2.38 ± 0.72 b	2.17 ± 0.31 b	2.9 ± 0.21 b

Note: Different letters in the row means statistical difference by Tukey test ($p < 0.05$), total thrombocytes and leucocytes including differential (×10^3 µl^{-1}).

Abbreviations: IAH10^4, injection with *A. hydrophila* at concentration 10^4; IAH10^6, injection with *A. hydrophila* at concentration 10^6; IAH10^8, injection with *A. hydrophila* at concentration 10^8 CFU ml^{-1}; ISSS, injection with sterile saline solution.

TABLE 4 Red blood cell (Mean values ±*SD*) of juvenile fish *Arapaima gigas* challenged with *Aeromonas hydrophila* (second experiment)

	Injection with *Aeromonas hydrophila* at concentration 10^6 UFC ml^{-1}			
Treatments	**CD-IAH10^6**	**MRSD-IAH10^6**	**D10^6-IAH10^6**	**D10^8-IAH10^6**
Er (×10^6 µl^{-1})	1.75 ± 0.09 b	1.15 ± 0.07 b	2.32 ± 0.30 a	2.31 ± 0.08 a
Ht (%)	32.00 ± 3.25 b	36.60 ± 4.50 b	44.92 ± 2.50 a	45.30 ± 2.16 a
Hg (g dl^{-1})	10.20 ± 1.26 b	11.00 ± 1.60 b	13.80 ± 0.57 a	14.10 ± 0.30 a
TPP (g dl^{-1})	4.25 ± 0.52 a	4.27 ± 0.50 a	4.73 ± 0.85 a	4.57 ± 0.46 a
MCV (fl)	156.90 ± 22.18 b	186.51 ± 26.15 b	365.00 ± 32.2 1 a	380.76 ± 35.8 6 a
MCH (pg)	52.75 ± 12.97 b	43.82 ± 8.93 b	89.40 ± 11.03 a	96.50 ± 5.39 a
MCHC (g dl^{-1})	25.83 ± 2.91 a	33.78 ± 2.22 a	30.81 ± 1.43 a	29.51 ± 2.90 a

Note: Different letters in the row means statistical difference by Tukey test ($p < 0.05$).

Abbreviations: CD, fish group without probiotic supplementation; D10^6, fish group supplemented with; D10^8, fish group supplemented with; Er, erythrocyte; Hg, haemoglobin; Ht, haematocrit; IAH10^4, injection with *A. hydrophila* at concentration 10^4; IAH10^6, injection with *A. hydrophila* at concentration 10^6; IAH10^8, injection with *A. hydrophila* at concentration 10^8 CFU.mL^{-1}; ISSS, injection with sterile saline solution; MCHC, mean corpuscular haemoglobin concentration; MCH–mean corpuscular haemoglobin; MCV, mean corpuscular volume; MRSD, fish group fed with MRS diet; TPP, total plasmatic protein.

Fish fed with probiotic at 10^6 CFU g^{-1} and 10^8 CFU g^{-1} (D10^6 and D10^8) showed increased levels of thrombocytes (14.30 ± 1.92 and 15.40 ± 2.10 cell × 10^3 µl^{-1}) and leucocytes (27.50 ± 1.49 e 26.45 ± 1.19 cell × 10^3 µl^{-1}) when compared with fish without probiotic supplementation (CD and MRSD groups) after pathogen injection (Table 5). The treatments with probiotic supplementation occurred increased values of monocyte (7.34 ± 1.44 and 6.39 ± 1.17 cell × 10^3 µl^{-1}) and neutrophil (6.25 ± 0.82 and 6.56 ± 0.61 cell × 10^3 µl^{-1}) respectively (Table 5).

TABLE 5 White blood cell (Mean values ± *SD*) of juvenile fish *Arapaima gigas* challenged with *Aeromonas hydrophila* (second experiment)

	Injection with *Aeromonas hydrophila* at concentration 10^6UFC ml^{-1}			
Treatments	**CD-IAH10^6**	**DMRS-IAH 10^6**	**D10^6-IAH 10^6**	**D10^8-IAH 10^6**
Thrombocytes	7.33 ± 1.48 b	8.20 ± 1.25 b	14.30 ± 1.92 a	15.40 ± 2.10 a
Leucocytes	18.88 ± 1.80 b	17.65 ± 1.70 b	27.50 ± 1.49 a	26.45 ± 1.19 a
Lymphocytes	12.39 ± 1.45 a	11.97 ± 1.25 a	12.75 ± 1.30 a	12.60 ± 2.04 a
Monocytes	3.14 ± 0.82 b	2.49 ± 1.67 b	7.34 ± 1.44 a	6.39 ± 1.17 a
Neutrophil	2.40 ± 0.41 b	2.20 ± 0.92 b	6.25 ± 0.82 a	6.56 ± 0.61 a
Basophil	0.71 ± 0.12 a	0.80 ± 0.20 a	0.70 ± 0.18 a	0.75 ± 0.21 a

Note: Different letters in the row means statistical difference by Tukey test ($p < 0.05$), total thrombocytes and leucocytes including differential ($\times 10^3$ μl^{-1}).

Abbreviations: ISSS, injection with sterile saline solution; IAH10^4, injection with *A. hydrophila* at concentration 10^4; IAH10^6, injection with *A. hydrophila* at concentration 10^6;IAH10^8, injection with *A. hydrophila* at concentration 10^8; CD, fish group without probiotic supplementation; MRSD, fish group fed with MRS diet; D10^6, fish group supplemented with 10^6 CFU g^{-1}; D10^8, fish group supplemented with 10^8 CFU g^{-1}.

TABLE 6 Red blood cell (Mean values ± *SD*) of juvenile fish *Arapaima gigas* challenged with *Aeromonas hydrophila* (third experiment)

	Injection with *Aeromonas hydrophila* at concentration 10^8UFC ml^{-1}			
Treatment	**CD-IAH10^8**	**DMRS-IAH10^8**	**D10^6-IAH10^8**	**D10^8-IAH10^8**
Er ($\times 10^6$ μl^{-1})	1.84 ± 0.82 b	2.18 ± 0.94 ab	2.33 ± 0.49 a	2.52 ± 0.29 a
Ht (%)	42.00 ± 1.20 b	46.10 ± 2.50 ab	47.50 ± 1.80 a	46.50 ± 2.50 a
Hg (g dl^{-1})	13.60 ± 1.30 b	13.15 ± 0.12 b	19.29 ± 1.69 a	19.82 ± 1.28 a
TPP (g dl^{-1})	4.20 ± 0.80 a	5.20 ± 1.10 a	5.81 ± 1.79 a	5.50 ± 1.30 a
MCV (fl)	187.80 ± 28.20 b	197.30 ± 40.43 b	296.80 ± 29.60 a	301.52 ± 26.30 a
MCH (pg)	58.90 ± 12.30 b	63.80 ± 13.42 ab	92.00 ± 7.16 a	102.19 ± 7.32 a
MCHC (g dl^{-1})	30.82 ± 2.13 b	32.30 ± 1.70 b	121.76 ± 5.41 a	122.00 ± 7.30 a

Note: Different letters in the row means statistical difference by Tukey test ($p < 0.05$).

Abbreviations: CD, fish group without probiotic supplementation; D10^6, fish group supplemented with 10^6 CFU g^{-1}; D10^8, fish group supplemented with 10^8 CFU g^{-1}; Er, erythrocyte; Hg, haemoglobin; Ht, haematocrit; IAH10^4, injection with *A. hydrophila* at concentration 10^4; IAH10^6, injection with *A. hydrophila* at concentration 10^6; IAH10^8, injection with *A. hydrophila* at concentration 10^8; ISSS, injection with sterile saline solution; MCHC, mean corpuscular haemoglobin concentration; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; MRSD, fish group fed with MRS diet; TPP, total plasmatic protein.

TABLE 7 White blood cell ($\times 10^3$ μl^{-1}) (Mean values ± *SD*) of juvenile fish *Arapaima gigas* challenged with *Aeromonas hydrophila* (third experiment)

	Injection with *Aeromonas hydrophila* at concentration 10^8UFC ml^{-1}			
Treatment	**CD-IAH10^8**	**MRSD-IAH10^8**	**D10^6-IAH10^8**	**D10^8-IAH10^8**
Thrombocytes	8.10 ± 1.19 b	8.14 ± 2.20 b	11.30 ± 1.30 a	12.60 ± 1.89 a
Leucocytes	25.90 ± 1.82 b	26.56 ± 2.26 b	29.50 ± 2.60 a	31.88 ± 2.33 a
Lymphocytes	15.95 ± 2.20 a	15.25 ± 2.65 a	14.35 ± 2.87 a	14.64 ± 2.65 a
Monocytes	2.71 ± 2.72 b	3.46 ± 1.06 b	5.82 ± 1.12 a	6.55 ± 1.30 a
Neutrophil	6.71 ± 0.33 b	6.97 ± 0.86 b	8.82 ± 0.95 a	9.86 ± 0.72 a
Basophil	0.40 ± 0.10 a	0.52 ± 0.100 a	0.40 ± 0.25 a	0.50 ± 0.25 a

Note: Different letters in the row means statistical difference by Tukey test ($p < 0.05$), total thrombocytes and leucocytes including differential ($\times 10^3$ μl^{-1}).

Abbreviations: CD, fish group without probiotic supplementation; D10^6, fish group supplemented with 10^6 CFU g^{-1}; D10^8, fish group supplemented with 10^8 CFU g^{-1}; IAH10^4, injection with *A. hydrophila* at concentration 10^4; IAH10^6, injection with Aeromonas hydrophila at concentration 10^6; IAH10^8, injection with *A. hydrophila* at concentration 10^8; ISSS, injection with sterile saline solution; MRSD, fish group fed with MRS diet.

3.3 | Experiment 3: Bacterial challenge using lethal concentration 100% (LC_{100}) in *A gigas* supplemented with probiotic

The lethal dose of *A. hydrophila* (IAH10^8, 1.2 × 10^8 CFU ml^{-1}) caused 100% mortality in fish without probiotic supplementation (CD), 75% in the MRSD group, and 25% in the probiotic groups. Regarding red blood cells, the probiotic groups (D10^6 and D10^8) promoted increased values of erythrocytes (2.33 ± 0.49 and 2.52 ± 0.29 cell × 10^6 µl^{-1}), haematocrit (47.50 ± 1.80 and 46.50 ± 2.50%), haemoglobin (19.29 ± 1.69 and 19.82 ± 1.28 g dl^{-1}), MCV (296.80 ± 29.60 and 301.52 ± 26.30 fl), MCH (92.00 ± 7.16 and 102.19±7.32 pg) and MCHC (121.76 ± 5.41 and 122.00 ± 7.30 g dl^{-1}) (Table 6).

After experimental infection, fish fed with higher probiotic supplementation (D10^6 and D10^8) showed increased values of thrombocytes (11.30 ± 1.30 and 12.60 ± 1.89 cell ×10^3 µl^{-1}), leucocytes (29.50 ± 2.60 and 31.88 ± 2.33 cell ×10^3 µl^{-1}), monocyte (5.82 ± 1.12 e 6.55 ± 1.30 cell ×10^3 µl^{-1}) and neutrophil (8.82 ± 0.95 and 9.86 ± 0.72 cell ×10^3 µl^{-1}) (Table 7).

In this experiment, phagocyted bacteria were observed in the cytoplasm of different leucocytes for the D10^6-IAH10^8 and D10^8-IAH10^8 treatments (Figure 3).

3.4 | Histological analysis

Arapaima gigas subjected to *A. hydrophila* injection (all fish in the first experiment and fish of CD group from second and third experiments) exhibited hepatic alteration (Table 8), lipid degeneration (Figure 4), necrosis, hyperaemia and structural disarrangement. However, fish subjected to the probiotic diet at a concentration 108 CFU g^{-1} showed reduced hepatic alterations.

4 | DISCUSSION

The use of probiotics in fish aquaculture has shown positive results, promoting intestinal modulation and an improved immunological system (Dias, Santos, et al., 2018; Dias, Abe, et al., 2018; Mouriño et al., 2015; Sousa et al., 2019; Yamashita et al., 2020). However, probiotic supplementation should be tested for real resistance of the host to bacterial infections. For this reason, bacterial challenges in the laboratory have become necessary to ensure the benefits of probiotic supplementation (Mohammadian, Jangaran-Nejad, et al., 2019; Mouriño et al., 2015). This study provided scientific data regarding the resistance of *A. gigas* fed with the probiotic *E. faecium* after experimental infection with *A. hydrophila*.

The use of pathogenic bacteria for sanitary challenges has been commonly used to confirm the resistance of fish previously subjected to essential oils, prebiotics or probiotic diets (Dias, Santos, et al., 2018; Dias, Abe, et al., 2018; Lima et al., 2019; Mouriño et al., 2015). However, determining an adequate concentration for pathogenic bacteria is fundamental to perform challenge experiments due to physiology differences among fish species (Dias et al., 2016; Dias, Santos, et al., 2018; Dias, Abe, et al., 2018).

This study determined the lethal dose 30% (1.0 × 10^6 CFU ml^{-1}) and 100% (1.2 × 108 CFU ml^{-1}) of *A. hydrophila* for pirarucu *A. gigas*. Our results differ from Dias et al. (2016), who found 86% mortality after 96 h at a concentration of 10^{10} CFU ml^{-1}. However, the fish in present study are smaller than used in Dias et al. (2016) that explained the difference in the lethal concentration, allied to virulence and pathogenicity of each strain used (Abdelhamed et al., 2019; Peatman et al., 2018).

Water quality parameters can provoke physiological or immunological alterations in fish; however, in this study, water quality did not influence (Triebskorn, 2002; Garver et al., 2016; Hobbs et al., 2016). In addition, the *sentinel* fish used for experiments has become an efficient strategy for monitoring water quality or any crossed infection. This strategy confirmed that all mortalities were related to the injection of *A. hydrophila* in sanitary challenge.

Aeromonas hydrophila infection caused rapid mortality of *A. gigas* fed without probiotic supplementation, mainly in the first and third experiments. This result was also observed for *Channa striata* (Munir et al., 2018), *Colossoma macropomum* (Dias, Santos, et al., 2018; Dias, Abe, et al., 2018), *Piaractus mesopotamicus* (Farias et al., 2016), and *Oreochromis niloticus* (Kuebutornye et al., 2020) after experimental infection with the same pathogen. This virulence is due to aerolisine, a protein with haemolytic activity (*β-hemolisine*), causing haemorrhage and consequently mortality (Oliveira et al., 2012; Kim et al., 2018).

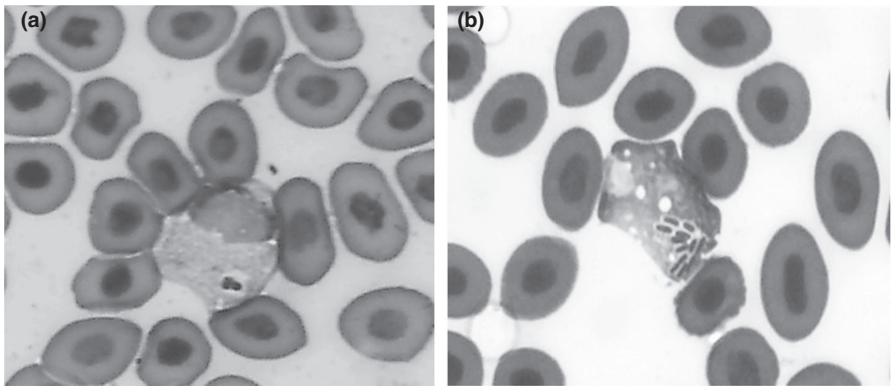


FIGURE 3 Phagocytes observed in the blood smears from *Arapaima gigas* after experimental infection with *Aeromonas hydrophila*. a—Neutrophil, b—Monocyte

All fish from experiments 1, 2 and 3 demonstrated similar behavioural signs, corroborating the results of Dias et al. (2016) and Bhat et al. (2021). In addition, most of the clinical signs observed in *A. gigas* have previously been reported in the literature, including haemorrhage, necrosis, ulceration, and inflammation (Laith & Najiah, 2013; El-Barbary, 2017; Alyahya et al., 2018). Some clinical signs occur 48 or 72 hours after the infection, development in chronic cases with greater ulceration and specific haematological alterations (Fernandes et al., 2019; Kumar et al., 2016).

TABLE 8 Hepatic alterations in *Arapaima gigas* after experimental infection

	Lipid degeneration	Necrosis	Cord-like structure disarrangement
Experiment 1			
ISSS	–	–	–
IAH10^4	+	+	+
IAH10^6	+	++	+
IAH10^8	++	+++	++
Experiment 2			
CD-IAH10^6	+	+	+
MRSD-IAH 10^6	+	–	+
D10^6-IAH 10^6		–	+
D10^8-IAH 10^6	–	–	+
Experiment 3			
CD-IAH 10^8	+++	+++	++
MRSD- IAH 10^8	+++	++	+
D10^6- IAH 10^8	++	–	+
D10^8- IAH 10^8	–	–	+

Note: (+) Signal of plus indicates the intensity of lesion, (–) signal of minus indicates light lesion or absence. Following Van Dyk et al. (2007).
Abbreviations: ISSS, injection with sterile saline solution; IAH10^4, injection with *A. hydrophila* at concentration 10^4; IAH10^6, injection with *A. hydrophila* at concentration 10^6; IAH10^8, injection with *A. hydrophila* at concentration 10^8; CD, fish group without probiotic supplementation; MRSD, fish group fed with MRS diet; D10^6, fish group supplemented with 10^6 CFU g^{-1}; D10^8, fish group supplemented with 10^8 CFU g^{-1}.

Particularly, anaemia microcytic hypochromic occurred in fish from experiment 2 and 3 (LC_{30} and LC_{100}) and has been cited in *Aeromonas hydrophila* infection for *Channa striata* (Munir et al., 2018), hybrid of surubim (*Pseudoplatystoma corruscans* ×P. *fasciatum*) (Silva et al., 2012), *Labea victorianus* (Ngugi et al., 2015) and *Oreochromis niloticus* (Manrique et al., 2019). Nonetheless, at the first experiment (*Lethality of Aeromonas hydrophila in A. gigas*), there was reduction in red blood cells and increase in MCV, however, without haemoglobin alteration, characterizing as anaemia macrocytic normochromic. This change is uncommon but was also observed by Dias, Santos, et al. (2018), Dias, Abe, et al. (2018) for *Colossoma macropomum* after *Aeromonas hydrophila* infection.

The liver alterations observed in present study are due to toxins produced by *A. hydrophila* related to virulence genes (Kim et al., 2018; Laith & Najiah, 2013). In *Clarias gariepinus*, the toxin Aerolysin caused clinical signs such as hyperaemia, cord-like disarrangement and necrosis (Hamid et al., 2018; Fivaz et al., 2001). Juveniles of red hybrid tilapia also showed necrosis and lipid degeneration (Yardimci & Aydin, 2011). It is important to note that the severity of hepatic alterations observed in present study was directly related to the time of infection as same reported by Dias et al. (2016).

However, in the present study, most of the clinical signs and severity levels were reduced in the probiotic groups. Similar results were obtained for *C. gariepinus*, *O. niloticus*, *Labeo rohita*, *Cyprinus carpio* and *C. macropomum* when subjected to experimental challenge with *A. hydrophila* after probiotic supplementation (Dias, Santos, et al., 2018; Dias, Abe, et al., 2018; Hamid et al., 2018; Jesus et al., 2017; Kanwal & Tayyeb, 2019; Mohammadin et al., 2020; Mouriño et al., 2015). The *A. gigas* supplemented with probiotic showed an increased in thrombocytes, monocytes and neutrophils, which act by phagocytizing foreign bodies (Figure 1a, b), reducing any damage due to infection and increasing survival (Mouriño et al., 2015; Jesus et al., 2017; Lima et al., 2019).

Probiotic supplementation can provoke increases in defence cells, phagocytic activity, immunoglobulin and lysozyme (Farias et al., 2016; Kanwal & Tayyeb, 2019). All of these benefits can promote resistance to bacterial infection without affecting the host (Dias, Santos, et al., 2018; Dias, Abe, et al., 2018; Jatobá et al., 2011; Mouriño et al., 2015; Nandi et al., 2017; Sousa et al., 2019; Tang et al., 2019). Another probably benefit would be the increase in antioxidant enzymes in fish that received probiotic supplementation. These antioxidants enzymes provide protection to cells from

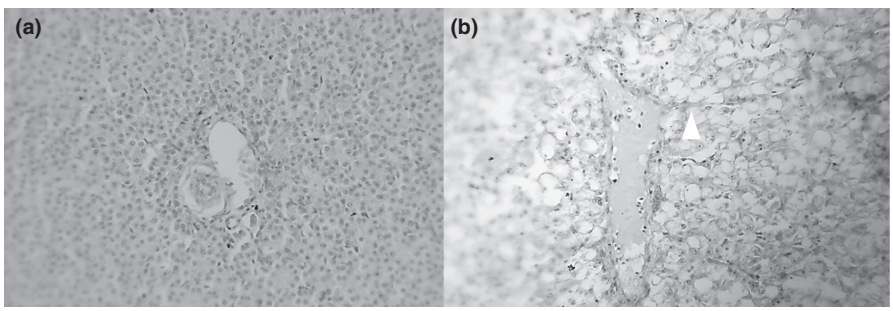


FIGURE 4 Histology of the liver for *Arapaima gigas* challenged by *Aeromonas hydrophila*. (a) Normal liver in the fish injected with sterile saline solution (b) lipid degeneration in the fish no supplemented (CD) injected with *A. hydrophila* (arrow)

reactive oxygen species (ROS) avoiding some tissues damage (Gobi et al., 2016, 2018).

This immunological improvement reflected in greater survival (75%) of fish submitted to probiotic diet (108 CFU g^{-1}) even after experimental infection by *A. hydrophila* at a lethal dose concentration (LC_{100}). For this reason, the use of a probiotic diet containing *E. faecium* is a suitable prophylactic method for the rearing of *A. gigas* in captivity.

5 | CONCLUSION

Probiotic supplementation with *Enterococcus faecium* at concentration 108 CFU g^{-1} promoted greater resistance for juvenile fish *Arapaima gigas* against *Aeromonas hydrophila* infection.

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CONFLICT OF INTEREST

The authors have no any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Marcia Valeria Silva do Couto: Conducting bioassay, revision of paper, data compilation, blood analysis. Natalino Costa Sousa: Conducting bioassay, revision of paper, blood analysis. Higo Andrade Abe: Conducting bioassay, revision of paper. Fernanda dos Santos Cunha: Conducting Bioassay, revision of paper. Peterson Emmanuel Guimarães Paixão: Conducting bioassay, revision of paper, blood analysis. José Luiz Pedreira Mourino: Revision of literature, revision of paper, statistical analysis. Mauricio Laterça Martins: Revision of literature, revision of paper, statistical analysis. Carlos Alberto Martins Codeiro: Revision of literature, revision of paper, statistical analysis. Rodrigo Yudi Fujimoto: Project's manager, draft paper, analysis of statistic, revision of paper.

DATA AVAILABILITY STATEMENT

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

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