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Probiotics feed additive promotes growth and health on stressed *Arapaima gigas*

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ABSTRACT

Arapaima gigas, an endemic fish species of the Amazon region, is characterized by rapid growth and acceptance of artificial feed despite being carnivorous. In fish farms, when submitted to stressful conditions, this fish is susceptible to various diseases. There is evidence that the health and zootechnical performance of farmed aquatic animals can be improved by the prophylactic use of probiotics. Therefore, this study aimed at investigating growth, physiology and stress resistance of *A. gigas* (35.22 ± 2.25 g) fed diets supplemented with commercial probiotic mix additive containing *Bacillus subtilis*, *Enterococcus faecium*, *Lactobacillus reuteri* and *Pediococcus acidilactici* (Aquastar®), at four concentrations: 0, 1, 5, or 10 g of Aquastar® per kg of diet. The daily growth index was higher in fish fed 10 g of probiotic mix per kg of diet. No changes were observed in hematocrit and hemoglobin. The albumin:globulin ratio increased in fish receiving 1 and 5 g probiotic mix, but significantly decreased in stressed fish fed 1 g kg^{-1} due to reduced albumin and increased total protein levels. Plasma total cholesterol and triglyceride levels decreased, and leukocytes number increased in *A. gigas* that received the probiotic mix, indicating beneficial effects on the immune system and health of this fish species. This is the first study providing hematological and biochemical parameters of *A. gigas* fed with probiotic mix additive supplement diets. Our results indicate that *A. gigas*, particularly when supplemented with 10 g kg^{-1} of commercial multi-cepa probiotic mix, presented improved growth, health and stress resistance.

KEYWORDS: feed additive, Amazonian fish, blood parameters, physiological resistance

Aditivos probióticos na alimentação promovem crescimento e saúde em *Arapaima gigas* estressados

RESUMO

Arapaima gigas, é uma espécie de peixe endêmica da região Amazônica, com rápido crescimento e aceitação de alimentação artificial, apesar de ser carnívoro. Quando exposto na piscicultura a condições estressantes mostra-se suscetível a doenças. Porém, temos evidências de melhoria da saúde e desempenho zootécnico de animais aquáticos com o uso profilático de probióticos. Assim, o objetivo foi investigar crescimento, fisiologia e resistência ao estresse de *A. gigas* (35,22 ± 2,25 g) alimentados com dietas suplementadas com mix de probióticos comercial contendo *Bacillus subtilis*, *Enterococcus faecium*, *Lactobacillus reuteri* e *Pediococcus acidilactici* (Aquastar®). O mix probiótico foi incorporado em quatro concentrações: 0, 1, 5 ou 10 g de Aquastar® kg^{-1} de ração. O índice de crescimento diário foi maior nos animais alimentados com 10 g de mix probiótico kg^{-1}. Não foram observadas alterações no hematócrito e na hemoglobina. A relação albumina:globulina aumentou nos peixes que receberam 1 e 5 g do mix probiótico, mas reduziu nos peixes alimentados com 1 g kg^{-1} de mix probiótico e estressados, devido à redução de albumina e aumento de proteínas totais. Colesterol total e triglicérides reduziram e os leucócitos aumentaram em *A. gigas* que receberam o mix probiótico, mostrando benefícios à saúde e ao sistema imunológico. Este é o primeiro estudo sobre parâmetros hematológicos e bioquímicos de *A. gigas* alimentado com mix probiótico. Nossos resultados indicam que a suplementação da dieta de *A. gigas*, especialmente com 10 g kg^{-1} do mix probiótico, leva à melhora do crescimento, saúde e resistência ao estresse dos animais.

PALAVRAS-CHAVE: aditivo alimentar, peixe amazônico, parâmetros sanguíneos, resistência fisiológica

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INTRODUCTION

Arapaima gigas (Schinz 1822), known as 'pirarucu', is a fish species endemic to the Amazon region. The interest in this fish for aquaculture has increased due to its rapid growth, reaching 10 kg in a year, and its ability to accept artificial feed despite being a carnivorous species (Cavero et al. 2003; da Costa Sousa et al. 2019). In addition, this species has a high nutritional quality flesh (Cortegano et al. 2017). In fish farming, the animals are exposed to stressful conditions and are susceptible to a variety of diseases (Balcázar et al. 2006). The use of probiotics could confer physiological modulation and improve fish immunological responses (Khattab et al. 2004; Balcázar et al. 2006), and there is growing evidence that the health and zootechnical performance of farmed aquatic animals can be improved by the prophylactic use of those products (Chauchevras-Durand and Durand, 2010; Chizhayeva et al. 2022). Besides that, probiotics are safe, eco-friendly live organisms that, when supplied in sufficient amounts, were shown to improve the health status of the fish (Eissa et al. 2022) by elevating favorable bacteria, augmenting metabolism, and strengthening the immune system against diseases (Nayak 2010; Brum et al. 2025).

Probiotics are one of the antimicrobial alternatives to antibiotics, since these may cause negative changes to the hematological, biochemical and antioxidant status of fish, and possess a potential risk to the ecosystems (De et al. 2014; Iftikhar and Hashmi, 2021). Moreover, probiotics can be effective in preventing some diseases and in reducing economic losses during feed changes, transportation or other types of stress by strengthening the immunological system of supplemented fish (Dias et al. 2018; da Costa Sousa et al. 2019; Chizhayeva et al. 2022). *Lactobacillus* and *Bacillus* species have been found to complement each other by exerting synergistic immunomodulatory responses in fish (Salinas et al. 2005; Ferrarezi et al. 2024). The use of lactic acid bacteria has antagonistic activity against opportunistic bacteria, viruses and fungi that cause fish and shellfish diseases (Ringø et al. 2020; Chizhayeva et al. 2022). Different probiotics, either on monospecies or multispecies supplementation, can eventually elevate phagocytic, lysozyme, complement system, respiratory burst activity as well as expression of various cytokines in fish (Nayak 2010; Telli et al. 2014; Hoshino et al. 2017, 2020; Dias et al. 2020). In addition, various factors such as source, type, dose, and duration of supplementation of probiotics can significantly affect their immunomodulatory activity (Nayak 2010).

In recent years, research on native Brazilian fish, such as tambaqui *Colossoma macropomum* (Souza et al. 2024); dourado *Salminus brasiliensis* (Oliveira et al. 2024); and hybrid surubim (*Pseudoplatystoma corruscans* x *P. reticulatum*) (Do Nascimento Veiga et al. 2020) has focused on enhancing immune responses through the supplementation of probiotic strains. While a few studies have examined probiotic supplementation in *A. gigas* (da Costa Sousa et al. 2019; do Vale Pereira et al. 2019; do Couto et al. 2022), they have only evaluated single-strain probiotics rather than multispecies. Therefore, the present study aimed to evaluate the dietary effects of a commercial multi-cepa probiotic mix (*Bacillus subtilis*, *Enterococcus faecium*, *Lactobacillus reuteri* and *Pediococcus acidilactici*) on the growth, physiology and stress resistance of *A. gigas*.

MATERIAL AND METHODS

This study was approved by the Committee for Animal Use of Federal University of Acre (No 23107009564/2014-29) and is registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge, SisGen (identification number A9FE48F).

Pirarucu fingerlings *A. gigas* (± 12 cm) were acquired from a commercial fish farm of Rio Branco, Acre State (Brazil). Fish were transported by air with all the documents, such as the transport guide, and received at Embrapa Amapá, Macapá, Amapá State (Brazil). Fish were acclimated at the Laboratory of Aquaculture and Fisheries in 1,000 L water tanks for 15 days, with aeration and continuous water flow. Fish specimens accepted the food a few days after arriving at the laboratory. Pirarucu fingerlings were fed *ad libitum* four times a day with a ration containing 45% crude protein (Nutripiscis, Presence®, Evialis do Basil Nutrição Animal Ltda., São Paulo, Brazil). Fish were kept in water tanks with dissolved oxygen (6.5 ± 0.07 mg L^{-1}), temperature (29.0 ± 0.06°C) and pH (5.7 ± 0.07) values, those were monitored daily using a multiparameter probe (Horiba Mod. U52, Kyoto, Japan).

Feeding trial and experimental diets

A multi-cepa feed additive containing probiotics such as *Bacillus subtilis*, *Enterococcus faecium*, *Lactobacillus reuteri* and *Pediococcus acidilactici*, named Aquastar® (Biomin, Austria), was used. This probiotic mix was added to extruded ration for carnivorous fish (45% crude protein, Presence, USA), at four different concentrations: 0 (control), 1, 5 or 10 g Aquastar® per kg^{-1} diet (manufacturer recommendation for aquaculture). In the case of diets with probiotic mix, soybean oil was used to incorporate the mix into each one kg of extruded commercial fish feed. All experimental diets were prepared with a volume of 50 mL of soybean oil. After that, the experimental diets were homogenized (ration and oil with probiotic mix or just ration and oil, in the case of control) and left to dry in a chamber heated to 60 °C for about 24 h. The analysis of the chemical composition of the experimental diets was performed in triplicate, according to the Association of Official Analytical Chemists (AOAC 1995) guidelines. The basal composition of commercial ration (manufacturer's data) and proximate composition of experimental diets containing different concentrations of the probiotic mix are shown in Table 1.

Table 1. Basal composition of commercial ration and proximate composition of experimental diets containing different concentrations of probiotic mix additive.

Parameters	Manufacturer's data	Diet (probiotic mix kg^{-1} feed)			
		0 g	1 g	5 g	10 g
Dry matter (%)	87	94.58±0.23	94.76±0.48	94.05±0.27	93.59±0.07
Crude protein (%)	45	45.37±0.41	45.11±0.40	43.95±1.38	45.83±0.78
Ether extract (%)	9	6.74±0.22	7.09±0.18	6.63±0.35	6.93±1.80
Ashes (%)	16	9.86±0.08	9.89±0.02	9.85±0.12	9.58±0.56
Phosphorus (%)	1	1.21±0.01	1.18±0.05	1.16±0.03	1.21±0.01
Calcium (%)	2-3	1.43±0.52	1.62±0.39	1.71±0.32	1.80±0.27

Data expressed as mean values ± standard deviation.

The fish (initial weight 35.22 ± 2.25 g and initial length 17.57 ± 0.46 cm) were distributed in 12 tanks of 100 L, with three replicates per treatment and eight fish per replicate. Fish were fed experimental diets, four times a day (8:30; 10:30; 14:30 e 16:30h), at 10% of biomass, for 30 days. On the 31st day of feeding, four fish were collected from each experimental unit for evaluation, totaling 12 fish per treatment. The fish were weighed, and blood samples were collected. The remaining fish were kept in the experimental units and subjected to handling stress, consisting of just one chasing of the fish with a net for five minutes (Hoshiba et al. 2009), thus simulating a daily management activity in fish farms (Brandão et al. 2006). Twelve hours after these fish had been subjected to stress, a blood sample was taken from each specimen and they were weighed and measured, obtaining values from non-stressed and stressed fish. After blood collection, the specimens were euthanized by spinal transection to obtain body measures; liver and viscera were removed and weighed.

Growth parameters

The data on initial weight (g) and final weight (g) were used to calculate fish performance parameters: Weight gain (WG, in g) = final weight - initial weight; Daily growth index (DGI, in %/day) = 100 x (final mean body weight$^{1/3}$ - initial mean body weight$^{1/3}$) / trial duration; Hepatosomatic index (HSI, in %) = 100 x (liver weight / body weight); and, Viscerosomatic index (VSI, in %) = 100 x (viscera weight / body weight) (Hoshino et al. 2020).

Blood parameters and respiratory activity of leukocytes

Blood samples were obtained by caudal vessel puncture using syringes containing sodium heparin (5,000 U.I. mL^{-1}). Each sample was used to determine hematocrit (Ht), hemoglobin concentration (Hb) and red blood cells count (RBC). These data were used to calculate mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Blood smears were prepared and panchromically stained with a combination of May Grünwald-Giemsa-Wright, for differential leukocyte count in up to 200 cells of interest and to determine the total number of leukocytes and thrombocytes (Ishikawa et al. 2008; Ranzani-Paiva et al. 2013).

The respiratory burst activity was determined according to Sahoo et al. (2005) and Biller-Takahashi et al. (2013), based on reactive oxygen species (ROS) produced by macrophage respiratory activity through colorimetric essay with nitroblue tetrazolium (NBT, Sigma, St Louis, MO, USA) mixed with n, n-dimetil formamida (DMF, Sigma, St Louis, MO, USA). The optical density of supernatant was determined on spectrophotometer (Biospectro SP-220, Curitiba, Brazil) at 540 nm. The remaining blood samples of each fish were centrifuged at 75 G for 10 min (Centrifuge Mod. 5424, Hamburg, Germany) and the plasma was used to determine glucose, total proteins, albumin, total cholesterol, and triglycerides levels, using specific colorimetric kits (Labtest®, MG, Brazil) for each metabolite, with absorbance readings at a spectrophotometer (Biospectro SP-220, Curitiba, Paraná, Brazil). Globulins content was determined by subtracting albumin from total protein levels to determine albumin:globulin (A:G) ratio (Mohapatra et al. 2012).

Statistical analysis

The data were first tested for normality and homoscedasticity using the Shapiro-Wilk and Levene methods, respectively, and analyzed using Kruskal-Wallis test and post hoc Dunn test comparing means. Differences were considered significant at 5% probability (Zar 2010). The tests were performed using the GraphPad Instat (Version 3.00, 1997) statistical software.

RESULTS

There were no changes in weight or body length in response to increased concentrations of the commercial probiotic evaluated (0, 1, 5 and 10 g probiotic mix additive per kg^{-1} of feed) in their diets, as compared to control (Table 2). The WG was higher ($p < 0.05$) for individuals that received 10 g probiotic mix additive kg^{-1} of feed, compared to the control group. Consequently, the DGI was significantly higher in the individuals fed with 10 g probiotic mix additive per kg^{-1} of feed than in those fed diets containing 0 or 1 g probiotic mix additive per kg^{-1} of feed. HSI and VSI were not affected by the addition of probiotics to the

fish diet ($p > 0.05$). However, after stress, no changes in weight or body length were observed. Both the control group and the group receiving 10 g probiotic mix additive per kg^{-1} of feed showed a reduction in HSI. Additionally, VSI was significantly smaller after induction of stress in the individuals that received the highest amount of the probiotic additive.

Several hematological and biochemical health markers were affected by the use of probiotics, or the combination of probiotics and stress (Table 3). Ht and Hb did not differ among probiotic treatments and control, either for animals subjected to chasing stress or not. RBC was higher ($p < 0.05$) for animals that received 1 or 5 g probiotic mix additive kg^{-1} per feed. In the groups receiving 0, 1 and 10 g kg^{-1} feed, a significant increase in the number of these blood cells was observed only after stress. The groups that received 1 and 5 g kg^{-1} probiotics had lower MCV values ($p < 0.05$), with stress causing a reduction in MCV values in the 1 and 10 g kg^{-1} probiotic treatments. The MCV values were significantly lower in the animals that received probiotics than in the group that did not receive supplementation. A reduction in MCH was observed with the

Table 2. Growth performance parameters of *Arapaima gigas* fed the experimental diets containing different concentrations of probiotic mix additive for 30 days, before and 12h after stress.

	0 g probiotic mix kg^{-1} feed		1 g probiotic mix kg^{-1} feed		5 g probiotic mix kg^{-1} feed		10 g probiotic mix kg^{-1} feed	
	Non-stressed	**Stressed**	**Non-stressed**	**Stressed**	**Non-stressed**	**Stressed**	**Non-stressed**	**Stressed**
Initial weight (g)	35.58 ± 2.06^{a}	-	35.20 ± 2.12^{a}	-	35.02 ± 2.45^{a}	-	35.10 ± 2.31^{a}	-
Final weight (g)	250.41 ± 45.67^{a}	253.56 ± 32.85^{A}	253.32 ± 30.65^{a}	247.69 ± 36.17^{A}	262.01 ± 38.01^{a}	270.83 ± 36.37^{A}	277.27 ± 44.15^{a}	248.68 ± 69.91^{A}
Initial length cm)	17.63 ± 0.47^{a}	-	17.56 ± 0.39^{a}	-	17.50 ± 0.56^{a}	-	17.58 ± 0.45^{a}	-
Final length (cm)	33.75 ± 2.41^{a}	34.38 ± 1.34^{A}	33.85 ± 1.60^{a}	34.04 ± 1.45^{A}	34.76 ± 1.49^{a}	35.01 ± 1.16^{A}	34.67 ± 1.97^{a}	34.92 ± 2.65^{A}
WG (g)	214.83 ± 27.64^{b}	271.98 ± 15.83^{A}	219.15 ± 7.08^{ab}	214.29 ± 22.33^{A}	227.44 ± 5.04^{ab}	236.77 ± 13.20^{A}	242.23 ± 3.79^{a}	213.59 ± 29.07^{A}
DGI (%/Day)	10.02 ± 0.79^{b}	10.13 ± 0.44^{A}	10.19 ± 0.23^{b}	10.05 ± 0.13^{A}	10.44 ± 0.13^{ab}	10.68 ± 0.36^{A}	10.82 ± 0.11^{a}	10.02 ± 0.82^{A}
HSI (%)	1.37 ± 0.18^{a}	$1.19\pm0.13^{A*}$	1.25 ± 0.06^{a}	1.24 ± 0.12^{A}	1.37 ± 0.18^{a}	1.19 ± 0.17^{A}	1.41 ± 0.17^{a}	$1.20\pm0.13^{A*}$
VSI (%)	6.18 ± 0.61^{a}	5.78 ± 0.62^{A}	5.79 ± 0.24^{a}	6.07 ± 0.50^{A}	6.08 ± 0.54^{a}	5.79 ± 0.65^{A}	6.41 ± 0.86^{a}	$5.67\pm0.55^{A*}$

Data expressed as mean values ± standard deviation. WG: weight gain, HSI: Hepatosomatic index, VSI: Viscerosomatic index, DGI: Daily growth index, Values followed by * indicate significant difference between stressed and not stressed fish ($p<0.05$). Values followed by different lowercase letters, on the same line, indicate difference between non-stressed animal groups by the Dunn test ($p<0.05$). Values followed by different uppercase letters, on the same line, indicate difference between stressed animal groups by the Dunn test ($p<0.05$).

Table 3. Hematological and biochemical parameters of *Arapaima gigas* fed experimental diets containing different concentrations of probiotic mix additive for 30 days, before and 12h after stress.

	0 g probiotic mix kg^{-1} feed		1 g probiotic mix kg^{-1} feed		5 g probiotic mix kg^{-1} feed		10 g probiotic mix kg^{-1} feed	
	Non-stressed	**Stressed**	**Non-stressed**	**Stressed**	**Non-stressed**	**Stressed**	**Non-stressed**	**Stressed**
Ht (%)	31.33 ± 2.08^{a}	32.23 ± 2.86^{A}	30.42 ± 2.01^{a}	31.87 ± 2.90^{A}	30.86 ± 1.82^{a}	30.83 ± 2.10^{A}	31.75 ± 2.15^{a}	31.21 ± 1.88^{A}
Hb (g dL^{-1})	11.92 ± 0.88^{a}	11.97 ± 1.12^{A}	11.57 ± 0.92^{a}	11.54 ± 0.66^{A}	11.11 ± 1.28^{a}	11.68 ± 1.27^{A}	11.21 ± 1.46^{a}	12.00 ± 1.01^{A}
RBC ($x10^{6}$ μL^{-1})	0.17 ± 0.05^{c}	$0.29\pm0.08^{B*}$	0.60 ± 0.23^{ab}	$0.97\pm0.23^{A*}$	0.93 ± 0.34^{a}	0.87 ± 0.30^{AC}	0.38 ± 0.10^{bc}	$0.50\pm0.13^{BC*}$
MCV (fL)	164.24 ± 73.61^{a}	$100.90\pm29.55^{A*}$	54.62 ± 17.16^{bc}	$33.71\pm9.60^{B*}$	37.24 ± 17.83^{b}	55.62 ± 23.69^{BC}	84.34 ± 22.08^{ac}	$65.39\pm16.59^{C*}$
MCH (g dL^{-1})	69.56 ± 27.32^{a}	$41.25\pm11.83^{A*}$	24.20 ± 7.39^{bc}	$13.03\pm3.23^{B*}$	12.91 ± 63.10^{b}	15.83 ± 5.43^{BC}	31.75 ± 7.70^{ac}	$24.95\pm6.13^{AC*}$
MCHC (g dL^{-1})	38.06 ± 1.71^{a}	37.25 ± 3.35^{A}	38.07 ± 2.58^{a}	36.38 ± 3.04^{A}	36.01 ± 3.66^{a}	37.77 ± 3.35^{A}	35.24 ± 3.84^{a}	$38.45\pm2.27^{A*}$
Burst (OD)	0.16 ± 0.03^{b}	$0.20\pm0.02^{B*}$	0.18 ± 0.03^{b}	$0.55\pm0.13^{A*}$	0.24 ± 0.06^{a}	0.23 ± 0.07^{B}	0.25 ± 0.04^{a}	0.24 ± 0.05^{B}
Glucose (mg mL^{-1})	148.20 ± 12.29^{a}	142.17 ± 11.67^{A}	26.32 ± 8.09^{c}	$60.12\pm18.19^{B*}$	43.75 ± 11.12^{b}	$60.42\pm17.10^{B*}$	34.20 ± 10.11^{bc}	$46.98\pm10.04^{B*}$
Total protein (g dL^{-1})	4.60 ± 0.14^{a}	4.56 ± 0.11^{A}	1.82 ± 0.48^{b}	$2.59\pm0.75^{B*}$	3.77 ± 0.90^{a}	$2.93\pm0.77^{B*}$	3.40 ± 0.99^{a}	3.21 ± 0.71^{B}
Albumin (g dL^{-1})	0.54 ± 0.19^{a}	0.58 ± 0.20^{AB}	0.51 ± 0.18^{a}	$0.24\pm0.05^{B*}$	0.64 ± 0.18^{a}	$0.94\pm0.23^{A*}$	0.58 ± 0.12^{a}	0.70 ± 0.20^{A}
A:G ratio	0.15 ± 0.01^{b}	0.16 ± 0.01^{BC}	0.41 ± 0.15^{a}	$0.11\pm0.04^{B*}$	0.23 ± 0.11^{ab}	$0.63\pm0.47^{A*}$	0.24 ± 0.12^{ab}	0.29 ± 0.11^{AC}
Total cholesterol (mg mL^{-1})	233.00 ± 17.70^{a}	$192.06\pm21.80^{A*}$	163.80 ± 25.052^{b}	142.95 ± 42.72^{B}	146.09 ± 37.49^{b}	164.43 ± 24.25^{AB}	106.76 ± 24.86^{c}	102.87 ± 24.71^{C}
Triglycerides (mg mL^{-1})	227.32 ± 21.81^{a}	$253.78\pm5.92^{A*}$	63.46 ± 15.01^{c}	73.35 ± 20.92^{B}	198.94 ± 18.83^{b}	$82.52\pm16.74^{B*}$	64.19 ± 15.37^{c}	78.11 ± 20.00^{B}

Mean values ± standard deviation. Ht: hematocrit, Hb: hemoglobin concentration, RBC: red blood cells count, MCV: Mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration. Burst: Leukocytes respiratory activity. A:G ratio: albumin and globulin ratio. Values followed by * indicate significant difference between stressed and not stressed fish ($p<0.05$) that received the same diet. Values followed by different lowercase letters, on the same line, indicate difference between non-stressed animal groups by the Dunn test ($p<0.05$). Values followed by different uppercase letters, on the same line, indicate difference between stressed animal groups by the Dunn test ($p<0.05$).

inclusion of 1 and 5 g probiotic mix kg^{-1} feed; however, stress also reduced these values in the control group and in the groups with 1 and 10 g probiotic mix kg^{-1} feed. The MCHC did not alter ($p > 0.05$) with probiotic supplementation between the groups with and without handling stress, except for the group that received 10 g kg^{-1} feed and was stressed, where a significant increase in MCHC was observed. In pirarucus fed 5 and 10 g of probiotic kg^{-1} diet, leukocyte respiratory activity (burst) was higher ($p < 0.05$); however, as expected, handling stress also increased leukocyte burst activity in fish supplemented with 0 and 1 g probiotic mix kg^{-1} diet.

Animals showed lower ($p < 0.05$) glycemic index values with the use of the probiotic mix from 1 g kg^{-1} feed onward. However, these plasmatic glucose concentrations increased significantly with the chasing stress, independently of the probiotic mix supplementation level (Table 3). Plasma total protein levels decreased ($p < 0.05$) with the use of the lowest concentration of the probiotic mix; at higher concentrations of the probiotic mix in the diets, total proteins remained similar to the control group. Handling stress significantly increased total protein levels in the group administered with 1 g probiotic mix kg^{-1} feed, however a reduction ($p < 0.05$) in the levels of plasma proteins in animals that received 5 g probiotic mix kg^{-1} feed was observed. Plasma albumin levels were generally low ($p < 0.05$), but were higher on animals fed with 5 g probiotic mix kg^{-1}, mainly after handling stress; conversely, animals that received 1 g probiotic mix kg^{-1} feed showed a decrease ($p < 0.05$) after stress. The A:G ratio was higher in animals that received 1 g probiotic mix in their diets for 30 days. With stress, animals that received 1 g probiotic mix kg^{-1} feed showed a significant reduction in this value due to the reduction in albumin and the increase in total proteins. Conversely, an increase ($p < 0.05$) in the A:G ratio occurred when fed 5 g probiotic mix kg^{-1} feed, possibly due to higher levels of both total proteins and albumin. Total plasma cholesterol levels decreased ($p < 0.05$) in animals fed diets supplemented in proportion to the amount added (Table 3). Handling stress caused a significant reduction in these levels only in the control group fish. Triglycerides decreased ($p < 0.05$) in the animals receiving the probiotic mix in the diets, with greater reduction when 1 and 10 g of probiotic mix kg^{-1} feed was used. There was an increase ($p < 0.05$) with the induction of chasing stress in these levels in the control group. Those who received 5 g of probiotic mix kg^{-1} feed and were subjected to stress showed a decrease in their triglycerides, at levels similar to the other groups that received the probiotics and were subjected to stress. However, they differed from the non-stressed animals, whose mean values were higher compared to the other supplemented groups.

Immunological responses after 30 days of experimentation also differed among concentrations of the probiotic mix (Table 4). Thrombocyte numbers were higher ($p < 0.05$) in groups that received 1 and 5 g kg^{-1} probiotic mix in the diets. With chasing stress, these values increased in all groups, except for the one that received 5 g probiotic mix kg^{-1} feed, which was already higher before handling stress and remained high afterward. The total number of leukocytes was higher ($p < 0.05$) in groups that received 1 and 5 g probiotic mix kg^{-1} feed. After handling stress, the leukocyte number decreased ($p < 0.05$) in animals that received 5 g probiotic mix kg^{-1} feed, but remained higher than the control group and the group that received 10 g probiotic mix kg^{-1} feed. Stress promoted a significant increase in leukocytes number in the control group. Lymphocytes, monocytes, and neutrophils were observed in most of the animals when differential leukocyte counts were done, and the fish that received the probiotic mix presented higher number than the control group, that received a diet without supplementation. With chasing-induced stress, the animals that received 5 g probiotic mix kg^{-1} feed showed a decrease ($p < 0.05$) in lymphocyte number, and those receiving 10 g probiotic mix kg^{-1} feed, and the control, showed a

Table 4. Total thrombocytes and leukocytes count and differential leukocytes number of *Arapaima gigas*, after 30 days feeding the experimental diets (with addition of probiotic mix additive), before and 12h after stress.

	0 g probiotic mix kg^{-1} feed		1 g probiotic mix kg^{-1} feed		5 g probiotic mix kg^{-1} feed		10 g probiotic mix kg^{-1} feed	
	Non-stressed	**Stressed**	**Non-stressed**	**Stressed**	**Non-stressed**	**Stressed**	**Non-stressed**	**Stressed**
Thrombocytes (x 10^3 μL^{-1})	1.77 ± 0.77^{b}	$5.43\pm2.23^{B*}$	9.88 ± 2.81^{ac}	$23.86\pm7.74^{A*}$	19.72 ± 7.39^{a}	20.99 ± 7.34^{A}	6.85 ± 2.14^{bc}	$12.68\pm3.28^{AB*}$
Leukocytes (x 10^3 μL^{-1})	3.02 ± 1.22^{b}	$4.20\pm1.42^{BC*}$	9.91 ± 5.60^{ac}	12.48 ± 4.42^{A}	14.32 ± 4.49^{a}	$10.72\pm2.73^{A*}$	5.65 ± 1.42^{bc}	5.89 ± 1.76^{C}
Lymphocytes (x 10^3 μL^{-1})	2.92 ± 1.13^{b}	3.81 ± 1.17^{B}	8.58 ± 2.64^{ac}	11.04 ± 3.83^{A}	13.74 ± 4.40^{a}	$9.53\pm2.42^{A*}$	5.37 ± 1.40^{bc}	5.31 ± 1.67^{B}
Monocytes (x μL^{-1})	46 ± 32^{b}	$225\pm213^{A*}$	206 ± 205^{a}	276 ± 148^{A}	185 ± 117^{a}	318 ± 201^{A}	97 ± 57^{ab}	$205\pm91^{A*}$
Neutrophils (x μL^{-1})	25 ± 46^{b}	$91\pm82^{B*}$	165 ± 193^{a}	$714\pm484^{A*}$	334 ± 218^{a}	754 ± 607^{A}	164 ± 110^{a}	259 ± 143^{AB}
LG-PAS (x μL^{-1})	5 ± 11^{a}	41 ± 72^{B}	29 ± 42^{a}	$392\pm494^{A*}$	23 ± 39^{a}	$78\pm78^{AB*}$	17 ± 32^{a}	$91\pm97^{AB*}$
Eosinophils (x μL^{-1})	3 ± 6^{b}	$17\pm17^{A*}$	15 ± 24^{ab}	49 ± 59^{A}	60 ± 58^{a}	42 ± 56^{A}	12 ± 15^{ab}	30 ± 66^{A}
Basophils (x μL^{-1})	0 ± 0^{a}	7 ± 21^{A}	0 ± 0^{a}	8 ± 28^{A}	4 ± 14^{a}	4 ± 14^{A}	0 ± 0^{a}	2 ± 5^{A}

Mean values ± standard deviation. LG-PAS: leukocyte granular-PAS positive. Values followed by * indicate significant difference between stressed and not stressed fish ($p<0.05$). Values followed by different lowercase letters, on the same line, indicate difference between non-stressed animal groups by the Dunn test ($p<0.05$). Values followed by different uppercase letters, on the same line, indicate difference between stressed animal groups by the Dunn test ($p<0.05$).

significant increase in monocyte count; as well as an increase in neutrophils in the control group and 1 g probiotic mix kg^{-1} feed group. The number of LG-PAS (leukocyte granular-PAS positive) and eosinophils did not show significant differences between the non-stressed animals. Handling stress caused an increase in LG-PAS counts in all the animals supplemented with the probiotic mix ($p < 0.05$), and eosinophils number increased only in the control group after stress. Basophils did not show significant counts or changes with the use of the probiotic mix and submission to chasing stress (Table 4).

DISCUSSION

The present study aimed to evaluate the dietary effects of commercial probiotic mix additive (*B. subtilis*, *E. faecium*, *L. reuteri* and *P. acidilactici*) on the growth, physiology and stress resistance of *A. gigas*. No significant changes were observed in final weight or body length, likely due to the short feeding trial period. However, weight gain and daily growth index were higher in animals fed with 10 g of probiotic mix kg^{-1} of feed compared to the group without supplementation. Similarly, *A. gigas* and *C. macropomum* fed a supplemented diet containing *E. faecium* or *E. faecium* plus *Bacillus cereus* showed enhanced productive performance (da Costa Sousa et al. 2019; Dias et al. 2022). The pirarucu did not show any changes in HSI or VSI, suggesting that the probiotic mix supplementation did not compromise liver health over a 30-day period. Collectively, these findings support the hypothesis that the utilization of probiotic mix additive could enhance the production of these important commercial fish species. Nevertheless, further research is needed, with higher levels probiotics mix added to fish diets. Moreover, handling stress caused a reduction in HSI and VSI in the animals that received the highest dose of supplementation, suggesting that probiotics modulate physiological and immunological responses in fish (Khattab et al. 2004; Balcázar et al. 2006; da Costa Sousa et al. 2019; El-Saadony et al. 2021), in addition to resistance to diseases (Nayak 2010; Dias et al. 2018; Gobi et al. 2018; Ringø et al. 2020) and stress (Dias et al. 2018; da Costa Sousa et al. 2019; Chizhayeva et al. 2022).

Hematological parameters are good indicators of stress and immune responses in fish. The Ht and Hb remained stable across all groups, even after stress exposure, suggesting that probiotic supplementation did not compromise oxygen transport. Increased hematocrit and hemoglobin were observed in red sea bream (Zaineldin et al. 2018) and in Nile tilapia (Telli et al. 2014) after *B. subtilis* diet supplementation. However, in relation to *A. gigas* these differences were probably due to rearing conditions, size and even to differences between fish species. The RBC increased using 1 or 5 g of probiotic mix kg^{-1} feed, since probiotic supplementation in fish can stimulate the hematopoietic organs, increasing RBC production and influencing the transport of oxygen and metabolites, as reported for *C. macropomum* (Dias et al. 2018), for *A. gigas* (da Costa Souza et al. 2019) and tilapia (Telli et al. 2014). Notably, handling stress increased RBC in all groups, except the 5 g kg^{-1}, aligning with studies that suggested dietary probiotics may mitigate stress effects (Chizhayeva et al. 2022). The MCV and MCH were generally lower in supplemented animals, contrasting with findings in Nile tilapia that reported increased MCH with probiotic inclusion of *B. subtilis*, at 5×10^6 CFU $g\ feed^{-1}$ (Telli et al. 2014). The MCHC remained stable, except for fish receiving 10 g kg^{-1} of probiotic mix, where it increased post-stress. Probiotics have been demonstrated to be essential for the development of the innate immunity system in various animals, including shrimp and fish, as they enable them to combat pathogenic microrganisms and environmental stressors (El-Saadony et al. 2021; Abdel-Latif et al. 2022; Monier et al. 2023). Consequently, the utilization of a probiotic mix additive could be beneficial for *A. gigas* health, even after a stressful situation such as fish management.

Probiotics have been shown to play a crucial role in regulating various metabolic functions, including glucose and lipid metabolism, as well as modulating metabolites associated with stress and immunity (Ringø et al. 2022). The inclusion of the probiotic mix in fish diets promoted a great reduction in glycemic, total cholesterol and triglycerides levels of *A. gigas*, showing stress relief (Barton and Iwama, 1991; Sopinka et al. 2016; Hoshino et al. 2020) and benefits to the fish lipid metabolism and health (Ringø et al. 2020, 2022), even after a stressful situation (Barton and Iwama 1991; da Costa Sousa et al. 2019; Chizhayeva et al. 2022). An increase in plasma glucose levels occurs after the initial rise in cortisol levels, which is triggered by stressors such as handling, temperature variation, and transportation (Sampaio and Freire, 2016; Jiang et al. 2017; Wijaya et al. 2019). Handling stress increased glucose levels in all groups, but levels remained lower in probiotic-fed fish than in control, suggesting modulatory effects of probiotics on glycemic responses. Similar glycemic reductions have been reported in Nile tilapia supplemented with *B. cereus* and *B. subtilis* (Marengoni et al. 2015). Mixed probiotics or herbal mixtures may exert a synergistic effect on the growth, biochemical parameters, and immune systems of *P. olivaceus*, enhancing their defense against the pathogen *Streptococcus parauberis* (Harikrishnan et al. 2011; Hoshino et al. 2020), and for *Pangasianodon hypophthalmus* against *Aeromonas hydrophila* (Abdel-Latif et al. 2023). Furthermore, protein metabolism was also influenced by probiotics supplementation. Total plasma protein levels were reduced in fish receiving 1 g kg^{-1} of probiotic mix, but at higher doses, levels remained similar to those of the control group. After handling stress, albumin and the A:G ratio decreased in the 1 g kg^{-1} group, potentially indicating an inflammatory process and immunosuppressive condition (Mohapatra et al. 2012, 2014). Conversely, fish supplemented with 5 g kg^{-1} of probiotic mix exhibited increased albumin and A:G ration after handling stress, suggesting immunostimulatory

effect. A similar response has been observed in olive flounder supplemented with multi-strain probiotics (MSP), which improved immune markers and increased GPx, lysozyme, and myeloperoxidase activities in low-fishmeal diets (Niu et al. 2019). However, additional studies evaluating antioxidants and enzymatic activity are needed to confirm these effects in *A. gigas*.

Leukocyte profile indicated that fish fed probiotics had higher total leukocyte and thrombocyte counts, suggesting enhanced immune function. This response is consistent with what was observed in *Labeo rohita* when MSP supplementation led to increased health status and disease resistance (Kumar et al. 2006; Mohapatra et al. 2014). Furthermore, an increase in lymphocytes, monocytes, and neutrophils numbers were observed in animals that received the probiotic mix (*B. subtilis*, *E. faecium*, *L. reuteri* and *P. acidilactici*). Similarly, *A. gigas* fed *E. faecium* showed increased thrombocytes, neutrophils, and monocytes number (da Costa Sousa et al. 2019). The observed leukocytes increase supports the role of probiotics in boosting innate immunity and disease resistance in fish (Nayak 2010). Understanding how probiotics influence cellular immune responses in fish could lead to more targeted probiotic applications in aquaculture.

CONCLUSIONS

This is the first study providing hematological and biochemical parameters of *A. gigas* supplemented with commercial multi-cepa probiotic mix additive. Our results indicate that diets containing *B. subtilis*, *E. faecium*, *L. reuteri* and *P. acidilactici* mix supplementation promoted growth, improved the health and stress resistance of pirarucu, mainly in the inclusion of 10 g kg^{-1} of diet.

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