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# Influence of lactic-acid bacteria feed supplementation on free amino acid levels in serum and feces of rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792)

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**Introduction:** The influence of intestinal microbiota, particularly lactic-acid bacteria, on digestion and nutrient metabolic activities has been extensively studied. In this research, we investigated the effects of supplementing the feed of rainbow trout (*O. mykiss*) with *P. acidilactici* or L. lactis probiotics. A Control group without probiotic supplementation was also included. The study aimed to evaluate growth, feed conversion indices, free amino acid levels in serum and feces, and the relative gene expression of amino acid solute carrier transporters (SLC).

**Methods:** Rainbow trout weighing 73.9±10 g were fed with a basal commercial diet supplemented with mono-strains of *P. acidilactici* or *L. lactis* at a dosage of 5 x 108 CFU per gram of feed for 60 days. The trout were reared in 100-liter tanks with independent closed recirculation systems, with a water replacement rate of 80% of the total volume daily and a controlled temperature of 15 ± 0.3 °C. The proximate composition of the basal diet consisted of crude protein (49.8%), fat (32.1%), fiber (1.31%), ash (13%), and moisture (8.3%) on a dry matter basis. The diet was provided at a rate of 2% of bodyweight per day.

**Results:** No significant differences ($p > 0.05$) were observed in growth and feed conversion indices between the experimental groups. However, significant differences in free amino acid levels in feces and serum samples ($p \leq 0.05$) were observed. The *P. acidilactici* group exhibited significantly higher levels of glutamate, lysine, proline, and tyrosine in feces samples, and higher levels of arginine, iso/leucine, phenylalanine, proline, serine, threonine, and valine in serum samples compared to the Control group. Additionally, the supplemented probiotic groups showed significant downregulation ($p \leq 0.05$) of theslc6a19 and slc7a9 genes, which encode transporters for neutral and cationic amino acids.

**Discussion:** The autochthonous rainbow trout strain, *P. acidilactici*, demonstrated more pronounced effects on amino acid levels in feces and serum compared to the non-autochthonous *L. lactis* strain. However, the increased amino acid levels, particularly in the serum, resulting from *P. acidilactici* supplementation, did not lead to improved trout growth or feed conversion. Nonetheless, lactic-acid bacteria supplementation induced measurable metabolic effects, as evidenced by elevated levels of specific amino acids in the serum and feces.

Future research should focus on evaluating these probiotic effects using specialized diets and considering the observed metabolic effects in this study.

KEYWORDS

**probiotics, amino acids, *Pediococcus acidilactici*, *Lactococcus lactis*, SLC genes, *Oncorhynchus mykiss***

## 1 Introduction

Cell proteins have structural, enzymatic, and signaling functions in organisms and represent 16–21% of the fish mass (Ahmed et al., 2022; Alberts et al., 2022). Given these critical functions, an exogenous supply of protein in animals is necessary. Aquaculture fish species have a specific protein requirement that must be supplied through aquafeeds (National Research, 2011a; Teles et al., 2020). Salmonid species in particular, such as rainbow trout (*Oncorhynchus mykiss*), require 35–50% wet matter protein in aquafeeds (National Research, 2011a; Teles et al., 2020), a much higher requirement compared to pigs and poultry (estimated at 19–24% wet matter) (Bowen, 1987).

Proteins are composed of chains of α-amino acids (α-AAs) linked by peptide bonds (Wu, 2013). α-AAs are organic molecules that contain an amino group and a carboxylic group attached to the same carbon; however, they have different side chains (R groups) that give different chemical properties and ionic states (Wu, 2013). α-AAs that have identification codons in nucleic acids (DNA-RNA) are classified as proteinogenic or primary (Ahluwalia et al., 2022; Alberts et al., 2022). There are 20 classic primary α-AAs (excluding selenocysteine and pyrrolysine), which in turn can be classified concerning their nutritional importance as essential or non-essential (Wu, 2013; Ahluwalia et al., 2022). Essential α-AAs cannot be synthesized *de novo* by fish or their synthesis rate is slower than the protein synthesis rate, thereby they have to supply by diet (National Research, 2011a; Mai et al., 2022). For rainbow trout, the essential α-AAs are arginine, lysine, leucine, isoleucine, valine, threonine, phenylalanine, tyrosine, methionine, cysteine, tryptophan, and histidine (Ogino, 1980). Thus, the non-essential α-AAs for rainbow trout are glycine, alanine, proline, serine, asparagine, glutamine, glutamic acid, and aspartic acid. Hence, animal protein requirements are α-AA requirements, not only in quantity but also proportion, especially to the essential α-AAs (National Research, 2011a).

A wide variety of protein ingredients of animal or vegetable origin are used in aquafeed formulations (Hardy and Brezas, 2022). In salmonid fish, these ingredients' proteins must be digested into basic units (i.e., α-AAs or tri-di peptides) for enterocyte absorption mainly in the proximal intestine (Dabrowski and Dabrowska, 1981; Bakke et al., 2010; Debnath and Saikia, 2021). This digestion is mediated by a broad enzyme complex secreted in different segments of the digestive tract (Bakke et al., 2010).

The intestinal absorption of α-AAs or tri-di peptides itself is mediated by transporters belonging to the superfamily of genes that code for solute carrier proteins (SLCs) (Poncet and Taylor, 2013; Zhang et al., 2019; Pizzagalli et al., 2021). Currently, 458 SLCs classified into 65 gene families have been described in humans (Pizzagalli et al., 2021); successively, 338 SLCs classified into 50 families have been described in fish (Verri et al., 2012). Each SLC can transport different α-AAs but is generally associated with the same ionic state (neutral, cationic, or anionic). Nevertheless, each transporter has a different α-AA specificity within each ionic state (Bröer, 2008; Gauthier-Coles et al., 2021). Tri-di peptides also have specific transporters, the best-known being PepT1, which has been found in fish (Verri et al., 2010). After α-AAs and oligopeptides are absorbed, they pass through the blood capillaries of the intestinal villi, which later form the portal system that carries these nutrients to the liver (Karlsson et al., 2006).

The salmon industry has had to replace ingredients such as fishmeal, which is considered a "gold standard" ingredient for the formulation of aquafeeds (Turchini et al., 2019), with other ingredients, some of which are of vegetable origin, such as soybean meal. However, these alternative ingredients have lower nutrient digestibility, including proteins (Glencross et al., 2004; Sørensen et al., 2021). Considering the replacement of these ingredients, the apparent digestibility of dry matter in these diets is approximately 63% (Sørensen et al., 2021), with a significant amount of protein organic matter eliminated as waste in the water (Buschmann et al., 1996; Wang et al., 2013). Organic matter

released into the water becomes a stressor in aquatic ecosystems, which increases the risk of aquatic ecosystem eutrophication (Streicher et al., 2021). In addition to the environmental impact, it is appropriate to consider that the organic matter wasted in the water are nutrients that represent a fish farming economic loss (Hua et al., 2019).

Among the factors that could contribute to nutrient digestion, absorption, and metabolization in mammals and fish, the microbiota has been frequently discussed (Ray et al., 2012; Portune et al., 2016; Chapagain et al., 2019; Debnath and Saikia, 2021). Researchers have especially described lactic-acid bacteria within the microbiota of salmonid fish (Ringø and Gatesoupe, 1998; Merrifield et al., 2010; Navarrete et al., 2010; Opazo et al., 2016; Villasante et al., 2019), this bacterial group has attractive qualities for probiotic use in aquaculture (Amenyogbe et al., 2020; Ringø et al., 2020; De Fátima Marques De Mesquita et al., 2021; Sumon et al., 2022). Probiotics in aquaculture have been proposed based on two main objectives: i) to stimulate the immune system or secretion of bacteriocins to prevent diseases (Pérez-Sánchez et al., 2011; Ramos et al., 2015; Araújo et al., 2016; Al-Hisnawi et al., 2019) and ii) to improve the growth or feed conversion performance in fish farming (Giannenas et al., 2015; Akbari Nargesi et al., 2020; Ali et al., 2020; Yeganeh et al., 2021).

The present study evaluated the effects of dietary supplementation with mono-strains of *Pedicoccus acidilacticiti* or *Lactococcus lactis* on rainbow trout (*O. mykkis*) for 60 days. This study examined various productive parameters including weight gain, length gain, specific growth rate (SGR), Fulton's condition factor (K), feed conversion ratio (FCR), and protein efficiency ratio (PER). Additionally, the study analyzed the levels of free amino acids in both feces and serum. Furthermore, the expression of genes encoding amino acid transporters in the proximal intestinal tissue, such as *slc6a19*, *slc7a9*, *slc15a1*, and *slc36a1*, was evaluated at the intestinal level. It is noteworthy that the *P. acilacticity* strain was isolate from rainbow trout intestine, making it autochthonous, while the *L. lactis* strain had non-autochthonous origins.

# 2 Material and methods

## 2.1 Study design and rainbow trout management

Fifty-four rainbow trout (*Oncorhynchus mykiss*) with an average weight of 73.9 ± 10.3 g were obtained from a Rio Blanco trout farm located in the Andes district of Chile's V region. The specimens were transported to the Instituto de Nutrición y Tecnología de los Alimentos (INTA) of the Universidad de Chile, as authorized by the Chilean National Fisheries and Aquaculture Service (Sernapesca) under movement health certificate no. 262119.

Initially, the trout were randomly distributed to nine 100-liter fiberglass experimental tanks (six fish per tank). The trout were acclimatized for 7 days to the experimental conditions without any manipulation. On day 8, an EM4305 microchip (Star, Shanghai, China) was implanted in each specimen in the subcutaneous area under the dorsal fin for tracking purposes. Subsequently, the trout were kept for another 7 days without any further manipulation. The tanks were divided into three experimental groups of 18 specimens each. Group 1 received a supplement of *Pediococcus acidilactici* (GenBank: OP806385) and Group 2 a supplement of *Lactococcus lactis* (GenBank: OP806386), each for 60 days. Both probiotic strains were mixed and administered with aquafeed at doses of $5 \times 10^8$ CFU $g^{-1}$. The study also included a control group that did not receive probiotic supplementation (Control Group). The study mortality was 5.5%, which corresponded to one fish from each experimental group; hence, the final number of trout per experimental group was 17 (n = 17).

Each tank featured an independent recirculation system containing one filter and heat exchanger to prevent the exchange of water, feces, and probiotic strains among the three groups' water systems. The water was regulated at a temperature of 15 ± 0.3°C. Daily, 80% of the system water was changed to maintain an ammonium level below 1 ppm. The tank water's pH, oxygen, and ammonia levels were evaluated three times per week. The tanks' room provided a photoperiod of 14 h light/10 h dark.

The trout were fed a PW250_A80 aquafeed (Biomar, Chile) at a rate of 2% of the specimens' average body weight. Supplementary Table 1 presents the chemical proximal analysis of the extruded commercial aquafeed. Aquafeed crude protein level of aquafeed was analyzed using the Kjeldahl method (AOAC Offical Method 976.05, 2023), to estimate the protein efficiency ratio (PER). The daily food rations were divided into three equivalent portions administered every 3 hours per day. In cases of trout from a specific tank showing lower appetite, the portion of feed was reduced, and this difference was subtracted from the total daily ration to record feed consumption per tank in detail. The weight and length of each trout were obtained before the start of probiotic supplementation (T0), after 30 days of probiotic supplementation (T30), and after 60 days of probiotic supplementation (T60). Due to the chip implanted in each fish, it was possible to assess these measurements individually throughout the study.

At the end of the supplementation period, six specimens from each experimental group were randomly selected, the euthanasia was performed using 30 mg $L^{-1}$ tricaine methanesulfonate (Virbac, Chile) for 10 min. Blood, feces, and proximal intestine tissue samples were obtained from the trout, which had been fasting for 12 h prior. Approximately 1 mL of blood was collected *via* puncture of the caudal vein and incubated for 30 min at 25°C for clotting, then centrifuged (Thermo Scientific, USA) at 2,000 × g for 10 min. The resulting blood serum was collected and transferred to a clean 1.5-mL polypropylene tube. The proximal intestine samples were obtained *via* dissection; Supplementary Figure 1 shows the specific proximal intestinal segment used for feces and tissue sampling. Proximal intestine tissue was first cleaned with sterile saline serum (NaCl 8.0 g, KCl 0.2 g, Na2HPO4 1.44 g, KH2PO4 0.24 g, and water q.s. 1 L), then placed in polypropylene tubes with RNAlater™ (Thermo Scientific, USA). Each fecal sample for amino acid analysis was collected in a 1.5 mL sterile polypropylene tube. Additionally, approximately 800 mg of each fecal sample was placed in a sterile 2.0 mL polypropylene tube and weighed. Next, 800 µL of sterile saline solution (previously described) was added. The feces were resuspended by pipetting and serially diluted four times at a factor

of 10 in saline solution. Dilutions of $10^{-2}$, $10^{-3}$, and $10^{-4}$ were plated in triplicate on MRS agar medium and incubated for 48 h at 20°C. The number of colony-forming units (CFU) per plate was counted. All samples of serum, tissue, or fecal for amino acid assessment were stored at -80°C until analysis.

The trout handling procedures and euthanasia were approved by the Comité Institucional de Cuidado y Uso de Animales of the Universidad de Chile (protocol certificate no. 19323-INT-UCH). All fish management procedures were carried out under the precepts and bioethical aspects associated with the management and care proposed by the Guide for the Care and Use of Laboratory Animals (National Research, 2011b).

## 2.2 Indices of trout growth and feed conversion performance

Through the data collected during the experiment, the following growth and feed conversion indices were calculated:

Weight gain (g) = *Wf (g) – Wi (g)*

Standard length gain (cm) = L*f (cm) - Li (cm)*

SGR = $\frac{Ln\ (\ Wf\ (g)) - Ln\ (Wi\ (g))}{Experimental\ period\ in\ days}$ x 100 (Hopkins, 1992)

Fulton's condition factor (K) = $\frac{Whole\ fish\ weight\ (g)}{(standard\ length\ (cm))^3}$ x 100 (Froese, 2006)

FCR = $\frac{Total\ feed\ intake\ (g)}{Weight\ gain\ (g)}$ (Charles Bai et al., 2022)

PER = $\frac{Weigh\ gain\ (g)}{Total\ protein\ intake\ (g)}$ (Charles Bai et al., 2022)

where *Wf* is the final wet weight in grams, *Wi* is the initial wet weight in grams, *Lf* is the final standard length in centimeters, and *Li* is the initial standard length in centimeters.

## 2.3 Probiotics strain origins and probiotic supplement formulation

The *Pediococcus acidilactici* probiotic strain (GenBank: OP806385) was previously isolated from the proximal intestine of rainbow trout (*Oncorhynchus mykiss*) while the *Lactococcus lactis* strain (GenBank: OP806386) was isolated from goat milk (*Capra hircus*). Both strains were isolated using an MRS agar medium (Merck, Germany) and incubated at 20°C (Memmert, USA). The strains were preserved in Cryobank™ cryovials (Copan innovation, USA) in a -80°C freezer.

The probiotic cultures were performed in a 250-mL Erlenmeyer flask containing 120 ml MRS broth medium (Merck, Germany) that was incubated at 20°C for 24 h in a shaking incubator (LabTech, Korea). The probiotic strain cultures were counted (number of bacteria cells/CFU per mL of culture medium) using an optical microscope (Motic, Xiamen China) and Neubauer improved chamber (Hirschmann, Germany).

Probiotics were supplemented in aquafeed at a rate of $5 \times 10^8$ CFU $g^{-1}$, a mixture that was prepared weekly during the study. The volume needed for each culture batch was calculated based on the count (explained previously) and in consideration of the total grams administered to each experimental group per week. Before mixing between feed and probiotics, each MRS batch was centrifuged (Thermo Scientific, USA) at 5000 × g for 10 min, and then the supernatant was resuspended in a volume of sterile physiological serum, which corresponded to approximately 4% (mL $g^{-1}$). Probiotic strains were supplied to the experimental aquafeed *via* spraying with 3% vegetable oil added as a coating. The Control Group feed received 4% physiological serum (without lactic-acid bacteria) and 3% vegetable oil. During their period of use, probiotic-supplemented feeds and the control feed were kept refrigerated at 5°C.

## 2.4 Free amino acid levels in trout feces and blood serum

### 2.4.1 Feces and blood serum sample preparation for amino acid analysis

Amino acids from 100-µL feces or serum samples (n = 6) were isolated using cold methanol (1:6 v/v aqueous/organic solvent) (Erben et al., 2021; Roper et al., 2022); this analysis was carried out on blind. The samples were then kept at -30°C for 15 min and centrifuged at 14,000 × g for 10 min at 4°C. The supernatants were collected and dried using a SpeedVac (Thermo Scientific, USA). The resulting metabolites were resuspended in a solution of labeled α-AAs (13C, 15N labeled at 1 nmol/mL; Cambridge Isotope Laboratories, USA) (Monnerat et al., 2019).

### 2.4.2 Flow injection analysis high-resolution mass spectrometry

The α-AAs were analyzed in quadruplicate using a Thermo Q Exactive Plus mass spectrometer (Thermo Scientific, USA) *via* direct flow injection analysis. The mobile phase used was ACN/H2O/MeOH (2:1:1 v/v/v) at a flow rate of 50 µL $min^{-1}$. The mass spectrometer was operated in Target-SIM data acquisition in positive mode. The spray voltage was set at 3.5 kV, capillary temperature at 280°C, and gas temperature at 150°C. The full scan was performed at a resolution of 70,000, AGC target of 5E5, isolation window of 1.0 *m/z*, maximum IT of 25 ms, and acquisition time of 2 min. The data obtained were processed using TraceFinder software (Thermo Scientific, USA). The intensity ratios of light to heavy α-AAs were calculated for each of their intensity value (L/H intensity) (Monnerat et al., 2019).

## 2.5 Analysis of the amino acid solute transporters' gene relative expression

The total RNA from the trout proximal intestinal tissue samples (n = 6) was obtained using TRIzol™ (Invitrogen, USA) according to the manufacturer's protocol. Quantification and quality of the total RNA were assessed *via* fluorometry using Qubit™ 4.0 (Thermo Scientific, USA) according to the manufacturer's protocol.

Reverse transcription was performed with 1 µg total RNA using the ImProm-II™ Reverse Transcription System kit (Promega, USA) according to the manufacturer's protocol. Four amino acids SLC transporter genes (*slc6a19*, *slc709*, *slc15a1*, and *slc36a1*) of rainbow trout were selected for relative gene expression (see Supplementary Table 2). Three genes *eef1a1a* (elongation factor 1

alpha 1a), *rps20* (ribosomal protein S20), and *ubb* (ubiquitin B) were evaluated for use as reference genes, with *eef1a1a* ultimately selected as the reference gene.

Quantitative polymerase chain reaction was performed with 2 µL cDNA for each sample using the FastStart™ Essential DNA Green Master kit (Roche, USA) according to the manufacturer's protocol in an AriaMx™ (Agilent, USA) Real-time PCR. The amplification program included 10 min of preincubation at 95°C, then 45 cycles of 10 s at 95°C, 15 s at 62°C, and 10 s at 72°C to finish with a melting analysis.

The relative gene expression ratio for each sample was calculated using the function proposed by Pfaffl (2001). The efficiency of the amplification curve was calculated using LinReg software (Brankatschk et al., 2012). Because Pfaffl's formula uses the experimental control group ($Cp_{ref}$) as part of the calculation function, the Control Group samples were duplicated from the RNA isolation to include this group in the statistical analysis and establish instrumental variation in the relative gene expression analysis. For each relative gene expression ratio, the natural logarithm was calculated to better visualize the upregulation versus downregulation of the gene expression among the experimental groups.

## 2.6 Statistical analysis

Normal distributions associated with parameters, growth indices, and feed conversion performance (n = 17) were analyzed using the Shapiro–Wilk test (Mishra et al., 2019). As the indices of Fulton's condition factor (K) and FCR did not fit a normal distribution, they were analyzed using the non-parametric Kruskal-Wallis (*H*) test; hence, the other indices were analyzed using one-way ANOVA (*F*) (Hoffman, 2019). A *p*-value of ≤ 0.05 was considered significant for both tests. The *post hoc* analysis for the parametric tests was Tukey's HSD (HSD) and for the non-parametric tests, Dunn's test (*Z*) (Kao and Green, 2008). In the *post hoc* test, the contrasts were considered significant with *p* adjusted to ≤ 0.1.

The Kruskal-Wallis (*H*) test was used to compare amino acid levels in the feces and blood serum samples among the experimental groups (n = 6) (Hoffman, 2019). In addition, the *post hoc* analysis featured Dunn's test (Kao and Green, 2008) with significance levels of $p \leq 0.05$ and *p* adjusted (p-adj.) to ≤ 0.1. The same statistical protocol was used to analyze the relative gene expression ratios among the experimental groups (n = 6). All statistical analyses were performed using the R statistical program (R. Core Team, 2021).

# 3 Results

## 3.1 Rainbow trout growth and performance indices

As a starting point for this study, it is appropriate to evaluate whether intervention with probiotics in the aquafeed could modulate the presence of lactic acid bacteria in the proximal intestine of trout. At the end of the supplementation period of 60 days, it was possible to isolate viable lactic-acid bacteria colonies only in the Petri dishes of experimental groups suplemented with *P. acidilactici* or *L. lactis*, which had an average count of 1.43 x 105 CFU g-1, and 1.19 x 104 CFU g-1, respectively. No colony forming units (CFU) were found in the dilutions analyzed in the Control group. The observed CFU per gram was approximately half the CFU per gram of the supplemented aquafeed. However, the administration method allowed viable lactic-acid bacteria colonies to be obtained from trout foregut feces.

The comparison of weight gain, length gain, and SGR distributions was not statistically significant between the experimental groups after 60 days of acid-lactic bacteria supplementation $p > 0.05$ (see Figures 1A–C). However, for the first 30 days of probiotic supplementation, significant differences in length gain distribution were observed between the experimental groups ($F_{(2,48)} = 4.33$, $p = 0.018$). *Post hoc* analysis showed a significant group contrast between the Control Group and Group 2 (HSD = 0.61, *p*-adj. = 0.014); Figure 1B. The basic statistical analysis and ANOVA test analyses for these indices are shown in Supplementary Table 3.

The differences in the associated FCR and PER distributions were not statistically significant between the experimental groups $p > 0.05$. The comparison of K distributions between experimental groups was not statistically significant at any of the data collection times, $p > 0.05$. The basic statistical analysis and significant test analyses for these indices are shown in Supplementary Table 4.

Nevertheless, the K comparison between data collection times was statistically significant within each experimental group (Control: $H_{(2)} = 28.28$, $p = 7.23e\text{-}07$; Group 1: $H_{(2)} = 29.53$, $p = 3.87e\text{-}07$; Group 2: $H_{(2)} = 2.41$, $p = 2.41e\text{-}08$). The *post hoc* analysis showed significant contrasts between T0 and T30 as well as T0 and T60, whereas that of T30 and T60 was not significant (see Supplementary Table 5).

## 3.2 The free amino acid concentration in rainbow trout feces and blood serum samples

The rainbow trout feces contained 17 α-AAs, of which eight are classified as essential (arginine, lysine, threonine, phenylalanine, iso/leucine, tryptophan, methionine, and valine) and nine as non-essential (asparagine, glutamine, serine, tyrosine, alanine, cystine, glycine, and proline). The basic statistical analysis of feces α-AA levels (nmol $mL^{-1}$, wet basis) per experimental group are shown in Table 1. The α-AAs with the six highest concentrations in feces were phenylalanine (551 ± 500.0 nmol $mL^{-1}$), iso/leucine (454.7 ± 176.2 nmol $mL^{-1}$), tyrosine (348 ± 420.0 nmol $mL^{-1}$), alanine (217.3 ± 90.4 nmol $mL^{-1}$), glutamine (179.2 ± 78.6 nmol $mL^{-1}$), and methionine (149.5 ± 54.2 nmol $mL^{-1}$).

The comparison of the total sum of each amino acid level (nmol mL-1) in the feces samples did not show statistical significance between the experimental groups ($H_{(2)} = 0.36$, $p = 0.8$). Among the 17 α-AAs identified in these samples, four exhibited statistically significant differences ($p < 0.05$) between the experimental groups:

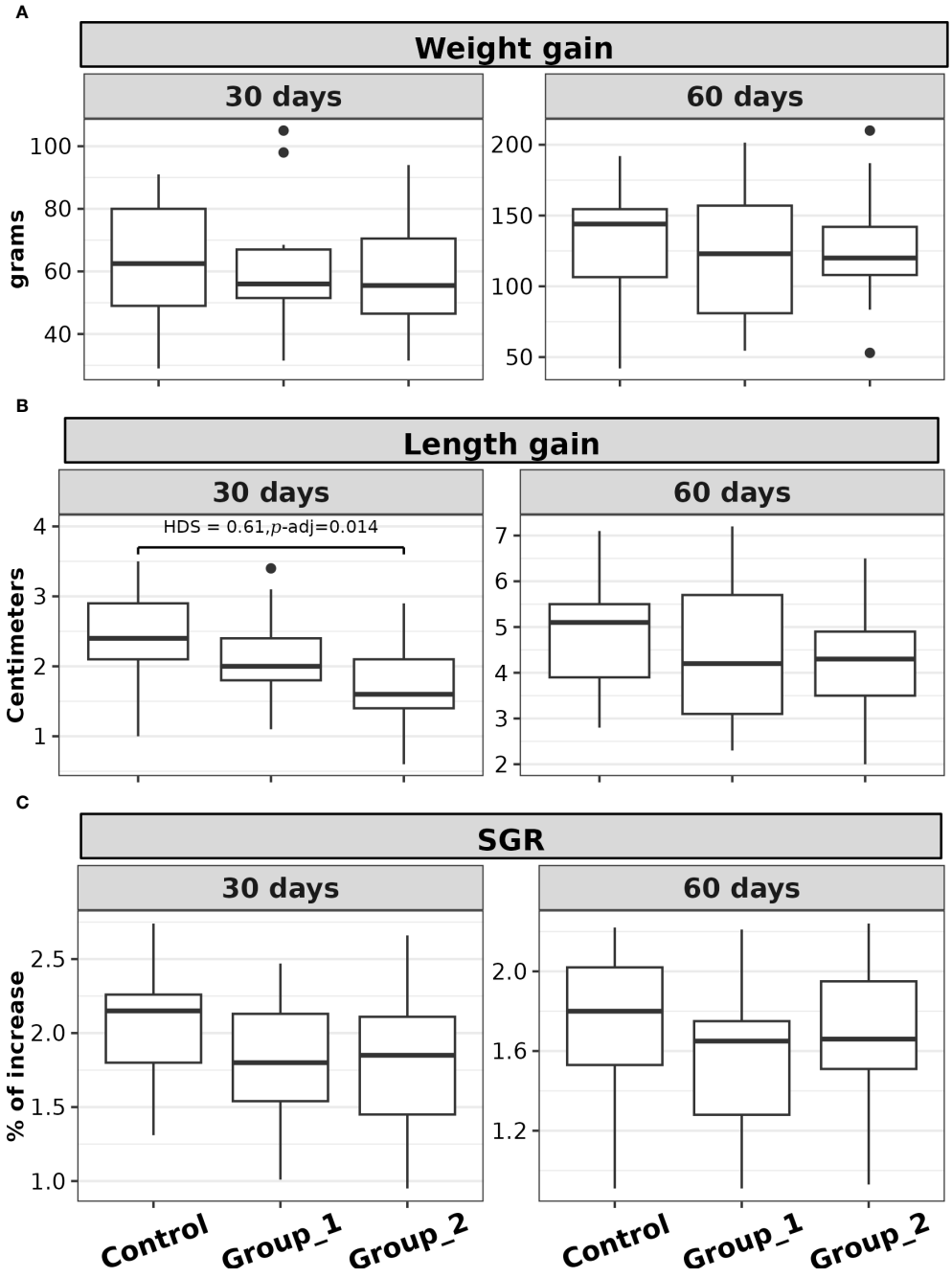


FIGURE 1
Box plots of the **(A)** weight gain, **(B)** length gain, and **(C)** SGR, of rainbow trout (*O. mykiss*) supplemented with *P. acidilactici* (Group 1) or *L. lactis* (Group 2) at a dosage of 5 x $10^8$ CFU $g^{-1}$ for either 30 or 60 days. The Control Group did not receive any probiotic supplements. The lines indicate statistically significant differences between the experimental groups, as determined by Tukey test. The distribution differences were considered significant at p< 0.05 and p-adjusted ≤ 0.1.

glutamate, lysine, proline, and tyrosine (see Table 1). Figure 2 depicts the *post hoc* analysis of α-AA levels in the feces samples. A significant contrast for glutamate emerged between Group 1 and Group 2 ($Z$ = 2.51, $p$ = 0.01, *p-adj.* = 0.035; Figure 2A); that for lysine was between Group 1 and the Control Group ($Z$ = 2.55, $p$ = 0.01, *p-adj.* = 0.02) and Group 2 and the Control Group ($Z$ = 3.24, $p$ = 0.001, *p-adj.* = 0.0035; Figure 2B); the significant contrast for proline was between Group 1 and Group 2 ($Z$ = 2.99, $p$ = 0.002, *p-adj.* = 0.008; Figure 2C); and the significant contrast for tyrosine emerged between Group 1 and the Control Group ($Z$ = 3.0, $p$ = 0.0027, *p-adj.* = 0.008; Figure 2D).

The same 17 α-AAs identified in the feces samples were also identified in the rainbow trout blood serum samples. The basic statistical analysis of serum α-AA levels (nmol $mL^{-1}$) for each experimental group is shown in Table 2. The α-AAs with the six highest concentrations in serum were phenylalanine (92.7 ± 21.4

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TABLE 1 Basic statistical analysis of free amino acid feces levels (nmol mL$^{-1}$) of rainbow trout after receiving mono-strain probiotic supplementation for 60 days.

| Amino acid | Groups | n[1] | mean | sd[2] | Median[6] | q25 | q75 | Kruskal-Wallis test (*H*) |
|---|---|---|---|---|---|---|---|---|
| Alanine | Control | 24 | 210.17 | 47.25 | 220.30 | 187.62 | 248.44 | |
| | Group 1 | 24 | 239.14 | 92.92 | 205.24 | 165.39 | 315.43 | $H_{(2)} = 5.09$, $p = 0.07$ |
| | Group 2 | 24 | 202.62 | 116.77 | 134.30 | 119.36 | 301.69 | |
| Arginine | Control | 24 | 42.64 | 9.28 | 43.53 | 41.92 | 46.69 | |
| | Group 1 | 24 | 43.02 | 9.28 | 43.26 | 34.09 | 50.47 | $H_{(2)} = 1.18$, $p = 0.55$ |
| | Group 2 | 24 | 42.45 | 13.14 | 39.19 | 33.53 | 47.89 | |
| Asparagine | Control | 24 | 18.22 | 4.60 | 18.42 | 15.63 | 20.54 | |
| | Group 1 | 24 | 17.35 | 5.48 | 17.25 | 13.62 | 19.56 | $H_{(2)} = 2.51$, $p = 0.28$ |
| | Group 2 | 24 | 16.14 | 6.34 | 14.46 | 11.11 | 20.42 | |
| Cysteine | Control | 24 | 0.14 | 0.08 | 0.12 | 0.09 | 0.15 | |
| | Group 1 | 24 | 0.10 | 0.02 | 0.10 | 0.08 | 0.11 | $H_{(2)} = 5.54$, $p = 0.06$ |
| | Group 2 | 24 | 0.11 | 0.02 | 0.11 | 0.10 | 0.13 | |
| Glutamate | Control | 24 | 36.06 | 21.10 | 30.77$^{ab}$ | 24.62 | 36.94 | |
| | Group 1 | 24 | 34.88 | 9.88 | 34.42$^{a}$ | 27.86 | 42.53 | $H_{(2)} = 6.45$, $p = 0.03$ |
| | Group 2 | 24 | 27.74 | 11.30 | 24.43$^{b}$ | 19.37 | 32.70 | |
| Glutamine | Control | 24 | 175.50 | 52.84 | 175.43 | 140.25 | 221.24 | |
| | Group 1 | 24 | 192.45 | 69.61 | 193.63 | 135.85 | 233.77 | $H_{(2)} = 2.16$, $p = 0.33$ |
| | Group 2 | 24 | 169.67 | 105.69 | 131.27 | 94.32 | 266.74 | |
| Glycine | Control | 24 | 9.47 | 9.41 | 6.88 | 4.32 | 9.60 | |
| | Group 1 | 24 | 6.40 | 3.81 | 5.47 | 4.58 | 7.80 | $H_{(2)} = 2.00$, $p = 0.36$ |
| | Group 2 | 24 | 7.12 | 6.78 | 4.53 | 3.33 | 9.79 | |
| Iso/Leucine | Control | 24 | 409.98 | 85.52 | 439.52 | 368.80 | 468.28 | |
| | Group 1 | 24 | 515.95 | 160.59 | 511.23 | 363.30 | 651.12 | $H_{(2)} = 2.52$, $p = 0.28$ |
| | Group 2 | 24 | 438.44 | 237.84 | 341.44 | 254.65 | 727.36 | |
| Lysine | Control | 24 | 51.46 | 20.16 | 46.10$^{a}$ | 40.07 | 59.99 | |
| | Group 1 | 24 | 80.17 | 34.89 | 74.19$^{b}$ | 45.70 | 115.58 | $H_{(2)} = 11.6$, $p = 0.002$ |
| | Group 2 | 24 | 91.42 | 46.70 | 86.41$^{b}$ | 47.80 | 132.51 | |
| Methionine | Control | 24 | 139.36 | 37.87 | 144.21 | 118.80 | 161.23 | |
| | Group 1 | 24 | 161.07 | 49.15 | 154.34 | 119.31 | 203.70 | $H_{(2)} = 2.02$, $p = 0.36$ |
| | Group 2 | 24 | 148.08 | 70.67 | 125.05 | 86.51 | 209.84 | |
| Phenylalanine | Control | 24 | 207.82 | 64.61 | 214.33 | 184.49 | 255.02 | |
| | Group 1 | 24 | 645.37 | 514.66 | 475.40 | 194.60 | 1058.68 | $H_{(2)} = 3.99$, $p = 0.13$ |
| | Group 2 | 24 | 799.91 | 944.88 | 257.64 | 116.94 | 1419.22 | |
| Proline | Control | 24 | 55.96 | 24.23 | 48.56$^{ab}$ | 39.32 | 71.76 | |
| | Group 1 | 24 | 68.68 | 27.96 | 57.56$^{a}$ | 48.79 | 81.49 | $H_{(2)} = 9.12$, $p = 0.01$ |
| | Group 2 | 24 | 47.92 | 21.71 | 39.42$^{b}$ | 35.78 | 67.90 | |
| Serine | Control | 24 | 92.56 | 30.57 | 87.16 | 78.97 | 107.79 | |
| | Group 1 | 24 | 77.98 | 30.37 | 70.02 | 51.02 | 95.36 | $H_{(2)} = 4.61$, $p = 0.09$ |

*(Continued)*

TABLE 1 Continued

| Amino acid | Groups | n[1] | mean | sd[2] | Median[6] | q25 | q75 | Kruskal-Wallis test (*H*) |
|---|---|---|---|---|---|---|---|---|
| | Group 2 | 24 | 75.60 | 40.61 | 54.81 | 48.41 | 96.91 | |
| Threonine | Control | 24 | 65.57 | 20.81 | 60.07 | 53.44 | 81.70 | |
| | Group 1 | 24 | 64.25 | 18.39 | 66.65 | 51.78 | 76.79 | $H_{(2)} = 3.23$, $p = 0.19$ |
| | Group 2 | 24 | 57.63 | 26.40 | 45.50 | 37.50 | 80.16 | |
| Tryptophan | Control | 24 | 51.13 | 14.33 | 48.78 | 39.79 | 62.50 | |
| | Group 1 | 24 | 61.46 | 19.25 | 59.82 | 44.24 | 77.65 | $H_{(2)} = 2.88$, $p = 0.23$ |
| | Group 2 | 24 | 57.55 | 24.89 | 52.27 | 34.47 | 82.65 | |
| Tyrosine | Control | 24 | 114.73 | 43.40 | 112.79[a] | 98.72 | 127.75 | |
| | Group 1 | 24 | 536.98 | 565.99 | 243.08[b] | 124.98 | 1074.89 | $H_{(2)} = 9.26$, $p = 0.009$ |
| | Group 2 | 24 | 392.56 | 357.35 | 276.40[ab] | 71.43 | 692.55 | |
| Valine | Control | 24 | 132.51 | 34.66 | 124.42 | 115.58 | 158.67 | |
| | Group 1 | 24 | 163.06 | 51.34 | 156.04 | 117.85 | 201.25 | $H_{(2)} = 3.47$, $p = 0.17$ |
| | Group 2 | 24 | 143.92 | 74.10 | 106.74 | 81.68 | 215.55 | |

[1] n=24: corresponds to 6 biological replicates of trout feces with 4 technical replicates.
[2] sd: standard deviation.
[3] Group 1 received a *P. acidilactici* supplementation of $5 \times 10^8$ CFU $g^{-1}$
[4] Group 2 received a *L. lactis* supplementation of $5 \times 10^8$ CFU $g^{-1}$
[5] Control did not receive probiotic supplementation.
[6] Superscript letters indicate significant differences between experimental groups by post hoc test.

nmol $mL^{-1}$), valine (70.1 ± 18.7 nmol $mL^{-1}$), methionine (57.9 ± 13.4 nmol $mL^{-1}$), alanine (54.4 ± 10.9 nmol $mL^{-1}$), iso/leucine (40.8 ± 9.1 nmol $mL^{-1}$), and threonine (25.5 ± 7.5 nmol $mL^{-1}$).

Comparison of the total sum of each amino acid level (nmol $mL^{-1}$) in the serum was statistically significant between the experimental groups ($H_{(2)} = 6.35$, $p = 0.04$). A significant contrast in the *post hoc* analysis emerged between Group 1 and the Control Group ($Z = 2.43$, $p = 0.001$, *p-adj.* = 0.04).

Of the 17 α-AAs identified in the serum, eight showed statistically significant differences between the experimental groups: proline, arginine, iso/leucine, phenylalanine, serine, threonine, and valine (see Table 2).

Proline was the only α-AA that showed statistically significant differences between the experimental groups in both the feces and blood serum matrices. *Post hoc* analysis of serum proline levels showed that the significant contrasts were between Group 1 and the Control Group ($Z = 4.31$, $p = 0.0000016$, *p-adj.* = 0.00004) and Group 1 and Group 2 ($Z = 2.89$, $p = 0.0036$, *p-adj.* = 0.007; Figure 2C).

Figure 3 shows the *post hoc* analysis of all serum α-AA levels between experimental groups, save proline. The significant contrasts for arginine were between Group 1 and the Control Group ($Z = 3.23$, $p = 0.0012$, *p-adj.* = 0.003) and Group 1 and Group 2 ($Z = 2.24$, $p = 0.025$, *p-adj.* = 0.05; Figure 3A); the significant contrasts for iso/leucine were between Group 1 and the Control Group ($Z = 2.63$, $p = 0.008$, *p-adj.* = 0.017) and Group 1 and Group 2 ($Z = 2.75$, $p = 0.0059$, *p-adj.* = 0.018; Figure 3B); the significant contrasts for phenylalanine were between Group 1 and the Control Group ($Z = 3.91$, $p = 0.00009$, *p-adj.* = 0.0003) and Group 1 and Group 2 ($Z = 3.04$, $p = 0.0023$, *p-adj.* = 0.005; Figure 3C); the significant contrasts for serine were between Group 1 and the Control Group ($Z = 2.98$, $p = 0.0028$, *p-adj.* = 0.008) and Group 1 and Group 2 ($Z = 2.01$, $p = 0.044$, *p-adj.* = 0.09; Figure 3D); the significant contrasts for threonine were between Group 1 and the Control Group ($Z = 2.76$, $p = 0.0057$, *p-adj.* = 0.01) and Group 1 and Group 2 ($Z = 3.88$, $p = 0.0001$, *p-adj.* = 0.0003; Figure 3E); and the significant contrasts for valine were between Group 1 and the Control Group ($Z = 4.1$, $p = 0.00004$, *p-adj.* = 0.0001), Group 2 and the Control Group ($Z = 1.73$, $p = 0.08$, *p-adj.* = 0.08), and Group 1 and Group 2 ($Z = 2.37$, $p = 0.017$, *p-adj.* = 0.035; Figure 3F).

## 3.3 Experimental groups' relative expression of amino acid SLC transporter genes

From the trout proximal intestine tissue samples, the relative expressions of the four genes that encode α-AA transporters (SLCs) were assessed. These were the *slc6a19* gene that encodes $B^0AT1$ transporter, the *slc7a9* gene that encodes $b^{0,+}$ transporter, the *slc15a1* gene that encodes PepT1 transporter, and the *slc36a1* gene that encodes PAT1 transporter.

Figure 4 shows the relative expressions of the α-AA SLC transporter genes. The relative gene expressions of *slc6a19* ($H_{(2)} = 8.25$, $p = 0.016$) and *slc7a9* ($H_{(2)} = 8.4$, $p = 0.014$) were statistically significant; however, the other two genes did not present statistically significant differences (*slc15a1*: $H_{(2)} = 2.4$, $p = 0.29$; *slc6a19*: $H_{(2)} = 2.56$, $p = 0.27$). *Post hoc* analysis showed that the contrasts with significant differences for *slc6a19* were between the Control Group

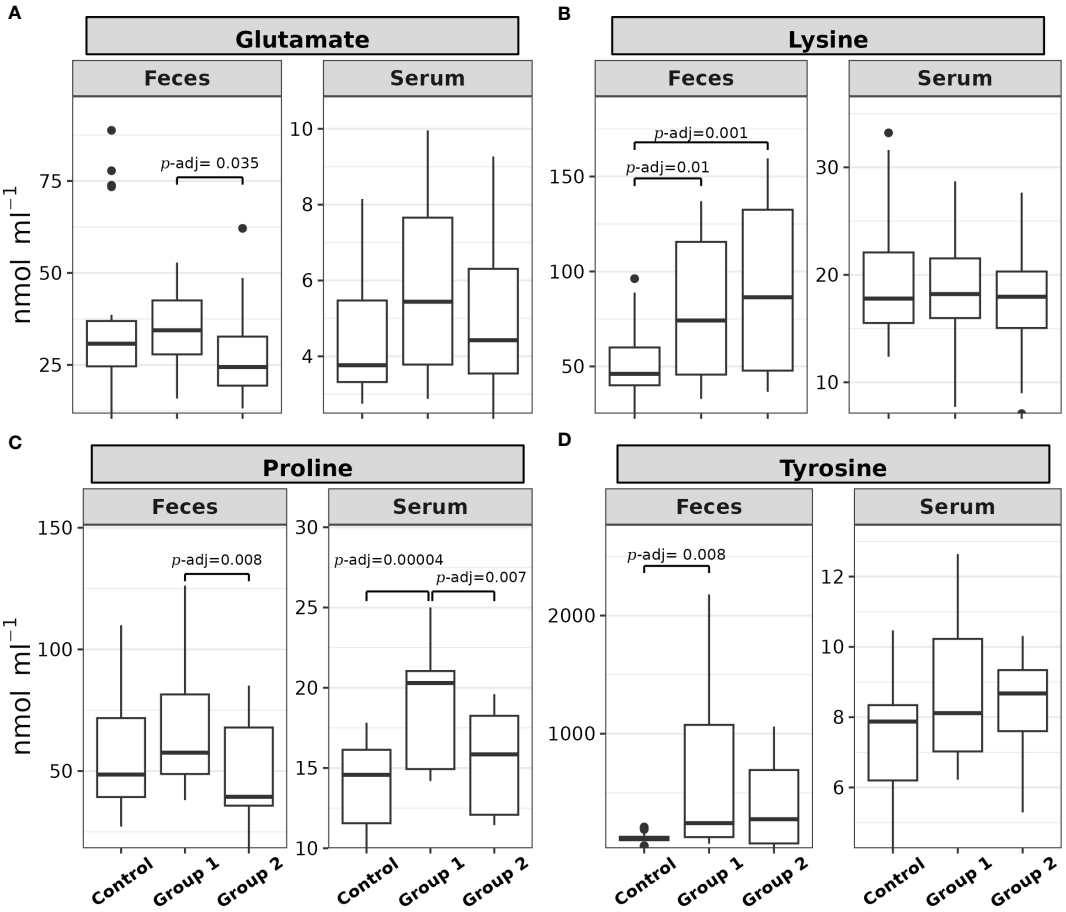


FIGURE 2
Box plots of the **(A)** glutamate, **(B)** lysine, **(C)** proline, and **(D)** tyrosine feces and serum levels (nmol mL$^{-1}$) of rainbow trout (*O. mykiss*) after a *P. acidilactici* (Group 1) or *L. lactis* (Group 2) supplementation of 5x10$^{8}$ CFU g$^{-1}$ for 60 days. The Control Group did not receive a probiotic supplement. The lines show statistically significant differences between the experimental groups, as derived *via* Dunn's test. The distribution differences were considered significant at $p < 0.05$ and $p$-adjusted $\leq 0.1$.

and Group 1 ($Z = 2.0$, $p = 0.04$, *p-adj.* = 0.09) and the Control Group and Group 2 ($Z = 2.78$, $p = 0.005$, *p-adj.* = 0.01; Figure 4A). To *slc7a9*, there were significant contrasts between the Control Group and Group 1 ($Z = 1.95$, $p = 0.05$, *p-adj.* = 0.1) and the Control Group and Group 2 ($Z = 2.84$, $p = 0.0045$, *p-adj.* = 0.01; Figure 4B).

Finally, the results obtained for the total sum of α-AA levels of the serum or feces samples were integrated with the relative expressions of *slc6a19* and *slc7a9* through correlation analysis. The relative expression of both SLC genes has a significant moderate negative correlation with the total sum of serum α-AA levels, -0.51 ($p = 0.03$) for *slc6a19 and -0.47* ($p = 0.04$) *for slc7a9*. However, the relative expression of both SLC genes versus the total sum of feces α-AA levels correlation, was not significant ($p > 0.05$).

## 4 Discussion

In the *post hoc* analysis contrast between the Control Group and Group 2 presented significant differences in length gain, but only in the first 30 days. This difference could be explained by the Control Group's compensatory growth (Morgan and Metcalfe, 2001; Ali et al., 2003), as a lower average standard length was observed in this group at the beginning of the study. However, the comparison of length and length gain was not statistically significant between the experimental groups at the end of the study.

The comparison of Fulton's condition factor (K) between data collection times was significant for the contrast between T0 and T30, but not for T30 and T60. Because the condition factor evaluates the relationship between weight and length (Froese, 2006), the observed increase in K indicates that the rainbow trout belonging to the three groups presented greater weight gain about length gain during the first 30 days.

Different studies have evaluated the potential of different bacterial strains as probiotic supplements for rainbow trout and other fish species (Amenyogbe et al., 2020; Ringø et al., 2020; De Fátima Marques De Mesquita et al., 2021). Mono-strain *P. acidilactici* supplementation has been assessed in a large number of studies, wherein researchers also found no significant differences between the experimental groups' growth rates or feed conversions (Aubin et al., 2005; Hoseinifar et al., 2017; Villumsen et al., 2020; Rasmussen et al., 2022). However, Ali et al. (2020) reported a significant increase in SGR and a significant decrease in FCR in

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TABLE 2 Basic statistical analysis of free amino acid blood serum levels (nmol mL$^{-1}$) of rainbow trout after receiving mono-strain probiotic supplementation for 60 days.

| Amino acid | Groups | n[1] | mean | sd[2] | Median[6] | q25 | q75 | Kruskal-Wallis test (*H*) |
|---|---|---|---|---|---|---|---|---|
| Alanine | Control | 24 | 52.31 | 7.62 | 52.83 | 47.86 | 57.78 | |
| | Group 1 | 24 | 57.30 | 10.24 | 57.62 | 48.13 | 63.72 | $H_{(2)} = 5.27$, $p = 0.07$ |
| | Group 2 | 24 | 52.84 | 13.89 | 48.25 | 44.62 | 51.97 | |
| Arginine | Control | 24 | 10.11 | 1.74 | 10.02[a] | 9.06 | 11.76 | |
| | Group 1 | 24 | 12.03 | 1.81 | 12.37[b] | 11.30 | 13.41 | $H_{(2)} = 10.96$, $p = 0.004$ |
| | Group 2 | 24 | 10.70 | 2.51 | 10.31[a] | 8.51 | 12.78 | |
| Asparagine | Control | 24 | 2.36 | 0.66 | 2.15 | 1.84 | 2.63 | |
| | Group 1 | 24 | 2.61 | 0.98 | 2.49 | 2.15 | 3.19 | $H_{(2)} = 1.36$, $p = 0.5$ |
| | Group 2 | 24 | 2.54 | 0.76 | 2.54 | 1.88 | 3.01 | |
| Cysteine | Control | 24 | 0.91 | 0.27 | 0.85 | 0.74 | 1.04 | |
| | Group 1 | 24 | 0.91 | 0.38 | 0.78 | 0.65 | 1.15 | $H_{(2)} = 3.63$, $p = 0.16$ |
| | Group 2 | 24 | 1.22 | 0.62 | 1.10 | 0.73 | 1.63 | |
| Glutamate | Control | 24 | 4.59 | 1.81 | 3.77 | 3.32 | 5.47 | |
| | Group 1 | 24 | 5.83 | 2.29 | 5.44 | 3.78 | 7.66 | $H_{(2)} = 4.14$, $p = 0.12$ |
| | Group 2 | 24 | 4.92 | 1.78 | 4.43 | 3.55 | 6.31 | |
| Glutamine | Control | 24 | 13.72 | 3.15 | 12.98 | 11.92 | 14.56 | |
| | Group 1 | 24 | 15.87 | 3.97 | 14.92 | 12.77 | 18.68 | $H_{(2)} = 4.81$, $p = 0.09$ |
| | Group 2 | 24 | 13.79 | 2.00 | 13.70 | 12.63 | 14.50 | |
| Glycine | Control | 24 | 10.83 | 5.87 | 8.53 | 7.67 | 11.98 | |
| | Group 1 | 24 | 17.07 | 25.36 | 10.76 | 6.92 | 17.83 | $H_{(2)} = 1.31$, $p = 0.51$ |
| | Group 2 | 24 | 13.93 | 9.54 | 11.77 | 7.33 | 16.33 | |
| Iso/Leucine | Control | 24 | 38.03 | 6.09 | 36.58[a] | 34.05 | 40.46 | |
| | Group 1 | 24 | 46.82 | 11.39 | 46.64[b] | 39.00 | 53.18 | $H_{(2)} = 9.66$, $p = 0.007$ |
| | Group 2 | 24 | 37.65 | 6.06 | 35.68[a] | 34.02 | 37.24 | |
| Lysine | Control | 24 | 19.59 | 5.87 | 17.79 | 15.52 | 22.08 | |
| | Group 1 | 24 | 18.48 | 5.42 | 18.21 | 15.98 | 21.53 | $H_{(2)} = 0.56$, $p = 0.75$ |
| | Group 2 | 24 | 17.40 | 5.21 | 17.96 | 15.06 | 20.31 | |
| Methionine | Control | 24 | 57.50 | 9.41 | 58.83 | 53.63 | 62.27 | |
| | Group 1 | 24 | 62.99 | 18.85 | 61.03 | 46.52 | 70.98 | $H_{(2)} = 5.08$, $p = 0.75$ |
| | Group 2 | 24 | 53.28 | 8.28 | 51.39 | 48.23 | 56.38 | |
| Phenylalanine | Control | 24 | 81.39 | 23.00 | 74.25[a] | 67.19 | 104.92 | |
| | Group 1 | 24 | 107.68 | 15.48 | 105.29[b] | 96.82 | 118.11 | $H_{(2)} = 16.86$, $p = 0.0002$ |
| | Group 2 | 24 | 89.05 | 16.44 | 89.70[a] | 80.01 | 102.70 | |
| Proline | Control | 24 | 14.09 | 2.40 | 14.58 | 11.56 | 16.14 | |
| | Group 1 | 24 | 19.22 | 3.64 | 20.30 | 14.94 | 21.04 | $H_{(2)} = 19.29$, $p = 0.0006$ |
| | Group 2 | 24 | 15.50 | 3.01 | 15.85 | 12.09 | 18.25 | |
| Serine | Control | 24 | 13.01 | 3.24 | 13.23[a] | 10.97 | 15.19 | |
| | Group 1 | 24 | 17.18 | 4.93 | 15.84[b] | 14.22 | 20.82 | $H_{(2)} = 9.23$, $p = 0.009$ |

*(Continued)*

TABLE 2 Continued

| Amino acid | Groups | n[1] | mean | sd[2] | Median[6] | q25 | q75 | Kruskal-Wallis test (*H*) |
|---|---|---|---|---|---|---|---|---|
| | Group 2 | 24 | 14.26 | 4.13 | 14.46[a] | 10.95 | 16.46 | |
| Threonine | Control | 24 | 23.80 | 4.60 | 23.64[a] | 20.19 | 25.90 | |
| | Group 1 | 24 | 30.88 | 9.12 | 27.42[b] | 25.51 | 38.55 | $H_{(2)} = 15.95$, $p = 0.0003$ |
| | Group 2 | 24 | 21.85 | 4.83 | 21.31[a] | 18.90 | 24.47 | |
| Tryptophan | Control | 24 | 5.29 | 0.98 | 5.13 | 4.74 | 6.02 | |
| | Group 1 | 24 | 6.31 | 1.88 | 6.13 | 4.82 | 7.31 | $H_{(2)} = 4.06$, $p = 0.13$ |
| | Group 2 | 24 | 5.42 | 1.31 | 5.67 | 4.72 | 6.00 | |
| Tyrosine | Control | 24 | 7.39 | 1.60 | 7.88 | 6.20 | 8.34 | |
| | Group 1 | 24 | 8.66 | 2.13 | 8.12 | 7.03 | 10.23 | $H_{(2)} = 4.30$, $p = 0.11$ |
| | Group 2 | 24 | 8.35 | 1.49 | 8.68 | 7.60 | 9.34 | |
| Valine | Control | 24 | 60.61 | 10.21 | 60.23[a] | 50.94 | 69.87 | |
| | Group 1 | 24 | 83.00 | 22.01 | 78.15[b] | 72.25 | 96.32 | $H_{(2)} = 16.07$, $p = 0.0002$ |
| | Group 2 | 24 | 66.88 | 14.89 | 63.32[c] | 53.21 | 78.45 | |

[1] n=24: corresponds to 6 biological replicates of trout feces with 4 technical replicates.
[2] sd: standard deviation.
[3] Group 1 received a *P. acidilactici* supplementation of $5 \times 10^8$ CFU $g^{-1}$
[4] Group 2 received a *L. lactis* supplementation of $5 \times 10^8$ CFU $g^{-1}$
[5] Control did not receive probiotic supplementation.
[6] Superscript letters indicate significant differences between experimental groups by post hoc test.

rainbow trout supplemented with 1 x $10^{10}$ CFU $g^{-1}$ *P. acidilactici* for 45 days. Other studies have proposed the use of multi-strain probiotic supplements, including *P. acidilactici*, such as Akbari Nargesi et al. (2020), who reported no significant differences between the experimental groups for SGR; nevertheless, a significant decrease in FCR was associated with the highest probiotic supplementation of 3 x $10^9$ UFC $g^{-1}$, though lower levels did not exhibit significant differences.

Although *L. lactis* has been described as a normal part of the rainbow trout microbiota (Navarrete et al., 2010), few studies have evaluated this bacterium as a probiotic supplement to improve trout farming performance. Although, Yeganeh et al. (2021) assessed *L. lactis* supplementation at a level of 1x$10^9$ CFU $g^{-1}$, but found no significant differences in SGR or FCR between the experimental groups.

The PER results associated with the supplementation of lactic-acid bacteria have been contradictory. Using diets with 52% or 57% crude protein, Rasmussen et al. (2022) described a significant decrease in PER in rainbow trout after supplementation with *P. acidilactici*. In contrast, Ali et al. (2020) used a diet with 40% crude protein and found a significant increase in PER in rainbow trout supplemented with *P. acidilactici*.

Amino acid levels in feces and serum of fish are mainly modulated by diet (Harper et al., 1970; Yamamoto et al., 1998; Mundheim et al., 2004; Larsen et al., 2012), nevertheless the intestinal microbiota also can influence the fecal metabolism of nutrients (Asakura et al., 2014; Lin et al., 2017). This situation presents a problem when comparing the results of different studies given the diversity of ingredients among diet formulations. Despite this problem, similar to Dabrowski and Dabrowska (1981), in this study, iso/leucine was one of the most prominent α-AAs in the feces samples. In blood serum samples, the influence of feed ingredients could be less direct because the liver and gut metabolism of amino acids can also influence their levels in the blood (Karlsson et al., 2006; Wu, 2013). In Schlisio and Nicolai (1978), four of the six highest concentrations of α-AAs in serum samples were consistent with our results (alanine, valine, threonine, and leucine); however, phenylalanine was one of the α-AAs with the lowest levels in this study.

Regarding the relative expression of the SLC genes, *slc6a19* and *slc7a9* genes showed downregulation in the groups supplemented with lactic-acid bacteria probiotics, besides that, *the slc15a1* and *slc36a1* gene did not present significant differences between the experimental groups. Although the *slc6a19* gene encodes a sodium-dependent transporter, $B^0AT1$ (symporter type), and the *slc7a9* gene encodes a non-sodium transporter, $b^{0,+}$ (antiporter type) (Gauthier-Coles et al., 2021), both present a similar specificity for transporting neutral and cationic α-AAs (Chairoungdua et al., 1999; Böhmer et al., 2005; Gauthier-Coles et al., 2021). Hence, considering that anionic α-AAs are generally only glutamic acid and aspartic acid (Bröer, 2008), it was observed that probiotic supplementation modulates serum levels of certain amino acids. This modulation was potentially linked to the downregulation of genes encoding SLC transporters with a wide range of substrate specificities, such as *slc7a9* and *slc6a19*. In contrast, the expression of *slc36a1*, an SLC transporter with limited substrate specificity, did not exhibit any variations between the experimental groups.

The probiotic supplementation downregulated SLC genes that encoded transporters with wide substrate specificity. On the other hand, the *slc36a1* gene encodes a proton-coupled amino acid transporter, PAT1, which transports only neutral α-AAs, specifically glycine, proline, hydroxyproline, and alanine

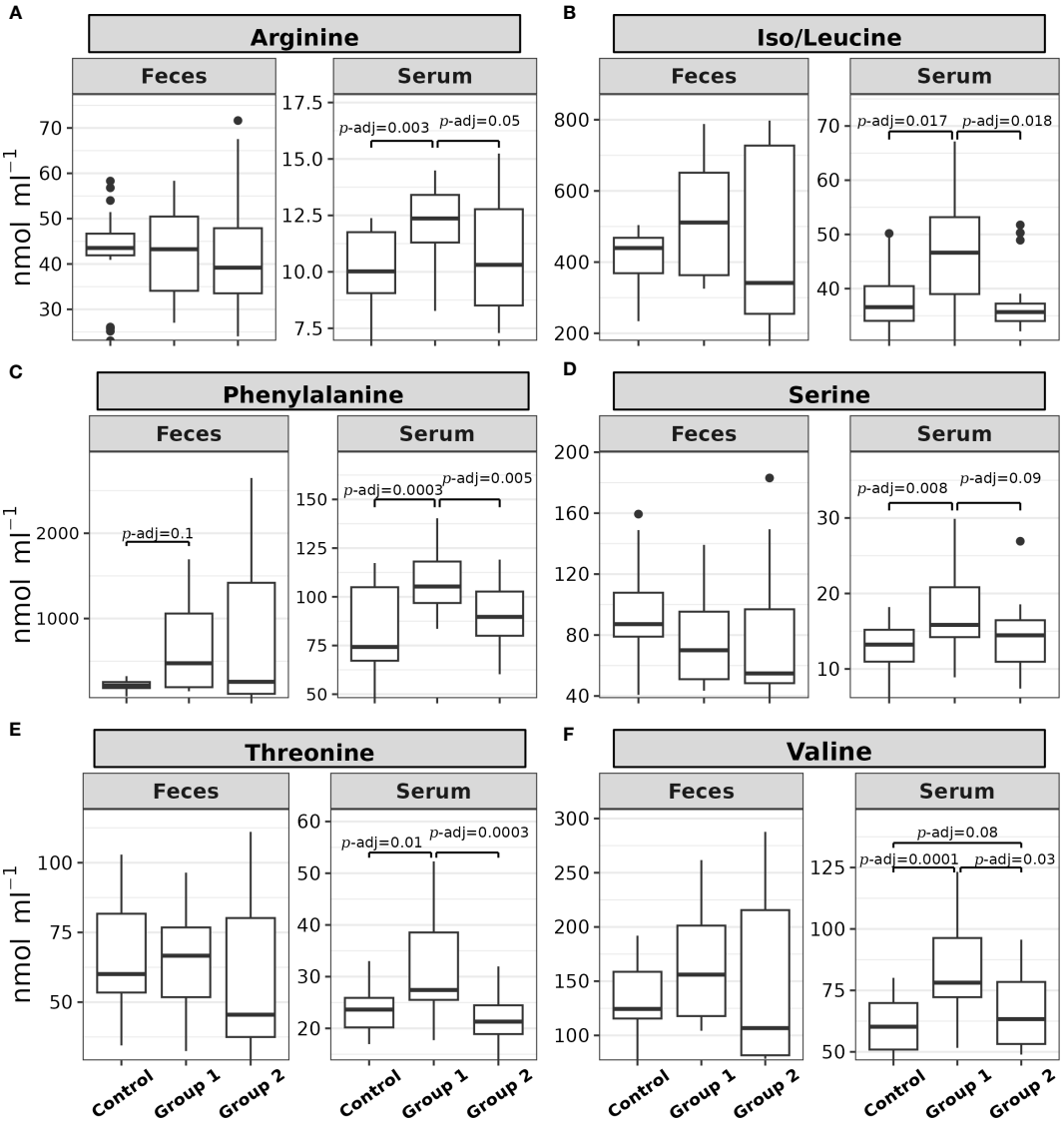


FIGURE 3
Box plots of the **(A)** arginine, **(B)** iso/leucine, **(C)** phenylalanine, **(D)** serine, **(E)** threonine, and **(F)** valine feces and serum levels (nmol mL$^{-1}$) of rainbow trout (*O. mykiss*) after a *P. acidilactici* (Group 1) or *L. lactis* (Group 2) supplementation of 5x10$^{8}$ CFU g$^{-1}$ for 60 days. The Control Group did not receive probiotic supplementation. The lines show statistically significant differences between experimental groups, as derived *via* Dunn's test. The distribution differences were considered significant at $p < 0.05$ and $p$-adjusted $\leq 0.1$.

(Thwaites et al., 1995; Anderson et al., 2004). Likewise, the *slc15a1* gene encodes the proton-coupled amino acid transporter PepT1, which transports tri-di peptides (Verri et al., 2010). In concordance with our results *slc15a1* gene did not show a relative expression difference between experimental groups in other dietary amino acid modulation studies (Jando et al., 2017; Wei et al., 2020).

*In vitro* and *in vivo* studies have established that starvation induces the upregulation of different α-AA SLC transporters in mammals and fish (Hatzoglou et al., 2004; Hellsten et al., 2017; Nitzan et al., 2017). Gazzola et al. (1981)has referred to this response as the "adaptive regulation of amino acid transporters transcription." In this study, the specimens fasted for 12 h upon euthanasia; hence, it is feasible that the transcriptional regulation of the SLCs in the trout was more strongly associated with α-AA levels. However, the total sum of α-AAs in the feces samples was not significant among the experimental groups, whereas the total comparison between the serum samples was significant, but only for Group 1 and the Control Group. Nevertheless, increased serum α-AA levels were moderated negatively correlated with the relative intestinal expression of *slc6a19* and *slc7a9*; hence, an increase of the serum α-AA levels could be associated with the downregulation of *slc6a19* and *slc7a9* in proximal intestinal tissue. Further research is necessary to confirm this relationship.

The present study compared the probiotic effects of an autochthonous *P. acidilactici* strain supplement with those of a non-autochthonous *L. lactis* strain supplement. When comparing the concentration of amino acids in feces and blood serum samples, the autochthonous strain yielded significantly positive differences, confirming that probiotics should ideally be isolated from the microbiota of the target species. This result could be associated

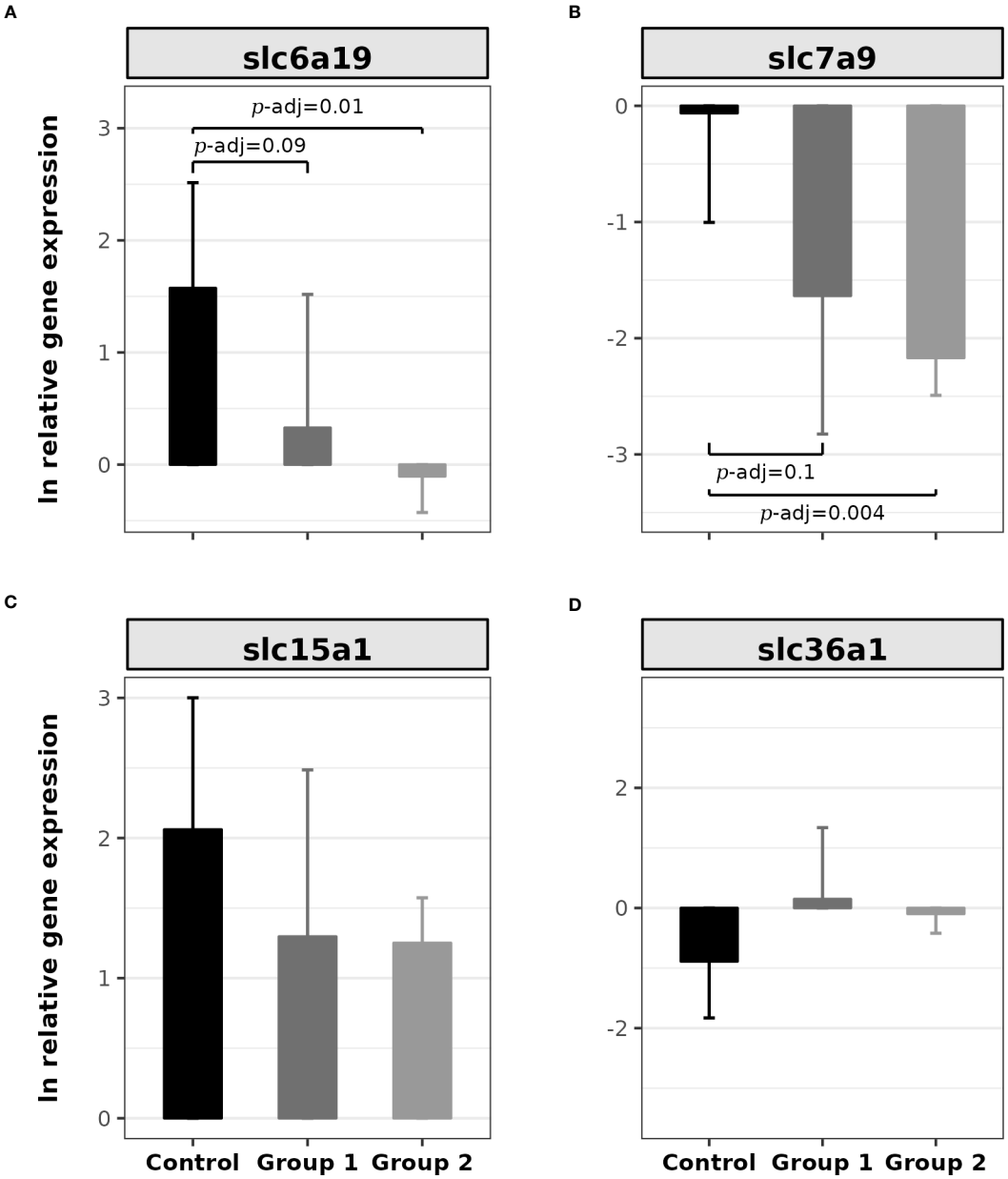


FIGURE 4
Bar plot of the relative gene expression of SLC transporters in the proximal intestine of rainbow trout (*O. mykiss*) after a *P. acidilactici* (Group 1) or *L. lactis* (Group 2) supplementation of 5x10$^8$ CFU g$^{-1}$ for 60 days. **(A)** *slc6a19* gene (B$^0$AT1), **(B)** *slc7a9* gene (b$^{0,+}$), **(C)** *slc15a1* gene (Pept1), and **(D)** *slc36a1* gene (PAT1) with bar expressed mean ± standard error of the mean (n = 6). The lines show statistically significant differences between experimental groups, as derived *via* Dunn's test. The distribution differences were considered significant at $p < 0.05$ and $p$-adjusted ≤ 0.1.

with the fact that, despite both strains being administered at the same supplementation doses of 5 x 10$^8$ CFU g$^{-1}$, the *P. acidilacticiti* strain exhibited counts almost 10 times higher than the *L. lactis* strain in feces. Additionally, *P. acidilactici* induced positive effects mainly associated with the increase of serum α-AA levels in Group 1 as compared to the Control Group that did not receive probiotic supplementation. However, these effects did not create significant improvements in growth or feed conversions.

This outcome can be further understood by assessing other research that defines the crude protein requirement for rainbow trout, such as Oliva-Teles (1989); Kim et al. (1991); Zeitoun et al. (1973), and Mahmud et al. (1996). These studies' researchers assessed diets formulated with increasing levels of wet or dry matter crude protein (approximately 25–60%. In general, the best PER performance was associated with diets containing 35–40% crude wet matter protein, also diets formulated with more than 40% crude protein did not have significant improvement in growth indices. The diet used in this study was formulated with 45% crude protein (as wet matter), which provided approximately 5% more crude protein than the rainbow trout requirements. This could explain the absence of improvement in growth or feed conversion indices as a result of the high availability of α-AA levels in feces or

serum associated with *P. acidilacticiti* supplementation, due to a permanent higher amino acid dietary contribution, which could cancel the increased amino acid levels in the serum produced by probiotics.

Understanding the effects of lactic acid bacteria as a probiotic supplement to enhance rainbow trout breeding opens an opportunity for future studies associated with diets specifically formulated for probiotic supplementation. Such diets should be formulated with lower crude protein requirements and even reduce the proportion of some essential and non-essential α-AAs.

## 5 Conclusions

Unlike *L. lactis*, *P. acidilactici* supplementation for 60 days significantly increased serum total α-AA levels (nmol $mL^{-1}$) and specific serum levels (nmol $mL^{-1}$) of proline, arginine, iso/leucine, phenylalanine, serine, threonine, and valine in rainbow trout (*Oncorhynchus mykiss*). *P. acidilactici* supplementation for 60 days also significantly increased the levels (nmol $mL^{-1}$) of glutamate, lysine, proline, and tyrosine in rainbow trout feces.

The autochthonous rainbow trout *P. acidilactici* showed better activity in improving certain α-AA levels (nmol $mL^{-1}$) in the blood serum and feces trout samples compared to the non-autochthonous *L. lactis*. This improved activity could be associated with the fact that *P. acidilactici* maintains higher viable cell counts (UFC g-1) than the *L. lactis* strain in feces after diet supplementation. When considering whether probiotics are a viable alternative to nutritional additives that offer benefits to the salmon industry, this study demonstrates that probiotic supplementation does indeed have beneficial effects on the increase of certain amino acids in the serum (nmol $mL^{-1}$), including four essential amino acids (iso/leucine, phenylalanine, threonine, and valine). However, our results did not allow us to quantify the actual productive and commercial impacts of probiotic supplementation, which is necessary for promoting their use in the salmon industry. Nonetheless, by comprehending the nutritional effects of probiotics, it is possible to propose new research that utilizes diets specifically formulated for probiotic supplementation with the aim of visualizing the productive impacts in the salmon industry.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GenBank: OP806385 and GenBank: OP806386.

## Ethics statement

The animal study was reviewed and approved by Comité Institucional de Cuidado y Uso de Animales of the Universidad de Chile (protocol certificate no. 19323-INT-UCH).

## Author contributions

GC: Investigation, software, methodology. GS: Formal analysis, software, writing - original draft. CO: Investigation, methodology. HP: Conceptualization, project administration, methodology, resources, supervision. GM: Conceptualization, methodology, software, writing - review & editing. RO: Conceptualization, investigation, formal analysis, supervision, writing - original draft, writing - review & editing, funding acquisition. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1216488/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Photograph of a rainbow trout (*Oncorhynchus mykiss*) proximal intestine, showing the intestine segment used to obtain feces and tissue samples.

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