BOLETIM DO INSTITUTO DE PESCA
Scientific Article

# Enrichment of *Artemia* sp. with autochthonous probiotics at different levels in larviculture of piauçu *Megaleporinus macrocephalus*

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## ABSTRACT

The research investigated the effect of dietary supplementation with *Artemia* sp. enriched with the autochthonous probiotic *Enterococcus faecium* on growth performance, microbiota modulation, intestinal morphology, and resistance to pathogenic bacteria of *Megaleporinus macrocephalus* larvae. The study evaluated four treatments (C: without probiotics; T1: $1 \times 10^4$; T2: $1 \times 10^6$; and T3: $1 \times 10^8$ CFU·mL$^{-1}$) in quadruplicates. The larvae (n = 160; weight = 5.3 ± 2.3 mg and length = 3.73 ± 0.4 mm) were distributed in 16 L containers at a density of 10 larvae·L$^{-1}$ for 20 days. The productive performance, survival, gut microbiology, and histology were measured. The larvae were also submitted to acute challenge against the pathogenic bacterium *Aeromonas hydrophila*. The results showed that supplementation with $1 \times 10^8$ CFU·mL$^{-1}$ promotes greater gain in length (13.78 ± 0.40 cm) and total weight (0.08 ± 0.002 g), higher counts of lactic acid bacteria and lower total heterotrophic in the intestines (7.11 ± 0.30; 0.12 ± 0.09 log CFU·g$^{-1}$, respectively) and larger villi (0.26 ± 0.03 µm). Diets containing probiotics influenced the animals' resistance to acute infection, with a lower accumulated mortality in T3 (33% ± 11.54%) and a higher one in C+ (93% ± 11.54%). Thus, probiotic supplementation with the autochthonous bacterium *E. faecium* ($1 \times 10^8$ CFU·mL$^{-1}$) provides zootechnical improvement, villus increase and greater resistance to infections.

**Keywords:** Growth performance; Live food; Sanity; Microbiology; Pathogen.

## Enriquecimento de *Artemia* sp. com probiótico autóctone em diferentes níveis na larvicultura de piauçu *Megaleporinus macrocephalus*

## RESUMO

A pesquisa investigou o efeito da suplementação dietética com *Artemia* sp. enriquecida com o probiótico autóctone *Enterococcus faecium* no desempenho do crescimento, modulação da microbiota, morfologia intestinal e resistência a bactérias patogênicas em larvas de *Megaleporinus macrocephalus*. O estudo avaliou quatro tratamentos (C: sem probióticos; T1: $1 \times 10^4$; T2: $1 \times 10^6$; e T3: $1 \times 10^8$ UFC·mL$^{-1}$) em quadruplicata. As larvas (n = 160; peso = 5,3 ± 2,3 mg e comprimento = 3,73 ± 0,4 mm) foram distribuídas em recipientes de 16 L na densidade de 10 larvas·L$^{-1}$ por 20 dias. Desempenho produtivo, sobrevivência, microbiologia e histologia intestinal foram medidos. As larvas também foram submetidas a desafio agudo contra a bactéria patogênica *Aeromonas hydrophila*. Os resultados mostraram que a suplementação com $1 \times 10^8$ UFC·mL$^{-1}$ promoveu maior ganho de comprimento (13,78 ± 0,40 cm) e peso total (0,08 ± 0,002 g), maior contagem de bactérias lácticas e menor de heterotróficos totais nos intestinos (7,11 ± 0,30; 0,12 ± 0,09 log UFC·g$^{-1}$, respectivamente) e maiores vilosidades (0,26 ± 0,03 µm). As dietas contendo probióticos influenciaram a resistência dos animais à infecção aguda, com menor mortalidade acumulada em T3 (33,33% ± 11,54%) e maior em C+ (93,33% ± 11,54%). Assim, a suplementação probiótica com a bactéria autóctone *E. faecium* ($1 \times 10^8$ UFC·mL$^{-1}$) proporciona melhora zootécnica, aumento de vilosidades e maior resistência a infecções.

**Palavras-chave:** Crescimento; Alimento vivo; Sanidade; Microbiologia; Patógeno.

**Received:** December 8, 2022 | **Approved:** July 25, 2023

## INTRODUCTION

The piauçu *Megaleporinus macrocephalus* is a neotropical species cultivated in South America and the 10th most produced species in Latin America (Ramirez et al., 2017; Pereira et al., 2020). This omnivore presents rapid growth in captivity, can reach up to 500 mm of total length, and is widely accepted among consumers (Tataje and Zaniboni-Filho, 2010; Soares Junior et al., 2013). However, studies on the nutrition of the species are scarce, but fundamental for the productive intensification of the animal, especially in the early stages of life.

To increase the productivity of sustainable intensive fish farming, the use of probiotic bacteria has been adopted to promote intestinal microbiota balance, improve immunity and growth performance, as well as resistance to pathogens (Hoseinifar et al., 2018; Ringø et al., 2020). For piauçu, the only respective study performed so far focused on the allochthonous probiotic *Saccharomyces cerevisiae*, although it did not improve the physiology nor the production system of this species (Lima et al., 2015).

Autochthonous probiotic bacteria are more suitable as they adhere better to the intestinal epithelium, colonising it and facilitating nutrient use, in addition to having a greater potential to inhibit pathogens (Sousa et al., 2019; Yamashita et al., 2020; Yeganeh Rastekenari et al., 2021; Hossain et al., 2022). However, for these benefits to occur, probiotics need to arrive in ideal amounts in the host's intestinal tract, with food being the most viable form of inclusion.

In the case of fish larvae, the introduction of probiotics by enriched live foods is an alternative (Ghorbani Vaghei et al., 2019; Ghoname et al., 2020; Oliveira et al., 2022) as it assists in the development of the intestine, balancing the microbiota (Comabella et al., 2013; Stephens et al., 2016), immune system development, and in the synthesis and absorption of amino acids and nutrients from the diet (Chung et al., 2012; Arrieta et al., 2014). Thus, in the larviculture of aquatic organisms, studies with bioencapsulated probiotics in live diets, such as artemia and rotifers, have promising results in aquaculture (Sun et al., 2013; Bhaheerathan et al., 2020; Ghoname et al., 2020; Samat et al., 2021; Oliveira et al., 2022).

Among the probiotic bacteria with potential to feed the piauçu larvae, the genus *Enterococcus* is a good option because of its high capacity for adhesion, growth and colonization of the host's intestinal tract compared to heterotrophic bacteria (Dias et al., 2019; Sousa et al., 2019) and can stimulate defense cells such as T lymphocytes, antibody production (IgA), macrophages and dentritic cells in the production of compounds such as nitric oxide (Khalkhali and Mojgani, 2017).

Thus, considering the potential of the species *M. macrocephalus* for aquaculture, the study evaluated dietary supplementation with the autochthonous probiotic *Enterococcus faecium* by enriching *Artemia* sp. Productive performance, microbiota modulation and intestinal histomorphometry were investigated, in addition to resistance to acute challenge with *Aeromonas hydrophila*.

## MATERIAL AND METHODS

### Experimental conditions

The present study was approved by the ethics committee in animal experimentation of the Universidade Federal do Pará, under the protocol CEUA no. 3991300420. The autochthonous probiotic strain was isolated from *M. macrocephalus* (n = 15 specimens) with the weight of 0.785 ± 0.12 kg and the length of 26.59 ± 0.23 cm, obtained from extensive fish farming. The selection criteria were as follows: gram-positive, catalase-negative and affinity to aniline blue dye. After the growth of blue colonies (lactic acid bacterium), the strains were kept in culture (Man Rogosa Sharpe Broth-MRS) at 35°C for 48 h and selected by *in-vitro* assays in different gradients of NaCl (0, 1.5, 3 and 4.5%), pH (4, 5, 6, 8 and 9), and bile salts (2.5, 5 and 7.5% weight·volume$^{-1}$) (Barros et al., 2022).

Colonies were grown in Man Rogosa Sharpe broth (35°C for 48 h), and the absorbance reduction was measured at 630 nm (Jatobá et al., 2008; Vieira et al., 2013). The strain was identified as *E. faecium* 20218_1 CHB by time-of-flight mass spectrometry with matrix-assisted laser desorption ionization (MALD-TOF), from the molecular weight of ribossomal proteins with 200 hz laser shots and score > 2 (Angeletti, 2017; Sousa et al., 2019).

The probiotic bacterium was grown in Falcon tubes containing broth medium (MRS broth + NaCl 0.65%) incubated to 35°C for 24 h, centrifuged at 1,800 g for 15 min and then resuspended in sterile saline (NaCl 0.65%). To determine the experimental concentrations, the strain was maintained in tubes containing MRS broth, and serial dilution (1:10) was performed to reach the experimental concentrations ($1 \times 10^4$; $1 \times 10^6$ and $1 \times 10^8$ UFC·mL$^{-1}$), according to the methodology of Jatobá et al. (2008). For the experiment, the strains were prepared every two days.

For the experiment, 160 piauçu larvae (weight = 5.3 ± 2.3 mg; length = 3.7 ± 0.4 mm), acquired two days after hatching and acclimatized in the laboratory. The *in-vivo* experiment was carried out in a completely randomized design with four

treatments: with *Artemia* without probiotics on control diet (C), and *Artemia* enriched the autochthonous probiotic *E. faecium* (T1: $1 \times 10^4$; T2: $1 \times 10^6$; and T3: $1 \times 10^8$ CFU·mL$^{-1}$), in four replicates, distributed in 16 aquarium (1-L) at a density of 10 larvae·L$^{-1}$. The animals were maintained in a static system with constant aeration over a period of 20 days. The feeding rate was 150 artemia nauplii per larva·day$^{-1}$ (Jomori et al., 2013), divided into four times a day (8; 11; 14 and 17 h). After the last daily feeding, food residues and excretes were removed with a siphon (± 30%) of the volume of water in each experimental unit (Abe et al., 2015; Sousa et al., 2020).

The water quality parameters were kept stable and ideal for the species during the entire period of experimentation, as follows: temperature (27.81 ± 0.35°C), dissolved oxygen (6.89 ± 0.14 mg·L$^{-1}$), pH (6.45 ± 0.36), total ammonia (0.28 ± 0.04 mg·L$^{-1}$), and electrical conductivity (185.12 ± 31.32 µS·cm$^{-1}$), measured with a Professional Plus YSI multiparameter, within the recommended values for aquaculture of continental species (Tavares-Dias et al., 2008).

## Preparation of live food and bacteriological analysis

To obtain *Artemia* sp. nauplii, the cysts were incubated for 24 h in 1-L aquariums with 30 g·L$^{-1}$ saline water, constant aeration, and light intensity of 15 W lux (Azevedo et al., 2016). After hatching, *Artemia* nauplii were washed with freshwater for further enrichment with probiotics and larvae feeding. *Artemia* nauplii were counted for the density estimate. For counting, 1 mL of the solution containing the nauplii was sampled, they were then kept in a formalin solution (4%), and the count was performed in squared petri dishes under a stereomicroscope with a 40x magnification, in quadruplicates (Abe et al., 2015).

For enrichment, after the incubation period, the *Artemia* nauplii were washed with freshwater in a beaker (50 mL). The autochthonous probiotic strain *E. faecium* was previously cultivated in Falcon tubes (MRS Broth + 0.65% NaCl), and incubated at 35°C for 24 h (Jatobá et al., 2008). Subsequently, the strain was centrifuged at 1.800 g for 15 minutes and resuspended in sterile saline solution (SSE + 0.65% NaCl), and then reduced by 1 mL to the final concentration ($1 \times 10^4$; $10^6$ and $10^8$ CFU·mL$^{-1}$), representing treatments (T1, T2 and T3, respectively). Right after, the enrichment was performed for the treatments (T1: $1 \times 10^4$; T2: $1 \times 10^6$; and T3: $1 \times 10^8$ CFU·mL$^{-1}$), where the concentrations were transferred to Falcon tubes with 10 mL v/v, during the period of 40 minutes. For each 1 mL, 150 *Artemia* nauplii were added, totaling a rate of 150 nauplii/larva in each experimental unit for each treatment, which were administered to the larvae directly in the culture water (Vázquez-Silva et al., 2016; Sousa et al., 2020). This process was performed at all feeding times.

The microbiology of the nauplii was evaluated in CFU·g$^{-1}$ after the enrichment period to determine the stable quantity of probiotics in the nauplii and confirmed as *E. faecium* by the MALD-TOF method. For this, the artemia were filtered through a 64-µm mesh filter, and the samples of each treatment were dried on filter paper. Subsequently, one specimen (artemia pool) was macerated in sterile 0.65% saline solution and passed through serial dilution (factor 1:10). An aliquot (100 µL) of each dilution ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$ CFU·mL) was seeded in petri dishes containing culture medium (MRS Agar) and incubated at 30°C for 48 h to obtain the counts of probiotic bacteria in CFU·g$^{-1}$. (adapted from Jatobá et al., 2008; Vázquez-Silva et al., 2016; Dias et al., 2022). The analyses were carried out in quadruplicate.

## Productive performance

At the end of the experiment, all post-larvae were subjected to biometry to record total length, standard length, and weight. Based on these data, the parameters were determined:

- 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);
- Uniformity (U) = (N ± 20%)/Nt;
- Nt = the total number of fish in each experimental unit;
- N ± 20% = number of animals with the parameter weight/length within ± 20% around the mean of the experimental unit (Gonçalves Júnior et al., 2013; Dias et al., 2022);
- relative condition factor Kr (ln final weight / b × ln initial length – a) (Le Cren, 1951).

## Microbiological analysis

To assess the intestinal bacterial microbiota (total probiotic × heterotrophic bacterium), samples (n = 24) of the post-larvae intestines were collected (pool of two post-larvae from each repetition), using the design described before. The fish were anesthetized by immersion in benzocaine solution, 20 mg·L$^{-1}$ (Pramod et al., 2010), and euthanized by medullary section. Intestinal samples were macerated using a mortar and pestle in a weight-to-volume ratio (w/v %) in 0.65% SSE for further serial dilutions at $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$ CFU·mL$^{-1}$. The aliquot

(100 µL) of each of the dilutions was used to inoculate petri dishes containing MRS agar for counting of probiotic bacteria, and another aliquot was seeded in tryptone soy agar (TSA) to obtain the count of total heterotrophic bacteria. The plates were then incubated at 35°C for 48 h followed by cell counting (CFU·g$^{-1}$) in the animal's intestine (Jatobá et al., 2008; Dias et al., 2018).

### Histological analysis

For the histological analysis of the intestines, eight post-larvae of each treatment were used. They were fixed in 10% formalin solution for 24 h and preserved in 70% alcohol (Azevedo et al., 2016). The intestine was removed, dehydrated in an alcohol series (70, 80, 90 and 100%), transferred to xylene and embedded in paraffin. Using a microtome, 5-µm cross sections were made and stained with hematoxylin eosin (HE). Subsequently, the histological sections were analyzed under a light microscope (Nikon, E600), and measurements of the morphometric parameters of the intestine sessions (total height, height, width, and thickness of the villi) were taken according to Silva et al. (2015).

### Immersion challenge with Aeromonas hydrophila

After the supplementation period, the fish were submitted to an acute bacterial challenge by the immersion method against the pathogen *A. hydrophila* to assess their ability to resist infections (adapted from Nikapitiya et al., 2018). The pathogenic bacteria were cultured in BHI broth and incubated at 30°C for 24 h according to Mouriño et al. (2017) and Dias et al. (2022). The bacteria were cultured to an optical density (OD) of 600 nm, sedimented by centrifugation (3,500 rpm at 4°C for 10 min) and resuspended in sterile buffered saline (0.65%). The bacterial suspension was then dispensed in Falcon tubes with a volume of 10 mL to reach the final concentration of $10^8$ CFU·mL$^{-1}$, and applied in 1-L volume post with new and innocuous water, for the challenge of infection by immersion. The control group (C- control larvae) was treated with sterile saline (0.65%) (Nikapitiya et al., 2018).

Therefore, 45 post-larvae were submitted to an acute challenge by *A. hydrophila* (lethal dose $1 \times 10^8$ CFU·mL$^{-1}$) in a completely randomized design, consisting of larvae that received *Artemia* enriched with *E. faecium* (T1: $1 \times 10^4$; T2: $1 \times 10^6$; and T3: $1 \times 10^8$ CFU·mL$^{-1}$), challenged with the pathogen, the group that received *Artemia* without probiotics (C+, positive control), infected with the pathogenic bacterium, and the group (C-, negative control) with sterile saline solution (0.65%) without *A. hydrophila*. Group C was used as a parameter for the experiment safety, as described by Dias et al. (2022).

The fish were kept in 1-L aquarium in a static system equipped with artificial aeration for 96 h. Every two h for four days, mortality was observed (Ina-Salwany et al., 2019). Dead fish (during the test time) and fish surviving at the end of 96 h were anesthetized by immersion in benzocaine solution 20 mg·L$^{-1}$ (Pramod et al., 2010), euthanized by medullary section and submitted to microbiological analysis to determine the Koch's postulate. After the growth of bacterial colonies, they were identified by the MALDI-TOF method (Evans, 1976; Angeletti, 2017).

For this experiment, the water variables were evaluated daily, analyzing temperature, electrical conductivity, dissolved oxygen, pH, and total ammonia, with the aid of the Professional Plus YSI multiparameter.

### Statistical analysis

The data was acquired, and the microbiological counts were converted into square roots, before being submitted to statistical tests. The mortality results were transformed to arcsin root ($x.100^{-1}$). After the data underwent normality and homoscedasticity tests (Shapiro-Wilk and Bartlett, respectively) and when heterogeneity of variance was observed, they were transformed into log 10 (x +1). Accordingly, the data were submitted to one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test ($p < 0.05$) to compare means.

## RESULTS

### Zootechnical performance

The productive performance of piauçu larvae (*M. macrocephalus*) supplemented with autochthonous probiotic *E. faecium*, treatment T3 ($1 \times 10^8$ CFU·mL$^{-1}$), presented the highest averages for total length 17.48 ± 0.30 mm (Fig. 1a) and weight-specific growth rate (SGRw – 25.14 ± 0.15%·day$^{-1}$) (Fig. 1b), differing significantly from the other treatments ($p < 0.0001$).

The highest means ($p < 0.05$) of length gain and weight gain were shown for fish that had received the highest concentration of probiotic T3 ($10^8$ CFU·mL$^{-1}$) in *Artemia* sp. (Table 1). In addition, the biomass values were the highest in T3. The probiotic promoted higher averages for treatments that had received artemia enriched with autochthonous *E. faecium*, with emphasis on the T3 group, with the highest average. On the contrary, the mortality rate of post-larvae was significantly increased ($p < 0.0001$) in the control group and in T1 ($10^4$ CFU·mL$^{-1}$) at the end of the experiment (Table 1).

## Microbiological analyses

The bacteria counting into *Artemia* nauplii showed statistic differences ($p < 0.05$) between groups, with the larger count in treatment T3 ($8.27 \pm 0.02 \times 10^8$ CFU·g$^{-1}$). In contrast, higher values total heterotrophic bacterial count was observed in the control ($4.34 \pm 1.2 \times 10^4$ CFU·g$^{-1}$) (Table 2).

Highest colony forming unit (CFU) levels of total heterotrophic bacteria were observed in the intestines of larvae

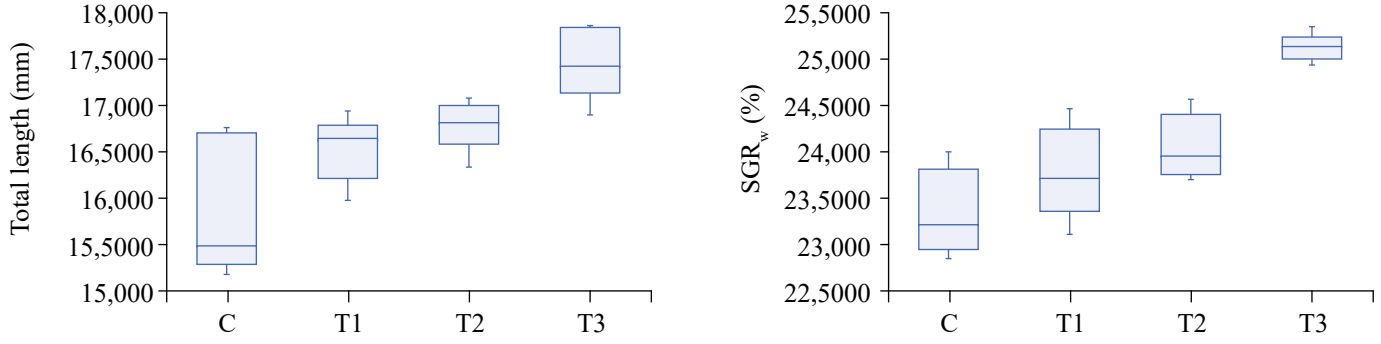



**Figure 1.** (a) Total length and (b) specific growth rate in weight of piauçu larvae (*Megaleporinus macrocephalus*) fed with control diet (C), *Artemia* sp. without probiotic and with *Artemia* sp. supplemented with autochthonous probiotic *Enterococcus faecium* (T1: $1 \times 10^4$; T2: $1 \times 10^6$; T3: $1 \times 10^8$ CFU.mL$^{-1}$). Different letters differ by Tukey's test ($p<0.05$).

**Table 1.** Growth performance of larvae of *Megaleporinus macrocephalus* fed a control diet (C), *Artemias* sp. not supplemented with probiotic and diets with *Artemia* sp. supplemented with different concentrations of autochthonous *Enterococcus faecium* probiotic (T1: $1 \times 10^4$; T2: $1 \times 10^6$; T3: $1 \times 10^8$ CFU·mL$^{-1}$).

| Parameters | Treatment | | | | p-value |
|---|---|---|---|---|---|
| | C | T1 | T2 | T3 | |
| FLG | 12.18 ± 0.77 b | 12.83 ± 0.36 b | 13.11 ± 0.28 ab | 13.78 ± 0.40 a | 0.0012 |
| LU | 93.14 ± 9.60 a | 96.67 ± 7.45 a | 97.50 ± 5.59 a | 100.00 ± 0.00 a | 0.5390 |
| TW | 0.06 ± 0.003 b | 0.06 ± 0.005 b | 0.07 ± 0.004 b | 0.08 ± 0.002 a | 0.0001 |
| WG | 0.06 ± 0.004 c | 0.06 ± 0.006 bc | 0.07 ± 0.005 b | 0.08 ± 0.002 a | 0.0001 |
| WU | 44.29 ± 18.68 b | 82.34 ± 5.36 a | 55.65 ± 4.39 a | 86.61 ± 5.02 a | 0.0331 |
| B | 0.36 ± 0.06 b | 0.45 ± 0.08 b | 0.65 ± 0.02 a | 0.72 ± 0.06 a | 0.0001 |
| SGRl | 7.27 ± 0.24 b | 7.48 ± 0.11 b | 7.56 ± 0.08 ab | 7.76 ± 0.12 a | 0.0014 |
| Kr | 0.86 ± 0.06 a | 0.85 ± 0.07 a | 0.86 ± 0.10 a | 0.95 ± 0.04 a | 0.1293 |
| S | 60 ± 7.07 b | 72 ± 8.37 b | 94 ± 8.94 a | 90 ± 7.07 a | 0.0001 |

FLG: final length gain (mm); LU: length uniformity (%); TW: total weight (g); WG: final weight gain (g); WU: weight uniformity (%); B: biomass; SGRl: length-specific growth rate (%.day$^{-1}$); Kr: relative condition factor; S: survival rate (%). Different letters on the same line differ by Tukey's test ($p<0.05$).

**Table 2.** Count of lactic acid bacterium and total heterotrophic bacterium (CFU·g$^{-1}$) in *Artemia* sp. after enrichment with probiotic *Enterococcus faecium*.

| Live food | Treatment | | | |
|---|---|---|---|---|
| | C | T1 | T2 | T3 |
| Artemia AL | $3.12 \pm 1.2 \times 10^2$ d | $3.95 \pm 0.0 \times 10^5$ c | $5.97 \pm 0.03 \times 10^7$ b | $8.27 \pm 0.02 \times 10^8$ a |
| Artemia HT | $4.34 \pm 1.2 \times 10^4$ c | $2.53 \pm 0.4 \times 10^3$ b | $1.20 \pm 0.09 \times 10^3$ b | $1.28 \pm 0.12 \times 10^2$ a |

Artemia AL: Artemia - lactic acid; Artemia HT: Artemia - total heterotrophic bacterium; C- control: no probiotic; T1: $1 \times 10^4$; T2: $1 \times 10^6$; T3: $1 \times 10^8$ CFU.mL$^{-1}$. Different letters on the same line differ by Tukey's test ($p < 0.05$).

without supplementation of the autochthonous probiotic *E. faecium* (control group). The largest means were observed in group C = 4.48 ± 0.15 and in T1 = 3.44 ± 0.42 log CFU·g$^{-1}$, which differed ($p < 0.0011$) from treatments T2 = 1.01 ± 1.07 and T3 = 0.12 ± 0.09 log CFU·g$^{-1}$, which presented the lowest counts of colonies of heterotrophic bacteria. In the lactic acid bacteria count, there was increase in count in treatments supplemented with probiotic (T1 = 2.50 ± 0.22; T2 = 4.43 ± 0.09; and T3 = 7.11 ± 0.30 log CFU·g$^{-1}$, respectively), stood out ($p < 0.0001$) to the control group (C = 0.34 ± 0.25 log CFU·g$^{-1}$) (Fig. 2).

After bacterial challenge with *A. hydrophila*, there were differences ($p < 0.05$) between treatments, with the highest colony counts of probiotics found in the intestines of fish from treatment T3 (5.34 ± 0.32 × 10$^7$ CFU·g$^{-1}$) and the lowest levels in the control (+). In the count of total heterotrophic bacteria in the intestines of fish after the challenge, there was also a significant difference between the groups, with lower bacterial counts in T3 compared to the other treatments. The C+ treatment showed the highest concentration of bacteria, the opposite was observed in C- (SSE 0.65%), that, in both cases, showed the lowest counts of lactic acid bacteria and total heterotrophs after acute challenge (Table 3).

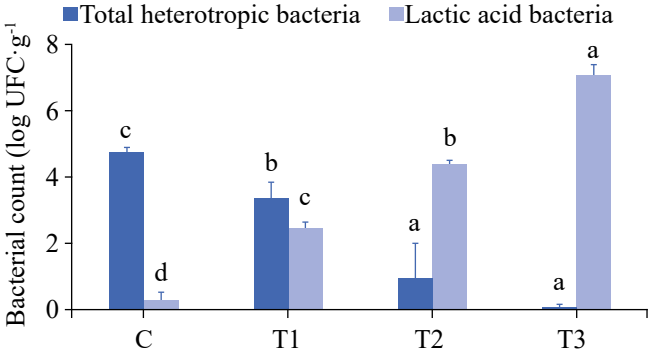



**Figure 2.** Total bacterial counts (heterotrophic × lactic acid) in the intestine of piauçu *Megaleporinus macrocephalus* larvae fed a control diet (C), *Artemia* sp. not supplemented with probiotic and diets with *Artemia* sp. supplemented with different concentrations of autochthonous *Enterococcus faecium* (T1: 1 × 10$^4$; T2: 1 × 10$^6$; T3: 1 × 10$^8$ CFU·mL$^{-1}$). Different letters in the columns indicate a significant difference ($p < 0.05$).

## Intestinal histomorphometry

Total villus height, villus height and intestinal mucosa thickness of fish fed a diet containing *E. faecium* at T3 (10$^8$ CFU·mL$^{-1}$) were significantly higher ($p < 0.01$; Table 4) compared to those of the other treatments. Regarding villus width and epithelium thickness, T2 and T3 showed higher values ($p < 0.01$), distinguishing them from the other groups (Table 4). Thus, the intestinal villi of the larvae were altered by probiotic supplementation, with T3 prominence, so that significantly higher than other treatments ($p < 0.05$; Fig. 3).

## Sanitary challenge

The acute infection challenge with *A. hydrophila* presented significant differences ($p < 0.001$) between groups challenged with pathogenic bacteria, with greater mortality in the control positive (93% ± 11.54%) and lower mortality for supplemented with artemia enriched with *E. faecium*, especially in treatment T3, 10$^8$ (33% ± 11.54%). Between diets supplemented with probiotics, T1 and T2, mortality rates were 66% ± 11.54% and 46 ± 11.54%, respectively, with the highest rates recorded between 48 and 72 h of the infection. For the control group negative, the survival rate was 100% (Fig. 4).

For bacterial challenge with the pathogen, Koch's postulate was confirmed by the presence of *A. hydrophila* in the intestines of animals challenged with the bacterium and that had died during the experimental period. After acute challenge, pathogenic strains were reisolated from infected fish and identified by the MALDI-TOF technique.

**Table 3.** Count of lactic acid bacterium and total heterotrophic bacterium in the intestine of fish after acute challenge with *Aeromonas hydrophila*.

| Site | Treatments | | | | |
|---|---|---|---|---|---|
| | C (-) | C (+) | T1 | T2 | T3 |
| Intestine AL | 0.45 ± 0.3$^2$ d | 1.26 ± 0.08 × 10$^3$ c | 2.31 ± 0.04 × 10$^3$ c | 3.28 ± 0.05 × 10$^5$ b | 5.34 ± 0.32 × 10$^7$ a |
| Intestine HT | 1.32 ± 0.9$^2$ a | 4.62 ± 2.34 × 10$^5$ c | 1.80 ± 0.25 × 10$^4$ b | 1.40 ± 0.48 × 10$^4$ b | 2.09 ± 0.32 × 10$^2$ a |

Intestine AL: count of lactic acid bacteria in the intestine; intestine HT: total heterotrophic bacterium count in post-larvae intestines after acute challenge with *Aeromonas hydrophila*; C (-) negative control: sterile saline solution (0.65%); C (+) positive control: not probiotic; T1: 1 × 10$^4$; T2: 1 × 10$^6$; and T3: 1 × 10$^8$ CFU.mL$^{-1}$. Different letters on the same line differ by Tukey's test (p<0,05).

**Table 4.** Effects of autochthonous probiotic *Enterococcus faecium* supplemented via *Artemia* sp. supers on intestinal histomorphology of larvae of *Megaleporinus macrocephalus*.

| Parameters | Treatment | | | | |
|---|---|---|---|---|---|
| | C | T1 | T2 | T3 | p-value |
| Total height of villus (µm) | 0.13 ± 0.02 d | 0.15 ± 0.02 c | 0.20 ± 0.01 b | 0.26 ± 0.03 a | 0.01 |
| Height of villus (µm) | 0.11 ± 0.02 d | 0.12 ± 0.02 c | 0.17 ± 0.02 b | 0.23 ± 0.03 a | 0.01 |
| Villus width (µm) | 0.06 ± 0.01 c | 0.08 ± 0.02 b | 0.09 ± 0.02 a | 0.09 ± 0.01 a | 0.01 |
| Epithelium thickness (µm) | 0.03 ± 0.01 c | 0.04 ± 0.03 b | 0.05 ± 0.02 a | 0.05 ± 0.01 a | 0.01 |
| Mucosal thickness (µm) | 0.025 ± 0.010 c | 0.027 ± 0.007 c | 0.034 ± 0.009 b | 0.038 ± 0.008 a | 0.01 |

Control: without probiotic; T1: $1 \times 10^4$; T2: $1 \times 10^6$; T3: $1 \times 10^8$ CFU·mL$^{-1}$. Data presented in mean ± standard deviation. Different letters in the lines mean statistical difference ($p < 0.05$).

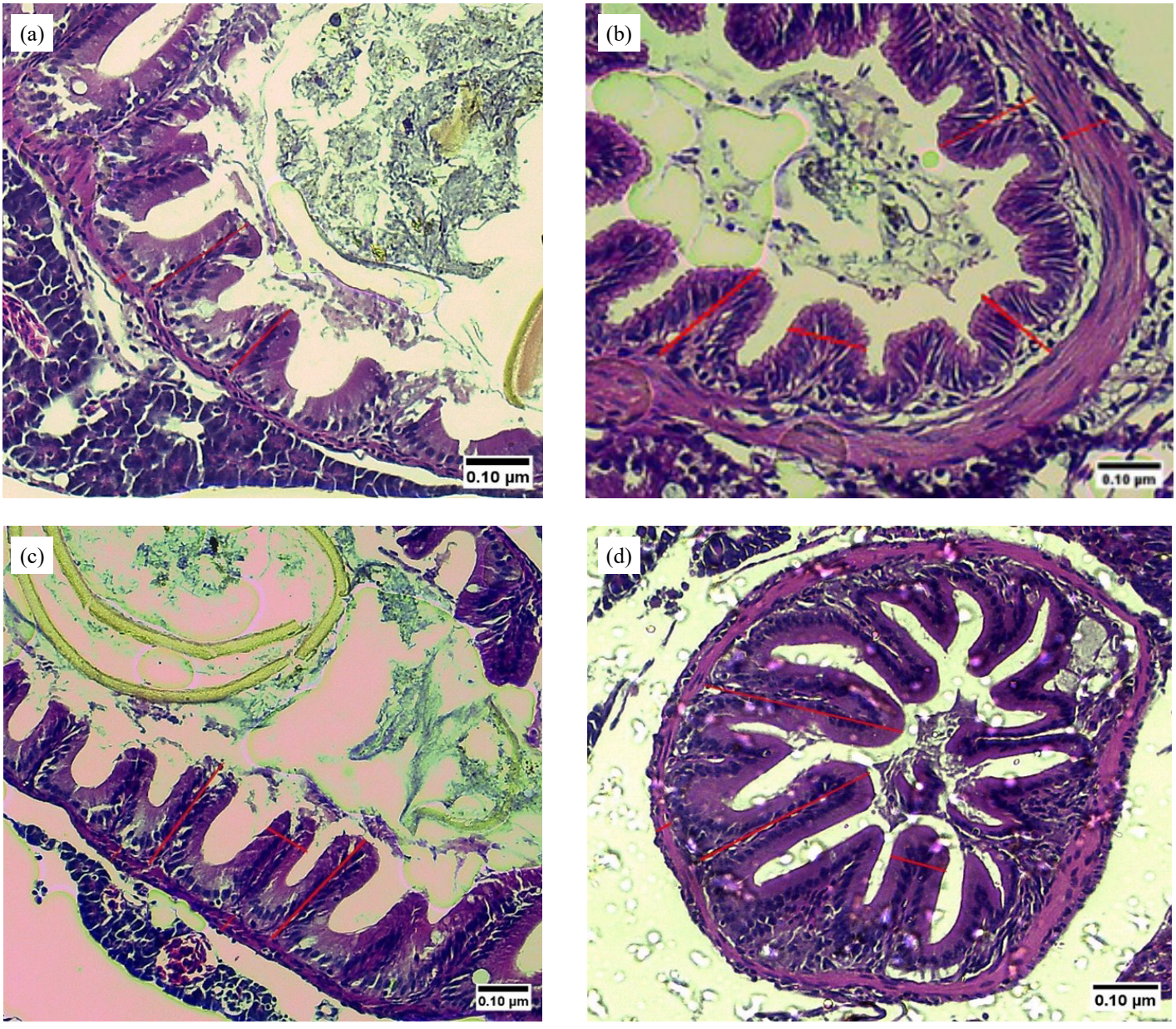
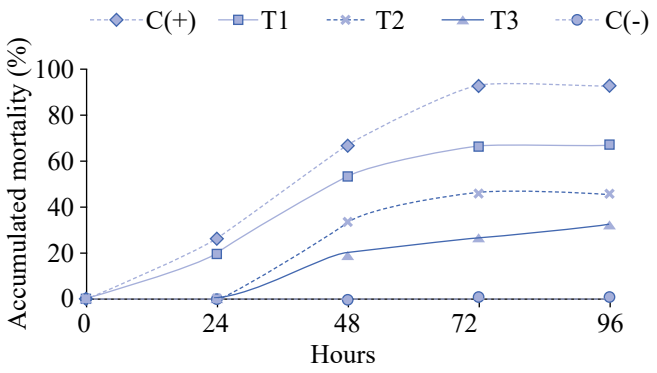



**Figure 3.** The structure of the intestine of (a), (b), (c) and (d) *Megaleporinus macrocephalus* gut represents the control (C), T1, T2 and T3, respectively. Tissue sections were stained with hematoxylin and eosin with visualization in the objective (20x).




Control diet (C+): without probiotic; T1: $1 \times 10^4$; T2: $1 \times 10^6$; T3: $1 \times 10^8$ CFU·mL$^{-1}$; negative control (C-): with sterile saline solution (0.65%).

**Figure 4.** Accumulated mortality up to 96 h after infection by *Aeromonas hydrophila* in piauçu (*Megaleporinus macrocephalus*) larvae, after 20 days of larviculture fed with *Artemia* sp. enriched with native *Enterococcus faecium* autochthonous.

## DISCUSSION

Supplementation with autochthonous probiotic bacterium has been reported for several fish species (Sun et al., 2013; Ghorbani Vaghei et al., 2019; Masduki et al., 2020; Sousa et al., 2020). Studies have demonstrated the effects of supplementation with probiotic monostrains on improving zootechnical performance and animal immunocompetence (Masduki et al., 2020; Sousa et al., 2020; Suphoronski et al., 2021; Hossain et al., 2022).

However, to date, there are no studies on probiotic supplementation in the diet of piauçu *M. macrocephalus* larvae. The findings of research indicate that the probiotic *E. faecium*, at $1 \times 10^8$ CFU·mL$^{-1}$ and administered via live food in *Artemia* sp., improved the productive performance of the microbiota and the intestinal morphometry, in addition to increasing resistance against the pathogen *A. hydrophila*. After 20 days of supplementation, improvements were observed in the growth performance of piauçu that were supplemented with *E. faecium* at the highest concentrations, T2: $1 \times 10^6$ and T3: $1 \times 10^8$ CFU·mL$^{-1}$.

The highlight was for fish from T3, which showed greater weight gain and final length (Table 1) and higher specific weight increment (Fig. 1b). These results may be related to the greatest presence of lactic acid bacteria in the intestines of the animals. Similar results were observed for tilapia larvae supplemented with *Bacillus pocheonensis* at the concentration of $5 \times 10^5$ CFU·mL$^{-1}$ in *Moina micrura* enrichment, conferring greater larval survival and resistance to *Streptococcus agalactiae* (Samat et al., 2021). The enrichment of *Artemia* nauplii with the probiotic *Bacillus subtilis* and inulin at the concentration of 7 g·L$^{-1}$ for 12 days also promoted a better gain in length and total height of *Pseudoplatystoma reticulatum* larvae (Oliveira et al., 2022).

The administration of probiotic via live foods such as artemia and rotifers, in the feeding of fish and crustacean larvae, has gained increasing interest in aquaculture (Lobo et al., 2018; Ghoname et al., 2020; Samat et al., 2021; Oliveira et al., 2022). Previous studies showed higher growth and survival rates, as well as higher protease, amylase, and lipase activities in *Macrobrachium rosenbergii* post-larvae supplemented with *Enterococcus durans* enriched in *Artemia franciscana* (Bhaheerathan et al., 2020), greater resistance against the pathogen *Vibrio harveyi* in larvae *Lates calcarifer* supplemented via *Artemia* with *Enterococcus hirae* (Masduki et al., 2020) and higher n-3 HUFA levels in *Solea senegalensis* larvae (Lobo et al., 2018). Overall, the introduction of probiotics through enrichment of live diets showed positive results, with higher growth and survival rates (Avella et al., 2010; Bhaheerathan et al., 2020), in addition to improving the immune responses of fish (Sun et al., 2013; Ghoname et al., 2020).

The colonization of the intestinal tract of piauçu larvae was evidenced by the increase in the intestinal lactic bacteria count in fish supplemented with autochthonous bacteria, with emphasis on the T3 ($7.11 \pm 0.30$ log CFU·g$^{-1}$) and lower counts at T1 and T2 ($2.50 \pm 0.22$ and $4.43 \pm 0.09$ log CFU·g$^{-1}$, respectively). This fall, in counts of lactic bacteria in T1 and T2, may be associated with insufficient amounts to maintain a stable optical density in the intestinal mucosa of the animal (Fig. 2). Opposite to the *Lactobacillus* sp. supplementation for *Astyanax bimaculatus*, where no differences were found between probiotics amounts, the effects of microbiological colonization act differently in each host (Jatobá et al., 2020). Many strains of LAB beneficially alter the intestinal tract, modulating the intestine microbiota according to the amounts of microorganisms supplemented in the diet (Dias et al., 2018).

Studies with dietary supplementation with *Lactobacillus* spp. via autochthonous feeding of *Astyanax bimaculatus* post-larvae also reported higher lactic acid bacterial counts ($\geq 1 \times 10^7$ CFU·g$^{-1}$) in relation to bacteria heterotrophic ones such as *Vibrio* spp., *Pseudomonas* spp. and *Staphylococcus* spp. (Moraes et al., 2018). The bacteria-host relation is an important factor in the viability of the selected strain, for the autochthonous bacterium have greater affinity for the adhesion of the intestinal epithelium of the host (Sousa et al., 2019; Yamashita et al., 2020; Yeganeh Rastekenari et al., 2021; Hossain et al., 2022). The report with supplementation with allochthonous and autochthonous probiotic bacteria (*Lactobacillus* sp. and *L. lactis*) in two species

of *lambari* (*Astyanax bimaculatus* and *Astyanax fasciatus*) highlight the groups of indigenous bacteria in tolerant changes in the microbiota of fish, with better adhesion to the epithelium in relation to allochthonous bacteria (Jatobá and Jesus, 2022).

The intestinal probiotic colonisation with *E. faecium* also promoted an intestinal modulation that influenced the increase in the length and width of the intestinal villi (Table 4; Fig. 3). Similar results were found for the supplementation of *Artemia* sp. enriched with *Bacillus subtilis* at the concentration of $10^6$ CFU·g$^{-1}$, in the feeding of *Pseudoplatystoma reticulatum*, with the highest values in total villus length of 24.4 ± 0.76 µm in the probiotic treatment compared to the control and symbiotic treatments (Oliveira et al., 2022). The application of *Planococcus* sp. ($1 \times 10^7$ CFU·mL$^{-1}$) bioencapsulated in Artemia, supplemented in the diet of *Sparus aurata*, significantly ($p < 0.05$) altered the villi length from 13 ± 30 to 35 ± 90 µm and the number of goblet cells from 20 ± 2 to 37 ± 75, but it did not affect the villus count for 20 days. Additionally, after 40 days of experiment, all parameters were changed by probiotic supplementation, to 126 ± 25 µm in villus length, 27 ± 2.5 for villus number and 96 ± 14.4 goblet cells (Ghoname et al., 2020). Thus, alteration in the villi may vary according to the period of supplementation and stage of larval development.

Changes in villus height and width are associated with the production and propagation of probiotics throughout the intestine; carbohydrates are used, generating short-chain fatty acids (SCFA) and therefore promoting the creation of peptides in the intestine and the formation of butyric acid. This balances the microbiota and maintains the integrity of the intestinal epithelial cells, facilitating nutrient absorption (Poolsawat et al., 2019; Ringø et al., 2020). Thus, probiotic supplementation increases the contact surface of the intestinal mucosa and, consequently, facilitates the use of dietary nutrients (Moraes et al., 2018; Deng et al., 2022).

The colonization, modulation and alteration of the intestinal villi reflected in improvements in the growth performance of the larvae. Similar results were reported for *Epinephelus coioides* larvae fed with autochthonous *Bacillus clausii* and *Bacillus pumilus* in the copepod *Pseudodiaptomus annandalei*, at the concentration of $1 \times 10^6$ CFU·mL$^{-1}$, which resulted in higher total length averages, increased total weight and the survival rate of 46.67% after 14 days of supplementation (Sun et al., 2013); however, survival was lower than that observed for piauçu larvae supplemented with *E. faecium*. A positive effect on growth and survival was also observed in *Chirostoma jordani* larvae fed with *Artemia franciscana* metanauplii for 40 min with *Lactobacillus johnsonii* ($2.3 \times 10^3$ CFU·mL$^{-1}$) and *Bifidobacterium animalis* ($2.06 \times 10^3$ CFU·mL$^{-1}$) (Vázquez-Silva et al., 2016).

The antagonistic activity against pathogens is one of the most important properties to be evaluated in a probiotic candidate. In the present study, the enrichment of *Artemia* with the autochthonous probiotic *E. faecium* in feed of *M. macrocephalus*, at concentrations T1: $10^4$, T2: $10^6$ and T3: $10^8$ CFU·mL$^{-1}$ after 20 days of supplementation, decreased the cumulative mortality rate during 96 h of acute infection with the pathogen *A. hydrophila.* The highest survival rate was observed for T3 (66.67%). These results corroborate those observed for the supplementation of Nile tilapia *Oreochromis* sp. larvae with *Bacillus pocheonensis*, administered via *Moina micrura* at concentrations of $10^4$ and $10^6$ CFU·mL$^{-1}$. The authors observed survival rates of 75 and 77%, respectively, after challenge with *Streptococcus agalactiae* (Samat et al., 2021). A study with autochthonous *E. faecium* showed increase of the fish immunity and decrease for mortality from 88.29% for the control group to 73.33% in the group with probiotic in tilapia infected by *S. agalactiae* (Suphoronski et al., 2021).

Generally, lactic acid bacteria can activate the host's immune system in situations of stress or acute illness. Among their characteristics, there is their ability to adapt to the gastrointestinal tract, which allows them to compete for specific binding sites in the intestinal layer and to dominate the bacterial community (He et al., 2017; Li et al., 2019). In addition, they inhibit pathogens both by competitive exclusion and the secretion of inhibitory substrates, such as hydrolytic enzymes (chitinases, proteases, cellulases, and β-1,3-glutanase), with antimicrobial properties capable of degrading the cell wall components of pathogenic microorganisms (Urdaci and Pinchuk, 2004; Allameh et al., 2017). This influence of probiotic strains on adhesion by exclusion and expressive reduction of potentially pathogenic bacteria has been widely proposed as a characteristic of the probiotic effect on the host. This mechanism of exclusion of these microorganisms is linked to the production of antimicrobial in the quorum sensing (Mouriño et al., 2017; Chauhan and Singh, 2019; Dias et al., 2022).

In addition, probiotic strains of the genus *Enterococcus* can increase intestinal mucosal immunity through a higher concentration of pro-inflammatory factors (tumor necrosis factor-α and interleukin-β) (Standen et al., 2016). Alternatively, they can sensitize macrophage pattern recognition receptors, such as dectin-1 and β-glucan, thus activating the immune system in the face of an infection (Hoseinifar et al., 2018). Thus, the use of the probiotic *E. faecium* in live food in the first stage of live of *M. mecrocephalus* was able to potentiate the weight gain and survival of the larvae. In addition, they modulate

the gut microbiota and villi, providing greater resistance to the pathogenic bacterium.

## CONCLUSION

Thus, this is the first report on the use of autochthonous probiotic bacteria in the feeding of piauçu *M. macrocepahlus* larvae. Food supplementation with *Artemia* sp. enriched with the probiotic bacteria *E. faecium* 20218_1 CHB, at the concentration of $1 \times 10^8$ CFU·mL$^{-1}$ (T3), for 20 days, promoted greater larval growth performance, modulated the gut microbiota and altered the villi intestinal tracts of supplemented fish, in addition to increasing resistance against the pathogen *A. hydrophila*.

## CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

## ETHICAL APPROVAL

The study was approved and conducted within all ethical standards, including approval by the animal experimentation ethics committee under protocol number: CEUA no. 3991300420.

## DATA AVAILABILITY STATEMENT

All dataset were generated or analyzed in the current study.

## FUNDING

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior Finance Code 001

## AUTHORS' CONTRIBUTIONS

**Conceptualization:** Barros FAL, Sousa NC, Cordeiro CAM, Fujimoto RY; **Investigation:** Barros FAL, Dias JAR; **Resources:** Costa Junior KS; **Methodology:** Barros FAL; **Data curation:** Barros FAL, Dias JAR, Abe HÁ, Sousa NC, Fujimoto RY; **Supervision:** Cordeiro CAM, Fujimoto RY; **Writing — original draft:** Barros FAL, Dias JAR, Sousa NC; **Writing — review & edition:** Barros FAL, Abe HA, Fujimoto RY.

## ACKNOWLEDGMENTS

Thank you all for participating in the research and for sharing your knowledge. In particular, Universidade Federal do Pará, Campus Bragança, Instituto de Estudos Costeiros, Universidade Federal Rural da Amazônia, EMBRAPA – Tabuleiros Costeiros and the Programa de Pós-Graduação em Ciência Animal for the infrastructure and equipment provided for the development of the research.

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