FISHERIES & AQUATIC LIFE (2024) 32: 155 - 165
Archives of Polish Fisheries
DOI 10.2478/aopf-2024-0014

sciendo

RESEARCH ARTICLE

Assessing the probiotic potential of *Lactococcus lactis* isolated from the intestine and gill of rainbow trout (*Oncorhynchus mykiss*) through in vitro analysis

Billy M. Lucano, Manuel A. Feria, Adrian E. Zatan, Odalis E. Toledo, Jorge L. Aguilar, Benoit M. Diringer, Arnaldo E. Castañeda

Received – 27 May 2024/Accepted – 28 September 2024. Published online: 30 September 2024;
Citation: Lucano, B. M., Feria, M.A., Zatan, A.E., Toledo, O.E., Aguilar, J.L., Diringer, B.M., Castañeda, A.E. (2024). Assessing the probiotic potential of *Lactococcus lactis* isolated from the intestine and gill of rainbow trout (*Oncorhynchus mykiss*) through in vitro analysis. Fisheries & Aquatic Life 32, 155-165

Abstract. The emergence of diseases and the indiscriminate use of antibiotics have had deleterious consequences in the aquaculture industry. Consequently, the growing emphasis on eco-friendly alternatives has taken center stage, and probiotics have emerged as a notable solution. This investigation aimed to identify and characterize potential probiotic bacteria present in the gills and intestine of rainbow trout (*Oncorhynchus mykiss* (Walbaum)). A total of 29 isolates were selected for in vitro evaluation. The assays included morphological evaluation, catalase, oxidase, hemolysis, pathogen antagonism, antimicrobial susceptibility, protease, and amylase tests. Seven isolates exhibited antagonism against one of the three pathogens tested (*Aeromonas hydrophila*, *Streptococcus agalactiae*, and *Lactococcus garvieae*). None of the isolates showed resistance to the antibiotics ampicillin, florfenicol, or tetracycline. Furthermore, all isolates showed proteolytic but not amylolytic activity. The isolates with the best characteristics were identified as *Lactococcus lactis* (n=7) by 16S rRNA gene sequencing.

Keywords: Autochthonous, gut, lactic acid bacteria, microbiota, fish health

B.M. Lucano
National University of Tumbes, Aquaculture Department, Tumbes/Tumbes, Peru (24001)

M.A. Feria, A.E. Zatán, O.E. Toledo, J.L. Aguilar, A.E. Castañeda [✉]
Pezbiotec SAC, Mz D Lote 24, Urb. Hermanos Carcamo, Piura/Piura, Peru (20009)
E-mail: arnaldoc.ve@gmail.com

B.M. Diringer
IncaBiotec SAC, Jr. Filipinas 212, Tumbes/Tumbes, Peru (24001)

Introduction

The aquaculture industry has grown exponentially spurred by the constant global demand for food, promoting sustainable production, and food security (Kuebutornye et al. 2020). However, this industry is threatened by outbreaks of viral, bacterial, and parasitic infections that cause significant economic losses (Wang et al. 2019). The global estimated losses, both direct and indirect, resulting from sea lice species in salmonid aquaculture, exceed $100 million annually (Johnson et al. 2004). In addition, for freshwater farmed fish in Brazil, the losses were estimated at US$ 84 million per year (Tavares-Dias and Martins 2017).

In Peru, fish farming is notable for the production of rainbow trout, *Oncorhynchus mykiss*

(Walbaum), primarily farmed in Puno, Pasco, and Junín (PRODUCE 2019). However, the spread of bacterial pathogens such as *Aeromonas*, *Yersinia*, *Weissella*, and *Flavobacterium* are a constant threat to fish farms (Távara et al. 2019, Medina et al. 2020a, Nuñure et al. 2021). Currently, antibiotic use has been established as a means of controlling these infections, but its frequent and inappropriate use has led to antibiotic resistance. For example, multi-resistant *A. hydrophila* isolated from *O. mykiss* was reported in Peru, threatening food safety, public health, and the environment (Moya-Salazar et al. 2022). Prophylactic alternatives such as the use of probiotics have been proposed (Wang et al. 2019, Liu et al. 2022).

Probiotics are defined as living microorganisms that improve water quality, digestion, and immune functions when administered directly to fishes or water. The positive impact of probiotics on the host digestive tract has been documented. They are known for performing various functions in the host and participating in nutritional and immune processes (Akhter et al. 2015, Lazado et al. 2021). Furthermore, they are used widely as nutritional additives and are characterized by the production of bacteriocins, siderophores, lysozymes, proteases, and amylases (Kuebutornye et al. 2020, Saadony et al. 2021).

Preferably, probiotic bacteria should be sourced from the intestinal microbiota and skin mucus of fishes (Gómez and Balcázar 2008, Bhatnagar and Rathi 2023). However, given the crucial role of the microbiota in maintaining gill health, it could be a valuable source of probiotic bacteria (Llewellyn et al. 2014). The genus *Lactobacillus* is the most common probiotic bacteria that is successfully isolated from the intestines of apparently healthy fish (Balcázar et al. 2008, Nathanailides et al. 2021). Multiple tests must be performed to qualify a bacterium as a potential probiotic, including hemolytic activity, antibiotic sensitivity, tolerance to acidic conditions and bile salts, adhesion capacity, antagonistic capacity, and viability in food (Ramírez et al. 2019). These assays are valuable for characterizing bacterial strains isolated from hosts of interest.

There is limited research on obtaining potential probiotic bacteria from rainbow trout gills despite their numerous immunological and physiological functions. This research aimed to characterize and identify bacteria with probiotic potential from the intestine and gills of *O. mykiss*. The exploration of new microorganisms will allow for significant advancements in the aquaculture industry, enhancing production and sustainability.

Materials and methods

The process for isolating, characterizing, and molecularly identifying potential probiotics is illustrated in Fig. 1.

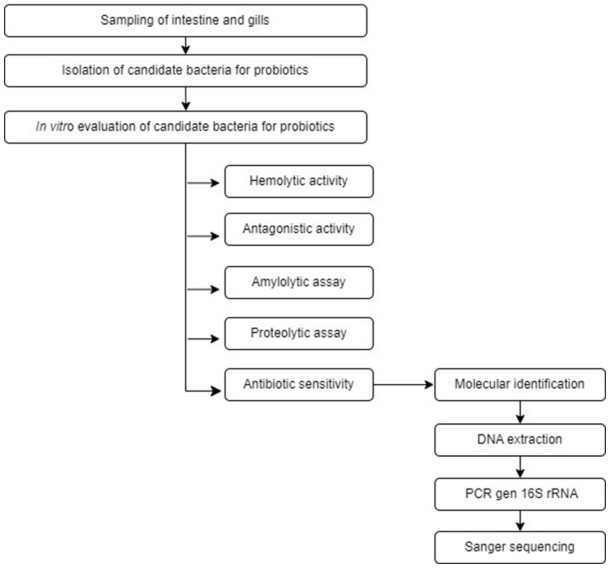

Figure 1. Schematic representation of the process for isolating, characterizing, and the molecular identification of potential probiotics from rainbow trout intestine and gill.

Isolation of potentially probiotic bacteria

Five commercially sized fish, with body weights of approximately 250 g each, were selected from a fish farm located in the province of Jaen, department of Cajamarca, Peru. The fish were euthanized with a 50 ppm overdose of eugenol (Keene et al. 1998) and subsequently disinfected with 70% ethanol (Alkofarma, Peru). The hindgut was aseptically removed, opened,

and washed with sterile saline solution to eliminate intestinal contents. Using dry swabs, smears were taken from the gills and intestine under sterile conditions, with the aid of a Bunsen burner. Both swab samples were placed in Falcon tubes containing Man Rogosa Sharpe (MRS) broth (HiMedia, India) and were cultured under aerobic conditions at 30°C for 24 h (Castañeda et al. 2018). The samples were subcultured on MRS agar at 30°C for 24 h. After the incubation period, pure colonies were selected and morphologically differentiated using Gram staining. Finally, they were preserved in 15% (v/v) glycerol (HiMedia, India) at -20°C for subsequent assays.

Catalase and oxidase assays

To assay catalase, a bacterial colony smear was placed on a slide with a drop of 3% hydrogen peroxide. Colonies that reacted by converting hydrogen peroxide into water and oxygen were considered positive. For the oxidase test, a small sample was taken from each colony and applied to strips impregnated with cytochrome c oxidase (Bactident®Oxidase); a cream-colored strip was considered a negative result, while a pinkish strip was interpreted as a positive result.

Hemolytic activity assay

The assay was carried out using Blood Agar Base (HiMedia, India) with 5% sheep blood. The bacteria candidates for probiotics were cultured and incubated under aerobic conditions at 30°C for 48 h (do Vale Pereira et al. 2017). Bacteria with hemolytic activity were classified as α-hemolytic (incomplete lysis) or β-hemolytic (complete lysis) based on the presence of clear zones around the colonies, while bacteria without hemolytic activity were classified as γ-hemolytic (no lysis).

Bacterial antagonism against pathogenic bacteria

The antagonistic activity against three previously isolated fish pathogens, *Aeromonas hydrophila*, *Lactococcus garvieae*, and *Streptococcus agalactiae*, was determined using the agar diffusion method adapted from Balcázar et al. (2008). The pathogens were subcultured in Trypticase Soy Broth (HiMedia, India) at 30°C for 24 h. Subsequently, 100 µl of each pathogen was streaked onto Nutrient Agar (HiMedia, India). Wells were made, and 30 µl (1×10^8 cfu mL^{-1}) of each candidate probiotic bacteria were added and incubated at 30°C for 24 h. The inhibitory activity was measured in millimeters (mm).

Antibiotic sensitivity

The antibiotic sensitivity test was performed on Mueller-Hinton Agar (HiMedia, India) following the Kirby-Bauer disk diffusion method (Bauer et al. 1966). Three antibiotics (Oxoid™, United Kingdom) were used: florfenicol (30 µg), ampicillin (10 µg), and tetracycline (30 µg). A total of 100 µl (1×10^8 cfu mL^{-1}) was streaked onto Mueller-Hinton Agar (HiMedia, India), the sensitivity discs were placed in the culture medium, and incubated at 30°C for 48 h. Results were interpreted based on the diameter of inhibition as sensitive (≥ 21 mm), intermediate sensitivity (17-20 mm), and resistant (≤ 16 mm).

Proteolytic and amylolytic assay

Proteolytic activity was assessed using 10% Skim Milk Agar (HiMedia, India) as the protein source. Bacterial candidates for probiotics were streaked, and the plates were incubated at 30°C for 24 h. Positive results were observed as a clear zone around the streaked bacteria. Amylolytic activity was determined using 2.5% Starch Agar (HiMedia, India). Bacterial streaking was performed on starch agar and incubated at 30°C for 24 h. After bacterial growth, several drops of 1% Lugol's iodine solution were added using a pipette until the entire plate was covered. Amylolytic activity was evident as a clear zone around the streak (Reda et al. 2018).

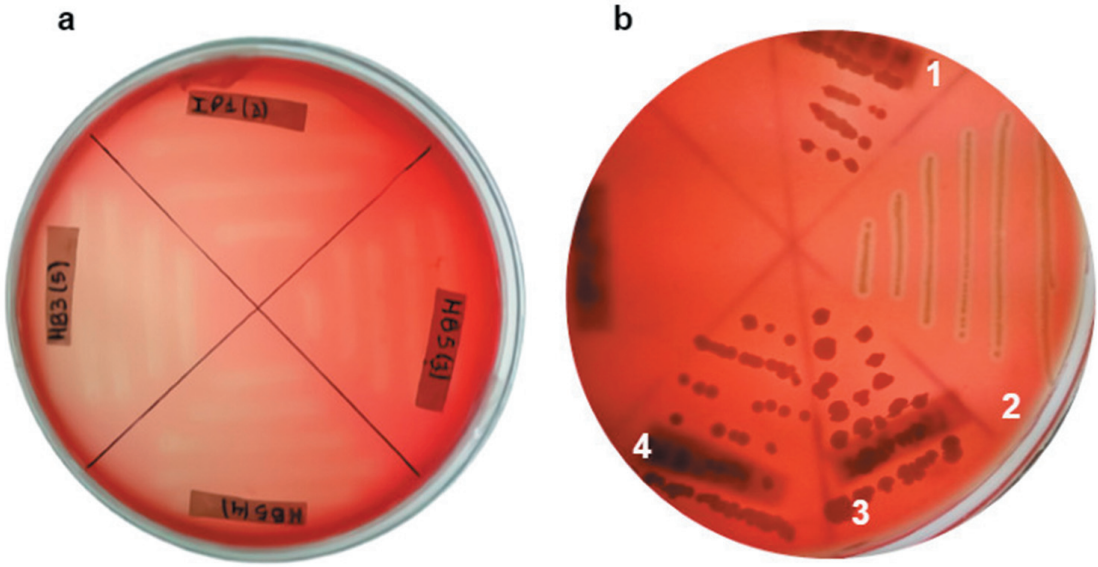

Figure 2. Hemolytic activity of bacterial isolates. (a) β-hemolysis in bacterial isolates; (b) γ-hemolysis in three bacterial isolates (1, 3, and 4) and β-hemolysis in one bacterial isolate (2).

Molecular identification of isolates

The isolates were identified by 16S rRNA gene sequencing. DNA was extracted using the boiling method (Ribeiro et al. 2016). The primers used for PCR were 27F (5'-CCAGAATTCAGAGTTTGATCMTGGCTCA-3') and 1492R (5'-ACCAAGCTTTACGGYTACCTTGTTAGGACTT-3') under the following conditions: an initial cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 58°C for 45 s, 72°C for 1 min, and a final cycle at 72°C for 10 min. PCR products were assessed in 1% (w/v) agarose gel and visualized via blue light transilluminator after staining with safe nucleic acid stain (Safeview™, Canada).

The nucleotide sequences obtained were processed with DNA Dragon – DNA Sequence Contig Assembler Software and then were compared to sequences from the NCBI database using the Nucleotide Basic Local Alignment Search Tool BLAST (NCBI, http://blast.ncbi.nlm.nih.gov/Blastn). The limit fixed for the identification of a bacterial species was 99–98% nucleotide identity for the 16S rRNA gene.

Results

A total of 29 bacteria were successfully isolated, 14 originating from the hindgut and 15 from the gills of rainbow trout on MRS medium. The Gram staining showed a total of 12 Gram-negative and 17 Gram-positive bacteria, with two morphologies observed under microscopy: cocci and coccobacilli.

In the hemolytic activity assay (Fig. 2), 11 isolates exhibited β-hemolysis, one displayed α-hemolysis, and 15 showed γ-hemolysis, and two isolates did not grow. The 15 isolates showing γ-hemolysis were selected as candidate probiotics due to their lack of virulence characteristics.

Antagonistic activity was assessed in 15 candidate probiotic bacteria, of which seven showed inhibitory activity against three pathogens: *S. agalactiae*, *L. garvieae*, and *A. hydrophila* (Table 1). The results demonstrated that isolates IP1-5 (*Lactococcus lactis*.), IP3-1 (*L. lactis*), and HB4-5 (*L. lactis*) inhibited the growth of *S. agalactiae* with inhibition zones measuring 6.3 mm, 3 mm, and 2.6 mm, respectively. Furthermore, isolates IP5-3 (*L. lactis*) and HB4-2 (*L. lactis*) inhibited the growth of *L. garvieae*, both with inhibition zones measuring 6.3 mm. Similarly, isolates IP3-1 (*L. lactis*), IP2-6 (*L. lactis*) and HB4-5 (*L. lactis*) exhibited inhibition zones against *A. hydrophila* measuring 4 mm, 3.3 mm, and 2.6 mm, respectively.

To ensure the absence of antibiotic-resistant profiles in the isolates, an antibiogram test was

Table 1
Antagonistic activity of probiotic candidate isolates

Isolates	Species	*Aeromonas hydrophila*	*Streptococcus agalactiae*	*Lactococcus garvieae*
IP5R1	*Lactococcus lactis*	-	-	+
IP5-3	*Lactococcus lactis*	+	+	++
IP2-6	*Lactococcus lactis*	+	+	+
HB4-5	*Lactococcus lactis*	+	+	+
HB4-2	*Lactococcus lactis*	+	++	+
IP3-1	*Lactococcus lactis*	++	+	+
IP1-5	*Lactococcus lactis*	+	++	++

(-) No inhibition zone, (+) Inhibition zone of 1 to 3 mm, (++) Inhibition zone of 4 to 7 mm.

Table 2
Antibiotic sensitivity of probiotic candidate isolates

Isolates	Species	Florfenicol (30 µg)	Tetracycline (30 µg)	Ampicillin (10 µg)
IP5R1	*Lactococcus lactis*	I	I	S
IP5-3	*Lactococcus lactis*	I	S	S
IP2-6	*Lactococcus lactis*	I	S	S
HB4-5	*Lactococcus lactis*	I	S	S
HB4-2	*Lactococcus lactis*	I	S	S
IP3-1	*Lactococcus lactis*	I	S	I
IP1-5	*Lactococcus lactis*	I	S	S

(S) Susceptible, inhibition zone ≥ 21 mm; (I) Intermediate Susceptible, inhibition zone of 17 to 20 mm; (R) Resistant, inhibition zone ≤ 16 mm.

conducted against florfenicol (30 µg), ampicillin (10 µg), and tetracycline (30 µg), which are antibiotics commonly employed in aquaculture. None of the isolates showed resistance to any of the antibiotics tested. All isolates exhibited sensitivity to tetracycline, except for IP5R1 (*L. lactis*), which showed intermediate sensitivity. Similarly, all isolates showed sensitivity to ampicillin, except for IP3-1 (*L. lactis*), which exhibited intermediate sensitivity (Table 2).

All isolates exhibited proteolytic activity, suggesting their potential role in food digestibility, facilitating nutrient absorption, and improving growth rates. However, the results of the amylase test revealed the absence of this enzymatic activity in all the isolates tested (Fig. 3).

The 16S rRNA nucleotide sequences blasted indicated high similarity (98–99%) to *Lactococcus lactis* (Table 3). However, the IP5R1 strain showed a percentage of similarity of 93.57% to *L. lactis,* which falls below the threshold required to define microbial species (≤ 97%) following Tu and Lin (2016).

Discussion

Probiotics, living microorganisms applied directly to fish or water, serve to enhance water quality, digestion, and immune functions. Native probiotics tend to be better adapted to the host environment, which may enhance their overall effectiveness compared to non-native strains (Wanka et al. 2018). Furthermore, when added to feed in either liquid or dry form, probiotics have been shown to improve the specific growth rate and disease resistance in fishes. These improvements in growth and survival rates lead to higher yields, making probiotic-enriched feeds a cost-effective option for fish farmers (Shelby et al. 2007, Saravanan et al. 2021).

In this study, lactic acid bacteria (LAB), which are Gram-positive, non-spore forming cocci, coccobacilli, or rods, and common potential probiotic bacteria, were isolated from rainbow trout. Pérez et al. (2011) isolated bacteria from intestinal mucosa, gills, and skin mucus of rainbow trout to

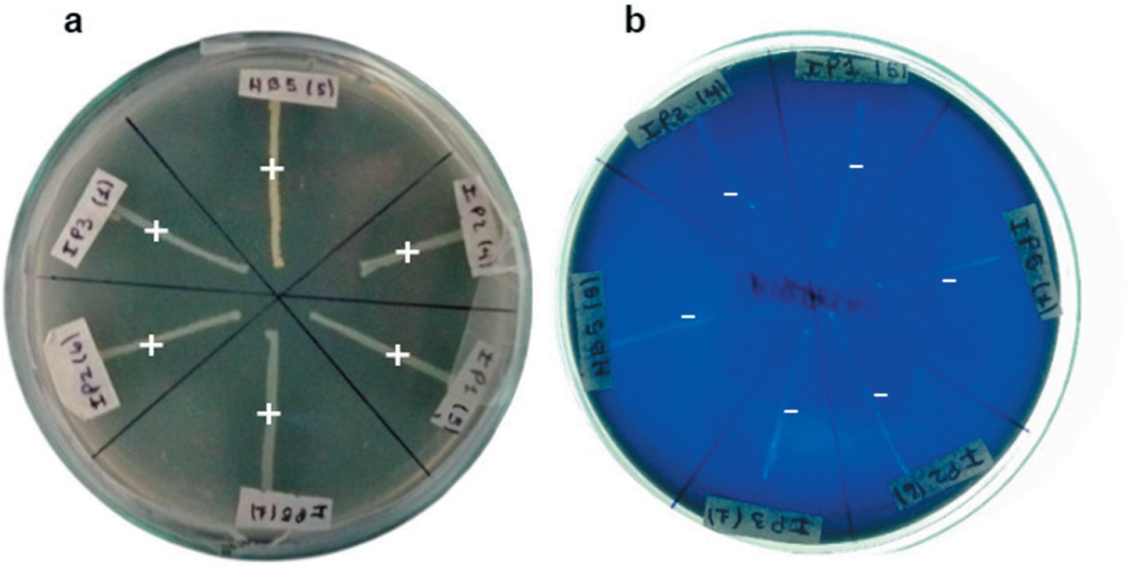

Figure 3. Proteolytic and amylolytic activity of bacterial isolates. (a) proteolytic activity in bacterial isolates; (b) negative results for amylolytic activity.

Table 3
Morphological description and molecular identification of the probiotic candidate isolates

Isolates	Source	Gram	Species	Similarity (%)	Access number
IP5R1	Intestine	Coccobacilli	*Lactococcus lactis*	93.57	MH620377.1
IP5-3	Intestine	Coccobacilli	*Lactococcus lactis*	99.06	NR_040955.1
IP2-6	Intestine	Coccobacilli	*Lactococcus lactis*	98.98	NR_113960.1
HB4-5	Gill	Coccobacilli	*Lactococcus lactis*	98.99	NR_040955.1
HB4-2	Gill	Coccobacilli	*Lactococcus lactis*	99.18	NR_113960.1
IP3-1	Intestine	Coccobacilli	*Lactococcus lactis*	99.12	NR_040955.1
IP1-5	Intestine	Coccobacilli	*Lactococcus lactis*	99.05	NR_113960.1

obtain a larger number of bacteria with potential probiotic properties. In addition, Quevedo (2020) isolated LAB on MRS agar from the intestinal mucosa of rainbow trout, a commonly used source for LAB isolation. Two morphologies were observed: cocci and coccobacilli. These characteristics did not affect the subsequent assays, in contrast to Gutiérrez (2016), who exclusively selected Gram-positive cocci as probiotic candidates to isolate bacteria from the genus *Lactobacillus* due to their extensive use as probiotics. However, Medina et al. (2020b) selected cocci and bacilli bacteria and identified *Bacillus amyloliquefaciens* and *Paenibacillus* spp. with potential probiotic properties in rainbow trout.

Probiotic bacteria do not have the capacity to produce hemolysis, a characteristic commonly found in pathogens. Hemolysin, produced by pathogens, is a compound that lyses host cells to release iron-rich compounds such as hemoglobin, which facilitates bacterial growth within the host organism (Argyri et al. 2013). Nandi et al. (2017), noted that hemolytic bacteria can cause anemia and are not suitable for selection as potential probiotics. In contrast, non-hemolytic bacteria were considered safe probiotic candidates for tilapia (Zapata et al. 2023), and they were evaluated for use in dietary supplementation for *Arapaima gigas* with good results (do Vale Pereira et al. 2017).

The production of antimicrobial compounds by LAB is a key aspect of their ability to exclude pathogenic bacteria. Extensive research has established that LAB produce several antimicrobial compounds

such as organic acids, hydrogen peroxide, and bacteriocins, among others (Choi et al. 2018). In this study, several probiotic bacteria showed antagonistic activity to common fish pathogens. The antagonistic capacity of Lactococcus species isolated, including *L. lactis*, can be explained by the production of bioactive molecules known as bacteriocins, such as nisins A, Z, Q, F, and U. These bacteriocins exhibit a bactericidal or bacteriostatic mode of action against sensitive Gram-positive and, in some cases, Gram-negative bacteria (Navale et al. 2023). Fotso Techeu et al. (2022) isolated *L. lactis* from *Cyprinus carpio* L., demonstrating its antagonistic activity against *Escherichia coli* and *Pseudomonas aeruginosa*. Additionally, results from Sequeiros et al. (2010), showed that *L. lactis* strains isolated from Patagonian fish exhibited inhibitory effects on the growth of Gram-positive bacteria, including *L. garvieae*, while showing no such impact on Gram-negative bacteria. However, these findings differ from the results of the present study, which demonstrated antagonistic effects against *A. hydrophila*, a Gram-negative bacterium.

The utilization of bacteria derived from antibiotic resistant strains presents inherent risks to fish, environmental ecosystems, and public health. Therefore, it is imperative to select probiotic candidates sensitive to antibiotics (Huang et al. 2017). The results of the antimicrobial susceptibility test could obtain probiotic bacteria candidates sensitive to florfenicol, ampicillin, and tetracycline. These results align with those of Quevedo (2020), who reported isolates sensitive to the antibiotics used in the present study. Moreover, it is suggested that sensitivity and intermediate sensitivity are intrinsic characteristic of probiotics, crucial to maintaining their role as beneficial members of the intestinal microbiota following antibiotic therapy (Panigrahi and Azad 2007). While *L. lactis* is widely used as a probiotic due to its beneficial characteristics, Mathur and Singh (2005) isolated a *L. lactis* strain from soft cheese containing a plasmid conferring multiple antibiotic resistance such as streptomycin, tetracycline, and chloramphenicol.

Extracellular enzymes produced by probiotic bacteria candidates, such as protease and amylase, play key roles in improving nutrient digestibility and enhancing animal health. Furthermore, Balcázar et al. (2006) showed that certain bacterial strains can stimulate the production of digestive enzymes like amylase, chitinase, lipase, and proteases, thereby enhancing food digestibility. Yeganeh et al. (2021) mentioned that specific strains of LAB are associated with antidiabetic effects, possibly related to their low amylolytic activity. Additionally, Mondal et al. (2010) demonstrated that amylolytic bacteria are predominantly in the foregut compared to the hindgut, which may explain the absence of this activity in the bacteria isolated in this study.

Lactococcus lactis is considered as a potential probiotic in aquaculture and has been isolated from various fish species, including *O. mykiss* (Mortezaei et al. 2020, Quevedo 2020), *Centropomus spp.* (Zatán et al. 2020), and *C. carpio* (Fotso Techeu et al. 2022). The *Lactococcus* genera is commonly found in the gastrointestinal tract of freshwater fish, as reported by Hagi et al. (2004). Furthermore, *L. lactis* was the most isolated species from the rainbow trout intestine (Araújo et al. 2015a). Studies have demonstrated its effectiveness in various in vivo assays, such as increasing the abundance of LAB in the intestine of *Rhamdia quelen* (Quoy & Gaimard) (Yamashita et al. 2020), decreasing the abundance of *S. agalactiae* in *Danio rerio* (Hamilton) (Tan et al. 2022), and enhancing survival in an experimental *Streptococcus* infection in *Paralichthys olivaceus* (Temmnick & Schlegel) when combined with β-glucooligosaccharides (Hasan et al. 2018). These findings highlight its ability in modulating the microbiota and stimulating the immune system in different fish species. In rainbow trout, *L. lactis* has been used as a dietary additive, offering benefits to the host such as low feed conversion rate, rapid growth, stimulation of enzyme activity, improved hematological levels, and resistance to pathogens such as *L. garvieae* and *Y. ruckeri* (Araújo et al. 2015b, Yeganeh et al. 2021).

This research highlights significant strengths, demonstrating the antagonistic capacity of native

probiotic strains against common pathogens in the aquaculture industry, such as *A. hydrophila*, *S. agalactiae*, and *L. garvieae*, showing a broad spectrum of activity. One of the key contributions of this study is the expansion of probiotic selection criteria to include strains obtained from the gills, unlike previous studies that focused exclusively on the intestines of rainbow trout (Kim and Austin 2008, Burbank et al. 2012, Medina et al. 2020b). This more comprehensive approach offers a fuller perspective for the identification of potential probiotics.

However, the study also presents certain limitations. While the in vitro results suggest potential efficacy of the selected strains, these do not guarantee the same results under in vivo conditions. Therefore, large-scale experiments under field conditions are recommended to validate the findings. Additionally, the study focused on a limited set of pathogens, without assessing its effectiveness against other relevant pathogens such as *Yersinia ruckeri*, which has a significant prevalence in Peru (Fernandez-Espinel et al. 2023).

This study presents significant implications for rainbow trout aquaculture and the aquaculture industry. The isolated probiotic strains, which have shown susceptibility to antibiotics and antagonistic activity against pathogens, could offer an effective solution for preventing disease outbreaks. If their effectiveness is confirmed under in vivo conditions, these strains may have the potential to reduce the reliance on antibiotics in aquaculture, a growing concern due to antimicrobial resistance. In general, the results of this study could contribute to developing more sustainable aquaculture practices, improving fish welfare, and reducing the environmental impact associated with the indiscriminate use of antibiotics.

Conclusions

Strains isolated from hind gut and gills of rainbow trout exhibit probiotic properties. Among the isolates, seven strains identified as *L. lactis* inhibited the growth of pathogens, showed proteolytic activity, and did not show resistance to the antibiotics tested. These findings highlight the significance of considering different sources, such as the gills, for the isolation of bacteria with specific probiotic capacities.

Author contributions. B.M.L. data curation, investigation, original draft; M.A.F. conceptualization, investigation, review and editing; A.E.Z. investigation, original draft; O.E.T. investigation, original draft; J.L.A. methodology, data curation; B.M.D. supervision, resources, review and editing; A.E.C. conceptualization, data curation, original draft, review and editing.

ORCID iD

Billy Merlin Lucano https://orcid.org/0000-0002-1395-3412
Manuel Alberto Feria https://orcid.org/0000-0001-6609-9232
Adrian Edu Zatán https://orcid.org/0000-0001-7926-6888
Odalis Epifanía Toledo https://orcid.org/0000-0001-7265-1433
Jorge Luis Aguilar https://orcid.org/0000-0002-6254-2505
Benoit Mathieu Diringer https://orcid.org/0000-0001-6129-1751
Arnaldo Edward Castañeda https://orcid.org/0000-0001-5108-8469

References

Akhter, N., Wu, B., Memon, A.M., Mohsin, M. (2015). Probiotics and prebiotics associated with aquaculture: a review. Fish and Shellfish Immunology, 45(2), 733-741.

Araújo, C., Muñoz-Atienza, E., Nahuelquín, Poeta, P., Igrejas, G., Hernández, P.E., Herranz, C., Ruiz-Zarzuela, I., Cintas, L.M. (2015). Inhibition of fish pathogens by the microbiota from rainbow trout (*Oncorhynchus mykiss*, Walbaum) and rearing environment. Anaerobe 32, 7-14.

Araújo, C., Muñoz-Atienza, E., Pérez-Sánchez, T., Poeta, P., Igrejas, G., Hernández, P.E., Herranz, C., Ruiz-Zarzuela, I., Cintas, L.M. (2015). Nisin Z production by *Lactococcus lactis* subsp. cremoris WA2-67 of aquatic origin as a defense mechanism to protect rainbow Trout (*Oncorhynchus mykiss*, Walbaum) against *Lactococcus garvieae*. Marine Biotechnology, 17, 820-830.

Argyri, A.A., Zoumpopoulou, G., Karatzas, K.A.G., Tsakalidou, E., Nychas, G.J.E., Panagou, E.Z. (2013). Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. Food Microbiology, 33, 282-291.

Balcázar, J., Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Muzquiz, J. (2006). The role of probiotics in

aquaculture. Veterinary Microbiology, 114(3–4), 173-186.

Balcázar J.L., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I., Muzquiz, J.L., Girones, O. (2008). Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. Aquaculture, 278(1–4), 188-191.

Bauer, A.W., Kirby, W.M., Sherris, J.C., Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology, 45(4), 493-496.

Bhatnagar, A., Rathi, P. (2023). Isolation and characterization of autochthonous probiotics from skin mucus and their in vivo validation with dietary probiotic bacteria on growth performance and immunity of *Labeo calbasu* (Hamilton, 1822). Fish Physiology Biochemistry, 49(2), 191-208.

Burbank, D.R., LaPatra, S.E., Fornshell, G., Cain, K.D. (2012). Isolation of bacterial probiotic candidates from the gastrointestinal tract of rainbow trout, *Oncorhynchus mykiss* (Walbaum), and screening for inhibitory activity against *Flavobacterium psychrophilum*. Journal of Fish Diseases, 35(11), 809-816.

Castañeda, A.E., Aguilar, J.L., Zatán, A.E., Toledo, O.E., Feria, M.A., Castillo, D. (2018). Molecular identification of lactic acid bacteria with probiotic properties isolated from the posterior intestine of Nile tilapia (*Orechromis niloticus*). Revista de Investigaciones Altoandinas, 20(4), 429-438 (in Spanish).

Choi, A.R., Patra, J.K., Kim, W.J. Kang, S.S. (2018). Antagonistic activities and probiotic potential of lactic acid bacteria derived from a plant-based fermented food. Frontier in Microbiology, 9, 1963.

do Vale Pereira, G., da Cunha, D.G., Pedreira Mourino, J.L., Rodiles, A., Jaramillo-Torres, A., Merrifield, D.L. (2017). Characterization of microbiota in *Arapaima gigas* intestine and isolation of potential probiotic bacteria. Journal of Applied Microbiology, 123(5), 1298-1311.

El-Saadony, M.T., Alagawany, M., Patra, A.K., Kar, I., Tiwari, R., Dawood, M.A., Dhama, K., Abdel-Latif, H.M. (2021). The functionality of probiotics in aquaculture: An overview. Fish and Shellfish Immunology, 117, 36-52.

Fernandez-Espinel, C., Medina-Morillo, M., Irgang, R., Sotil, G., Araya-León, H., Flores-Dominick, V., Romalde, J.L., Avendaño-Herrera, R., Yunis-Aguinaga, J. (2023). Co-existence of two *Yersinia ruckeri* biotypes and serotype O1a retrieved from rainbow trout (*Oncorhynchus mykiss*) farmed in Puno, Peru. Journal of Fish Diseases, 46(2), 157-163.

Fotso Techeu, U.D., Kaktcham, P.M., Momo, H.K., Foko Kouam, E.M., Tchamani Piame, L., Ngouenam, R. J., Zambou Ngoufack, F. (2022). Isolation, characterization, and effect on biofilm formation of bacteriocin produced by *Lactococcus lactis* F01 isolated from *Cyprinus carpio* and application for biopreservation of fish sausage. BioMed Research International, 2022, 8437926.

Gómez, G.D., Balcázar, J.L., (2008). A review on the interactions between gut microbiota and innate immunity of fish. FEMS Immunology & Medical Microbiology, 52(2), 145-154.

Gutiérrez, L.A. (2016). Characterisation of *Bacillus* sp. and lactic acid bacteria strains with probiotic activity in the digestive tract of red tilapia (*Oreochromis* sp.) as a potential consortium for microencapsulation processes. PhD Thesis, Universidad Nacional de Colombia. 86 p. https://repositorio.unal.edu.co/handle/unal/56859 (in Spanish).

Hagi, T., Tanaka, D., Iwamura, Y., Hoshino, T. (2004). Diversity and seasonal changes in lactic acid bacteria in the intestinal tract of cultured freshwater fish. Aquaculture. 234, 335-346.

Hasan, T., Jang, W.J., Tak, J.Y., Lee, B.J., Kim, K.W., Hur, W., Han, H.S., Kim, B.S., Min, D.H., Kim, S.K., Kong, I.S. (2018). Effects *of Lactococcus lactis subsp. lactis* I2 with β-glucooligosaccharides on growth, innate immunity and Streptococcosis resistance in olive flounder (*Paralichthys olivaceus*). Journal of Microbiology and Biotechnology, 28(9), 1433-1442.

Huang, L., Xu, Y.B., Xu, J.X., Ling, J.Y., Chen, J.L., Zhou, J.L., Zheng, L., Du, Q.P. (2017). Antibiotic resistance genes (ARGs) in duck and fish production ponds with integrated or non-integrated mode. Chemosphere, 168, 1107-1114.

Johnson, S.C., Treasurer, J.W., Bravo, S., Nagasawa, K., Kabata, Z. (2004). A review of the impact of parasitic copepods on marine aquaculture. Zoological Studies, 43: 229-243.

Keene, J.L., Noakes, D.L., Moccia, R.D., Soto, C.G. (1998). The efficacy of clove oil as an anaesthetic for rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquaculture Research, 29(2), 89-101.

Kim, D.H, Austin, B. (2008). Characterization of probiotic carnobacteria isolated from rainbow trout (*Oncorhynchus mykiss*) intestine. Letters in Applied Microbiology, 47(3), 141-147.

Kuebutornye, F.K., Abarike, E.D., Lu, Y., Hlordzi, V., Sakyi, M.E., Afriyie, G., Wang, Z., Li, Y., Xie, C.X. (2020). Mechanisms and the role of probiotic Bacillus in mitigating fish pathogens in aquaculture. Fish Physiology and Biochemistry. 46(3), 819-841.

Lazado, C.C., Caipang. C.M., Brinchmann, M.F., Kiron, V. (2011). In vitro adherence of two candidate probiotics from Atlantic cod and their interference with the adhesion of two pathogenic bacteria. Veterinary Microbiology, 148(2–4), 252-259.

Liu, Y., Lv, H., Xu, L., Zhang, K., Mei, Y., Chen, J., Wang, M., Guan, Y., Pang, H., Wang, Y., Tan, Z. (2022). The effect of dietary lactic acid bacteria on intestinal Microbiota and

immune responses of crucian carp (*Carassius auratus*) under water temperature decrease. Frontiers in Microbiology, 13, 847167.

Llewellyn, M.S., Boutin, S., Hoseinifar, S.H., Derome, N. (2014). Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Frontiers in Microbiology, 2(5), 207.

Mathur, S., Singh, R. (2005). Antibiotic resistance in food lactic acid bacteriaa – a review. International Journal of Food Microbiology, 105(3), 281-295.

Medina, M., Fernandez-Espinel, C., Sotil, G., Yunis-Aguinaga, J., Flores-Dominick, V. (2020a). First description of Weissella ceti associated with mortalities in farmed rainbow trout (*Oncorhynchus mykiss*) in Peru. Aquaculture, 529, 735608.

Medina, M., Sotil, G., Flores, V., Fernández, C., Sandoval, N. (2020b). In vitro assessment of some probiotic properties and inhibitory activity against *Yersinia ruckeri* of bacteria isolated from rainbow trout *Oncorhynchus mykiss* (Walbaum). Aquaculture Reports, 18, 100447.

Mondal, S., Roy, T., Ray, A.K. (2010). Characterization and identification of enzyme-producing bacteria isolated from the digestive tract of bata, *Labeo bata*. Journal of The World Aquaculture Society, 41(3), 369-377.

Mortezaei, F., Royan, M., Allaf Noveirian, H., Babakhani, A., Alaie Kordghashlaghi, H., Balcázar, J.L. (2020). In vitro assessment of potential probiotic characteristics of indigenous *Lactococcus lactis* and *Weissella oryzae* isolates from rainbow trout (*Oncorhynchus mykiss* Walbaum). Journal of Applied Microbiology, 129(4), 1004-1019.

Moya-Salazar, J., Díaz, C.S., Cañari, B., Badillo, R.X., Verano-Zelada, M., Chicoma-Flores, K., Contreras-Pulache, H. (2022). Detection of pathogenic *Aeromonas hydrophila* from two rainbow trout (*Oncorhynchus mykiss*) farms in Peru. Brazilian Journal of Veterinary Medicine, 44, e000922.

Nandi, A., Dan, S.K., Banerjee, G., Ghosh, P., Ghosh, K., Ringř, E., Ray, A.K. (2017). Probiotic potential of autochthonous bacteria isolated from the gastrointestinal tract of four freshwater teleosts. Probiotics and Antimicrobial Proteins, 9(1), 12-21.

Nathanailides, C., Kolygas, M., Choremi, K., Mavraganis, T., Gouva, E., Vidalis, K., Athanassopoulou, F. (2021). Probiotics have the potential to significantly mitigate the environmental impact of freshwater fish farms. Fishes, 6(4), 76.

Navale, V.D., Borade, B.R., Krishna, G.R., Vamkudoth, K.R., Kontham, R. (2023). Metabolites from *Lactococcus lactis* subsp. lactis: Isolation, Structure Elucidation, and Antimicrobial Activity. ACS Omega, 8, 36628-36635.

Nuñure, O.J., Sandoval, C.N., Estrella, O.M., Ramos, E.F., Herrera, R.A., Valera, A.A., Manchego, S.A., (2021). Identification and characterization of pathogenic *Aeromonas* sp isolated from clinically diseased rainbow trout (*Oncorhynchus mykiss*) raised in Peruvian fish farms. Revista de Investigaciones Veterinarias del Perú, 32(4), e20927 (in Spanish).

Panigrahi, A., Azad, I.S. (2007). Microbial intervention for better fish health in aquaculture: the Indian scenario. Fish Physiology and Biochemistry, 33(4), 429-440.

PRODUCE (2019). Anuario estadístico Pesquero y acuícola 2019. Ministerio de la Producción, Lima, Perú. https://ogeiee.produce.gob.pe/index.php/en/shortcode/oee-documentos-publicaciones/publicaciones-anuales/item/949-anuario-estadistico-pesquero-y-acuicola-2019

Quevedo, K.G. (2020). Isolation and characterisation of *Lactobacillus* sp with in vitro probiotic potential against rainbow trout pathogens. Master thesis, Lima. Perú, Universidad Cayetano Heredia. 69 p. https://repositorio.upch.edu.pe/handle/20.500.12866/7752

Ramírez, J.A., Orozco, D.I., del Carmen Monroy, M., Handam, A., Bustos, J.A., Hernández, L.H., Castro, J. (2019). Desarrollo de probióticos para peces dulceacuícolas. Universidad Autónoma de Metropolitana. México, 144 p. https://www.casadelibrosabiertos.uam.mx/contenido/contenido/Libroelectronico/probioticos_peces.pdf

Reda, R.M., Selim, K.M., El-Sayed, H.M., El-Hady, M.A. (2018). In vitro selection and identification of potential probiotics isolated from the gastrointestinal tract of Nile Tilapia, *Oreochromis niloticus*. Probiotics and Antimicrobial Proteins, 10(4), 692-703.

Ribeiro J.C., Tamanini, R., Soares, B.F., de Oliveira, A.M., Silva, F.G., da Silva, F.F., Augusto N.A., Beloti, V. (2016). Efficiency of boiling and four other methods for genomic DNA extraction of deteriorating spore-forming bacteria from milk. Semina: Ciências Agrárias, 37(5), 3069-3078.

Saravanan, K., Sivaramakrishnan, T., Praveenraj, J., Kiruba-Sankar, R., Haridas, H., Kumar, S., Varghese, B. (2021). Effects of single and multi-strain probiotics on the growth, hemato-immunological, enzymatic activity, gut morphology and disease resistance in Rohu, *Labeo rohita*. Aquaculture, 540, 15, 736749.

Sequeiros, C., Vallejo, M., Marguet, E.R., Olivera, N.L. (2010). Inhibitory activity against the fish pathogen *Lactococcus garvieae* produced by *Lactococcus lactis* TW34, a lactic acid bacterium isolated from the intestinal tract of a Patagonian fish. Archives of Microbiology, 192, 237-245.

Shelby, R. A., Lim, C., Yildirim-Aksoy, M., Klesius, P. H. (2007). Effects of probiotic bacteria as dietary supplements on growth and disease resistance in young channel catfish, *Ictalurus punctatus* (Rafinesque). Journal of Applied Aquaculture, 19(1), 81-91.

Tan, C., Li, Q., Yang, X., Chen, J., Zhang, Q., Deng, X. (2022). *Lactococcus lactis*' effect on the intestinal microbiota of

Streptococcus agalactiae-infected zebrafish (*Danio rerio*). Microbiology Spectrum, 10(5), e01128-22.

Távara, P.C., Serrano-Martínez, E., Tafalla, C., Burga, C.C., Llanco, A.L. (2019). Molecular identification of *Flavobacterium psychrophilum* and characterization of cutaneous alterations in rainbow trout (*Oncorhynchus mikyss*) from Puno, Peru. Revista de Investigaciones Veterinarias del Perú, 30(4), 1762-1769 (in Spanish).

Tavares-Dias, M., Martins, M.L. (2017). An overall estimation of losses caused by diseases in the Brazilian fish farms. Journal of Parasitic Diseases. 41(4), 913-918.

Tu, Q., Lin, L. (2016). Gene content dissimilarity for subclassification of highly similar microbial strains. BMC Genomics, 17, 647. https://doi.org/10.1186/s12864-016-2991-9.

Wang, A., Ran, C., Wang, Y., Zhang, Z., Ding, Q., Yang, Y., Olsen, R.E., Ringø, E., Bindelle J., Zhou, Z. (2019). Use of probiotics in aquaculture of China–a review of the past decade. Fish and Shellfish Immunology, 86, 734-755.

Wanka, K.M., Damerau, T., Costas, B., Krueger, A., Schulz, C., Wuertz, S. (2018). Isolation and characterization of native probiotics for fish farming. BMC Microbiology, 18, 119, https://doi.org/10.1186/s12866-018-1260-2

Yamashita, M.M., Ferrarezi, J.V., do Vale Pereira, G., Júnior, G.B., da Silva, B.C., Pereira, Martins, M.L., Mouriño, J.L. (2020). Autochthonous vs allochthonous probiotic strains to *Rhamdia quelen*. Microbial Pathogenesis, 139, 103897.

Yeganeh, S., Adel, M., Nosratimovafagh, A., Dawood, M. A. (2021). The Effect of *Lactococcus lactis subsp. lactis* PTCC 1403 on the growth performance, digestive enzymes activity, antioxidative status, immune response, and disease resistance of Rainbow trout (*Oncorhynchus mykiss*). Probiotics and Antimicrobial Proteins, 13(6), 1723-1733.

Zapata, A., López-Alcántara, R., Leticia Bravo-Olivas, M., Chávez-Dagostino, R., Ramos-Miranda, J., Miranda-Hernández, D.U., Lara-Flores, M. (2023). Selection of native lactic acid bacteria with probiotic potential from Nile tilapia (*Oreochromis niloticus*) gastrointestinal tract. International Aquatic Research, 15, 135-144.

Zatán Valdiviezo, A.E., Castillo Chunga, D., Castañeda Vargas, A.E., Feria Zevallos, M.A., Toledo Valdiviezo, O.E., Aguilar Zavaleta, J.L., Cueva Távara, M.D., Motte, E. (2020). Characterization of the intestinal microbiota in snook (*Centropomus* sp.) and isolation of potential probiotic bacteria. Revista de Investigaciones Veterinarias del Perú, 31(3), e16036 (in Spanish).