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Lactobacillus brevis FROM GAMITANA (*Colossoma macropomum*) SHOWS PROBIOTIC POTENTIAL WITH ANTAGONISTIC ACTIVITY AGAINST *Lactococcus garvieae* PATHOGEN

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ABSTRACT

Autochthonous probiotics derived from the host's native microbiota can enhance gastrointestinal colonization, feed efficiency, disease resistance, and stress tolerance in aquaculture species. This study evaluated bacteria isolated from the intestine and gills of gamitana (*Colossoma macropomum*) based on key probiotic selection criteria, including tolerance to acidic conditions and bile salts, antagonistic activity against pathogens, antibiotic susceptibility, biofilm formation, and enzymatic activity. A total of 69 bacterial isolates were obtained using Man, Rogosa, and Sharpe (MRS) broth and Trypticase Soy Broth (TSB). Five isolates (7.2%) met all selection criteria and were molecularly identified as *Lactobacillus brevis* (IPIFAB2, IPIFAC1, BRALA1) and *Lactococcus garvieae* (G25, G23). *L. brevis* isolates showed moderate acid tolerance, with survival rates between 31 ± 2.04% and 36.4 ± 0.17% at pH 4. All selected strains tolerated bile salts; however, *L. brevis* maintained high viability (≥78% at 0.8%), while *L. garvieae* exhibited marked reductions at higher concentrations. Antagonistic activity was significantly greater in *L. brevis,* producing inhibition halos of 2.3 ± 0.1 to 3.9 ± 0.1 mm, compared with 1.2 ± 0.21 to 1.9 ± 0.12 mm for *L. garvieae*. Proteolytic activity was observed in three *L. brevis* strains, and six isolates demonstrated biofilm-forming capacity. All selected strains were susceptible to the antibiotics tested. Among them, *L. brevis* IPIFAB2 exhibited the strongest

probiotic profile. Overall, *L. brevis* strains show considerable potential as autochthonous probiotics for *C. macropomum* aquaculture, contributing to improved fish health, disease prevention, and sustainable production.

KEYWORDS: Tambaqui, amazon fish, gut, probiotic

Lactobacillus brevis DE GAMITANA (*Colossoma macropomum*) MUESTRAN POTENCIAL PROBIÓTICO CON ACTIVIDAD ANTAGONISTA CONTRA EL PATÓGENO *Lactococcus garvieae*

RESUMEN

Los probióticos autóctonos derivados de la microbiota nativa del huésped pueden mejorar la colonización gastrointestinal, la eficiencia alimentaria, la resistencia a enfermedades y la tolerancia al estrés en especies acuícolas. Este estudio evaluó bacterias aisladas del intestino y las branquias de la gamitana (*Colossoma macropomum*) con base en criterios clave de selección de probióticos, incluyendo tolerancia a condiciones ácidas y sales biliares, actividad antagonista contra patógenos, susceptibilidad a antibióticos, formación de biopelículas y actividad enzimática. Se obtuvo un total de 69 aislados bacterianos utilizando caldo Man, Rogosa y Sharpe (MRS) y caldo de soya tripticasa (TSB). Cinco aislados (7,2%) cumplieron con todos los criterios de selección y fueron identificados molecularmente como *Lactobacillus brevis* (IPIFAB2, IPIFAC1, BRALA1) y *Lactococcus garvieae* (G25, G23). Los aislados de *L. brevis* mostraron una tolerancia moderada al ácido, con tasas de supervivencia entre 31 ± 2,04% y 36,4 ± 0,17% a pH 4. Todas las cepas seleccionadas toleraron las sales biliares; sin embargo, *L. brevis* mantuvo una alta viabilidad (≥78% al 0,8%), mientras que *L. garvieae* exhibió reducciones marcadas a concentraciones más altas. La actividad antagónica fue significativamente mayor en *L. brevis*, produciendo halos de inhibición de 2,3 ± 0,1 a 3,9 ± 0,1 mm, en comparación con 1,2 ± 0,21 a 1,9 ± 0,12 mm para *L. garvieae*. Se observó actividad proteolítica en tres cepas de *L. brevis*, y seis aislados demostraron capacidad de formación de biopelículas. Todas las cepas seleccionadas fueron susceptibles a los antibióticos probados. Entre ellos, *L. brevis* IPIFAB2 exhibió el perfil probiótico más fuerte. En general, las cepas de *L. brevis* muestran un potencial considerable como probióticos autóctonos para la acuicultura de *C. macropomum*, contribuyendo a mejorar la salud de los peces, la prevención de enfermedades y la producción sostenible.

PALABRAS CLAVE: Tambaqui, pez amazónico, intestino, probiótico

INTRODUCTION

The species *Colossoma macropomum*, commonly known as gamitana or tambaqui, is a neotropical fish of high market value and increasing export demand in Latin America (Tomalá *et al.*, 2014). It is also one of the most widely cultivated species in Peru, particularly in the Amazon region (PRODUCE, 2023). Gamitana farming offers several advantages, including adaptability to captivity, high prolificacy, rapid growth, omnivorous feeding habits, and excellent meat quality (Hilsdorf *et al.*, 2022).

However, production intensification has increased the incidence of bacterial and parasitic diseases, leading to substantial economic losses for fish farmers (Dias *et al.*, 2021; Morey *et al.*, 2023). To address these challenges, antibiotics have become widely used in aquaculture; nevertheless, their excessive application has raised concerns about the emergence of antibiotic-resistant bacteria, posing risks to public health and the environment (Jo *et al.*, 2021; Okeke *et al.*, 2022).

In response, probiotics have gained attention as a sustainable alternative to antibiotics in fish farming, particularly for preventing and controlling bacterial infections in gamitana (Costa *et al.*, 2021; Kotzent *et al.*, 2021; Dias *et al.*, 2022). Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host by improving intestinal microbiota balance and enhancing the immune responses (Zorriehzahra *et al.*, 2016; Mondal *et al.*, 2022). These microorganisms can be isolated from various sources, including the natural microbiota of aquatic organisms (Wu *et al.*, 2012). In fish, the intestine and gill surfaces represent especially rich reservoirs of potential probiotic candidates due to their continuous interaction with the external environment and their ability to colonize host tissues and modulate immunity (Lucano *et al.*, 2024; Llewellyn *et al.*, 2014). Nevertheless, the isolation and characterization of autochthonous bacteria from Amazonian fish species remain limited.

Autochthonous probiotic bacteria, those naturally occurring in the gastrointestinal tract of specific host species, have shown promising results in improving fish health and performance (Kanwal *et al.*, 2021). These microorganisms are considered ideal probiotic candidates because they are already adapted to the host´s physiology and local environmental conditions. However, information on autochthonous probiotics from gamitana remains scarce. Previous work by Kotzent *et al.* (2021) demonstrated beneficial effects of autochthonous *Bacillus* and Enterococcus isolates, but their study did not include other important bacterial groups commonly associated with probiotic activity in fish, such as *Lactobacillus* spp. (Amenyogbe at al., 2021). Furthermore, no studies have evaluated the antagonistic activity against *Lactococcus garvieae,* an emerging pathogen in Amazonian aquaculture.

To address these gaps, the present study applied widely accepted probiotic selection criteria, including morphological characterization, bile salt resistance, pathogen antagonism, antimicrobial susceptibility, 16S rRNA gene sequencing, and host safety assessment (Palanivelu *et al.*, 2022). Given the limited characterization of autochthonous probiotics in gamitana, we hypothesized that autochthonous lactic acid bacteria would exhibit tolerance to gastrointestinal conditions and antagonistic activity against pathogens such as *L. garvieae*. Therefore, this study aimed to isolate and evaluate intestinal and gill bacteria using key probiotic criteria including acid and bile tolerance, pathogen inhibition, antibiotic susceptibility, biofilm formation, and enzymatic activity, to identify strains suitable for Amazonia aquaculture.

MATERIALS AND METHODS

PROBIOTIC CANDIDATE BACTERIAL ISOLATION

Healthy gamitana (n=11) at different developmental stages (fingerlings, juveniles, and adults) were collected from earthen ponds in the districts of Nieva and Río Santiago, Condorcanqui Province (Amazonas, Peru) (Figure 1). Fish were euthanized by immersion in an overdose of eugenol (50 ppm), following international animal welfare guidelines. The entire intestine was aseptically removed, opened longitudinally, and rinsed with sterile saline solution (0.85%). Intestinal tissue and gill swabs were inoculated into Man, Rogosa and Sharpe (MRS) broth and Trypticase Soy Broth (TSB) (HiMedia®, India) and incubated at 30 °C for 24 h. Three serial 1:10 dilutions were prepared, and 100 µL of each dilution was plated onto MRS and Trypticase Soy Agar (TSA) (HiMedia®, India) followed by incubation at 30 °C for 24 h. Colonies were selected primarily based on morphological characteristics such as size, color, and shape. Selected colonies were re-streaked at least once on fresh MRS agar to obtain purified isolates.

HEMOLYTIC ACTIVITY

Hemolytic activity was assessed following the protocol of Do Vale Pereira *et al.* (2017). Isolates were streaked onto Blood Agar Base (HiMedia®, India) supplemented with 5% sheep blood and incubated at 30 °C for 48 h. Hemolysis was determined by the presence of transparent or discolored zones around colonies. Non-hemolytic isolates were retained for subsequent analyses.

ANTIBIOTIC SENSITIVITY

Antibiotic susceptibility was tested on Mueller–Hinton (MH) agar (HiMedia®, India) following the Kirby–Bauer disk diffusion method (Bauer *et al.*, 1996). The following antimicrobial disks (OxoidTM®, United Kingdom) were used: chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), oxytetracycline (30 µg), penicillin (10 µg), and tetracycline (30 µg). A 100 µL aliquot of each bacterial suspension (1 × 108 cfu/mL) was spread onto MH agar plates. Antibiotic disks were placed on the surface, and plates were incubated at 30 °C for 48 h. Inhibition zone diameters were interpreted as follows: ≤ 14 mm = resistant (R), ≥ 20 mm = susceptible (S), and 15–19 mm = intermediate (I) (Sharma *et al.*, 2017).

BILE SALT AND pH RESISTANCE ASSAYS

Bile salt tolerance was assessed following Ramírez *et al.* (2019) with slight modifications. Each isolate was inoculated into MRS broth or TSB supplemented with bile salts at concentrations of 0.3%, 0.5%, 0.8%, and 1%. Cultures were incubated at 30 °C for 48 h, and absorbance at 600 nm was measured. Optical density values were converted to cells per mL to calculate bile salt resistance (%) using the following formula:

$$\text{Bile salt resistance}\left(\%\right) = \frac{\left[\left(\frac{\text{cfu}}{\text{mL}}\right)\text{treatment with bile sales}\right]}{\left(\frac{\text{cfu}}{\text{mL}}\right)\text{control}} \text{x } 100$$

For the pH resistance assay, isolates were inoculated into MRS broth or TSB adjusted to pH 1, 2, 3, or 4. Following incubation at 30 °C for 48 h, absorbance at 600nm was measured and converted to cells per mL. pH resistance (%) was calculated as:

$$\text{pH resistance}\left(\%\right) = \frac{\left[\left(\frac{\text{cfu}}{\text{mL}}\right)\text{modified pH treatment}\right]}{\left(\frac{\text{cfu}}{\text{mL}}\right)\text{control}} \text{x } 100$$

BIOFILM PRODUCTION

Biofilm formation was evaluated following the method described by Freeman *et al.* (1989). Isolates were streaked onto Brain Heart Infusion (BHI) agar (HiMedia®, India) supplemented

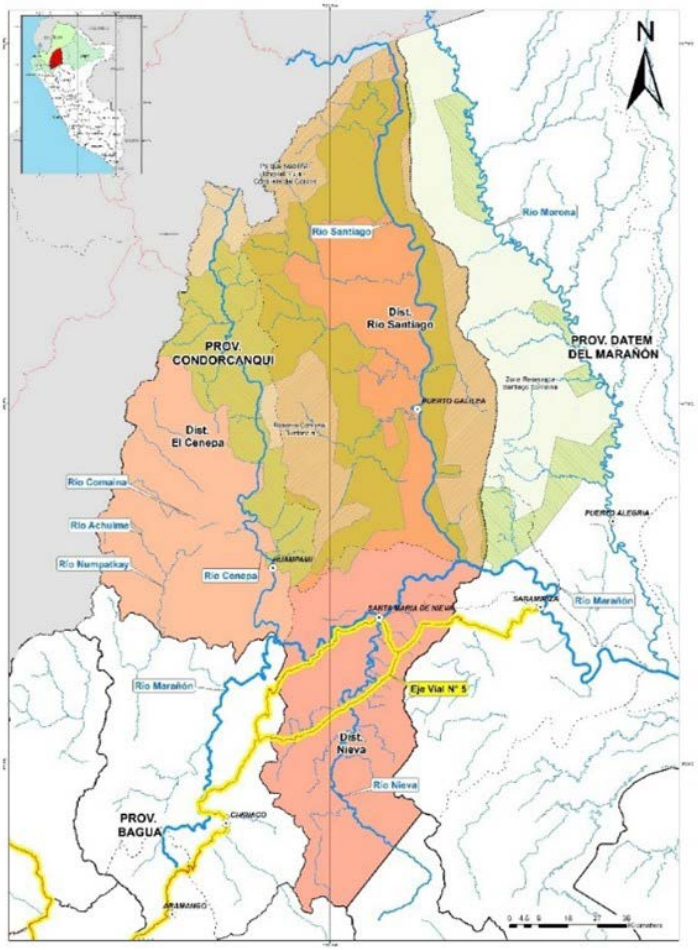

Figure 1. Geographic location of gamitana collection sites in the districts of Nieva and Río Santiago, Condorcanqui province, Amazonas, Peru.

with 36 g/L sucrose (HiMedia®, India) and 0.8 g/L Congo red (Oxoid®, USA), and incubated at 37 °C for 48 h. After incubation, black colonies were considered indicative of biofilm production.

BACTERIAL ANTAGONISM AGAINST PATHOGENIC BACTERIA

Antagonistic activity against fish pathogenic bacteria (*Aeromonas hydrophila, Lactococcus garvieae,* and *Streptococcus agalactiae*), previously isolated from outbreaks in Peruvian aquaculture, was assessed using the agar diffusion method described by Zatán *et al.* (2018). Pathogens were subcultured in TSB (HiMedia®, India) at 30 °C for 48 h, after which 100 µL of each suspension was spread onto Nutrient Agar plates (HiMedia®, India). Wells were aseptically created in the agar, and 30 µL of each isolate (1 × 108 cfu/mL) was added. Plates were incubated at 30 °C for 48 h. Antagonistic activity was quantified by measuring the diameter (mm) of inhibition halos around the wells.

PROTEOLYTIC AND AMYLOLYTIC ASSAYS

Proteolytic activity was assessed following Reda *et al.* (2018) using Skim Milk Agar (5%) (HiMedia®, India) as a protein substrate. Isolates were streaked onto plates and incubated at 30 °C for 48 h. The presence of clear halos surrounding the colonies indicated proteolytic activity. For

amylolytic activity, isolates were streaked onto Starch Agar (2.5%) (HiMedia®, India) and incubated at 30 °C for 48 h. Plates were subsequently flooded with Lugol's iodine solution (1%), and transparent zones surrounding the streaked areas were interpreted as positive for starch hydrolysis.

MOLECULAR IDENTIFICATION OF THE ISOLATES

Molecular identification was performed by sequencing the 16S rRNA gene. Bacterial DNA was extracted from liquid cultures using the boiling method described by Ribeiro *et al.* (2016). PCR amplification was conducted using primers 27F (5'-CCAGAATTCAGAGTTTGATCMTGGCTCA-3') and 1492R(5'-ACCAAGCTTTACGGYTACCTTGT TAGGACTT-3').

PCR products were visualized under blue light after staining with Safeview™ nucleic acid stain (ABM, Canada). Resulting nucleotide sequences were assembled using DNA Dragon (DNA Sequence Contig Assembler Software) and compared against the GenBank database using BLAST (National Centre for Biotechnology Information, USA).

STATISTICAL ANALYSIS

Statistical analyses for bile salt tolerance, pH resistance, and antagonistic activity were performed using one-way ANOVA in RStudio (version 3.2.5). Tukey´s honest significant difference (HSD) test was applied to compare means, and statistical significance was established at $P<0.05$.

RESULTS AND DISCUSSION

A total of 69 bacteria isolates were obtained from the intestines (n=40) and gills (n=29) of gamitana at different life stages and were subjected to further analysis. Hemolysis testing revealed alpha hemolysis (n=14), beta hemolysis (n=9), and gamma hemolysis (n=30), while 16 isolates did not grow. Hemolytic isolates were excluded from subsequent analysis due to their potential pathogenicity (Figure 2). Hemolysins

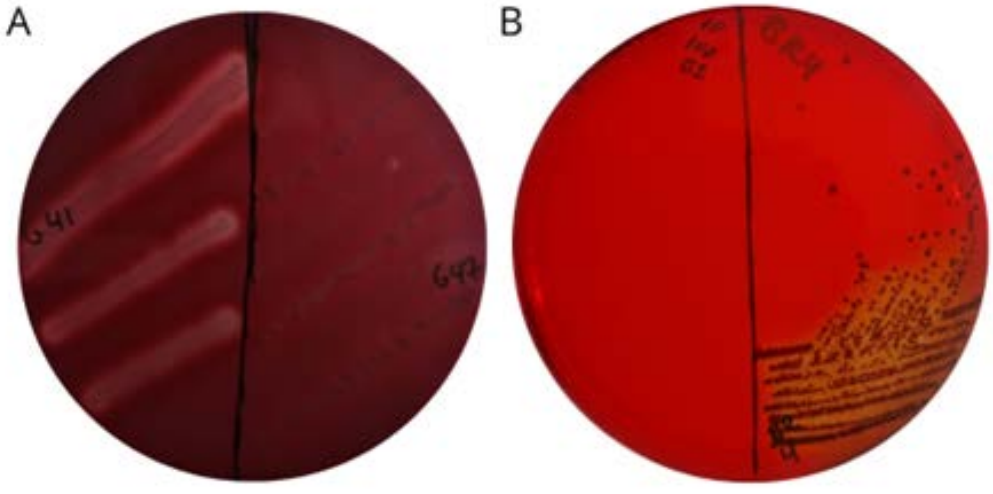

Figure 2. A: Beta-hemolysis (G41) and Gamma-hemolysis (G47). B: No biofilm production (IPIFA-B2) and biofilm production (BR4).

are known virulence factors capable of causing tissue damage in the host, making the exclusion of hemolytic bacteria essential in probiotic candidate selection (Kotzent *et al.*, 2021; Syahidah, 2021).

Acid and bile tolerance assays (Table 1; Figures 3–4) showed that only 12 isolates survived at least one acidic condition (pH 1–4), confirming their ability to withstand gastrointestinal acidity. Among these, isolates BRVG5T, BRVG2T, IPVG5T, and IPVG2T exhibited the greatest acid tolerance, surviving at pH 2–3 (5.4 ± 0.75% to 11.9 ± 1.1%) and demonstrating moderate survival at pH 4 (23.1 ± 3.07% to 36.4 ± 0.17%). These values were significantly higher than those of isolates IPIFAB2, IPIFAC1 and BRALA1, which survived only at pH 4 (31.0–36.4%) and failed to grow at pH ≤3. In the bile salt resistance assay, all isolates survived exposure, although degrees of tolerance varied. The most bile-resistant isolates (G22a, BRVG2T, BRVG5T, IPVG2T, IPVG5T, and G49) maintained high survival rates (≥ 90%) even at 0.8% bile, a concentration known to inhibit many bacteria. In contrast, isolates G23, IPIFAB2, and IPIFAC1 displayed markedly reduced viability (46–50%) at 0.8%. Although G49 and G50 tolerated bile, their lack of acid tolerance limits their probiotic potential. Overall, these findings

Table 1. Resistance to bile salts and low pH in potentially probiotic bacteria isolated from gamitana

Isolates code	Survival rate (%) at different conditions						
	pH concentration				Bile salts concentration		
	pH 4	pH 3	pH 2	pH 1	0.3%	0.5%	0.8%
G22a	105.3 ± 2.49^{a}	0 ± 0.00^{d}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	84.1 ± 0.93^{e}	89.7 ± 1.64^{c}	99.1 ± 0.93^{a}
G25	17.9 ± 0.15^{ef}	0.3 ± 0.04^{d}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	90.7 ± 0.33^{d}	80.85 ± 1.96^{e}	73.6 ± 0.19^{g}
BRVG2T	15.5 ± 0.55^{f}	7.0 ± 0.3^{a}	7.1 ± 0.63^{a}	11.9 ± 1.1^{a}	100.3 ± 0.15^{b}	96.0 ± 0.51^{b}	91.6 ± 0.30^{c}
BRVG5T	16.7 ± 0.66^{f}	5.4 ± 0.75^{b}	6.6 ± 0.4^{a}	11.0 ± 0.53^{a}	100.6 ± 0.15^{b}	94.4 ± 0.40^{b}	86.5 ± 0.50^{e}
IPVG5T	10.7 ± 0.60^{g}	5.4 ± 0.75^{b}	6.7 ± 0.67^{a}	11.1 ± 3.08^{a}	97.4 ± 1.10^{c}	99.0 ± 0.41^{a}	89.7 ± 0.61^{d}
IPVG2T	23.1 ± 3.07^{d}	5.6 ± 0.77^{b}	6.2 ± 0.80^{a}	11.2 ± 0.88^{a}	99.1 ± 0.30^{bc}	99.6 ± 0.31^{a}	97.4 ± 0.30^{b}
G50	1.0 ± 0.15^{h}	0.4 ± 0.06^{d}	0.1 ± 0.00^{b}	0.0 ± 0.00	0.9 ± 0.09^{g}	0.6 ± 0.02^{g}	0.9 ± 0.09^{j}
G23	21.6 ± 0.62^{de}	4.0 ± 0.34^{c}	6.0 ± 0.58^{a}	10.9 ± 0.50^{a}	85.0 ± 0.50^{e}	80.4 ± 0.41^{e}	46.7 ± 0.14^{i}
IPIFAB2	36.4 ± 0.17^{b}	0.0 ± 0.00^{d}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	112.4 ± 0.31^{a}	79.6 ± 0.25^{e}	46.7 ± 0.19^{i}
IPIFAC1	31.0 ± 2.04^{c}	0.0 ± 0.00^{d}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	78.6 ± 0.34^{f}	65.4 ± 0.17^{f}	50.0 ± 0.11^{h}
BRALA1	34.7 ± 2.77^{bc}	0.0 ± 0.00^{d}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	98.2 ± 0.88^{c}	87.3 ± 0.16^{d}	77.9 ± 0.24^{f}
G49	0.8 ± 0.11^{h}	0.0 ± 0.00^{d}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}	97.4 ± 0.14^{c}	98.3 ± 0.14^{a}	98.2 ± 0.33^{ab}

Values with different letters differ significantly ($p < 0.05$) according to Tukey´s test and one-way ANOVA.

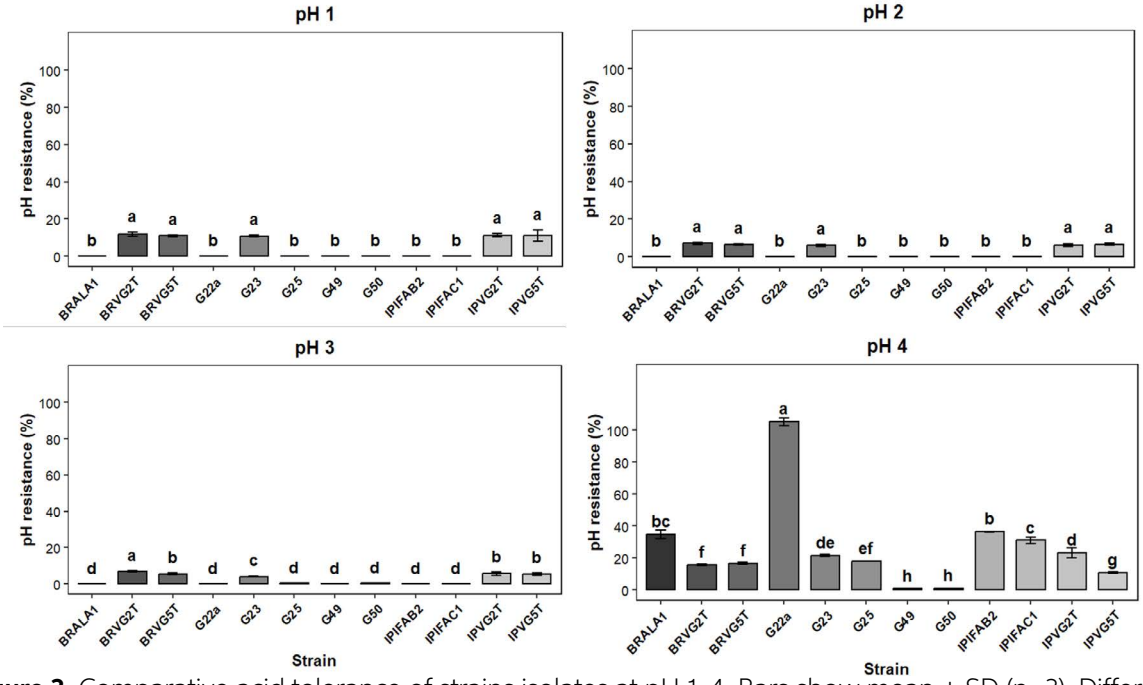

Figure 3. Comparative acid tolerance of strains isolates at pH 1-4. Bars show mean ± SD (n=3). Different letters indicate significant differences among strains within each pH (Tukey's HSD, p< 0.05).

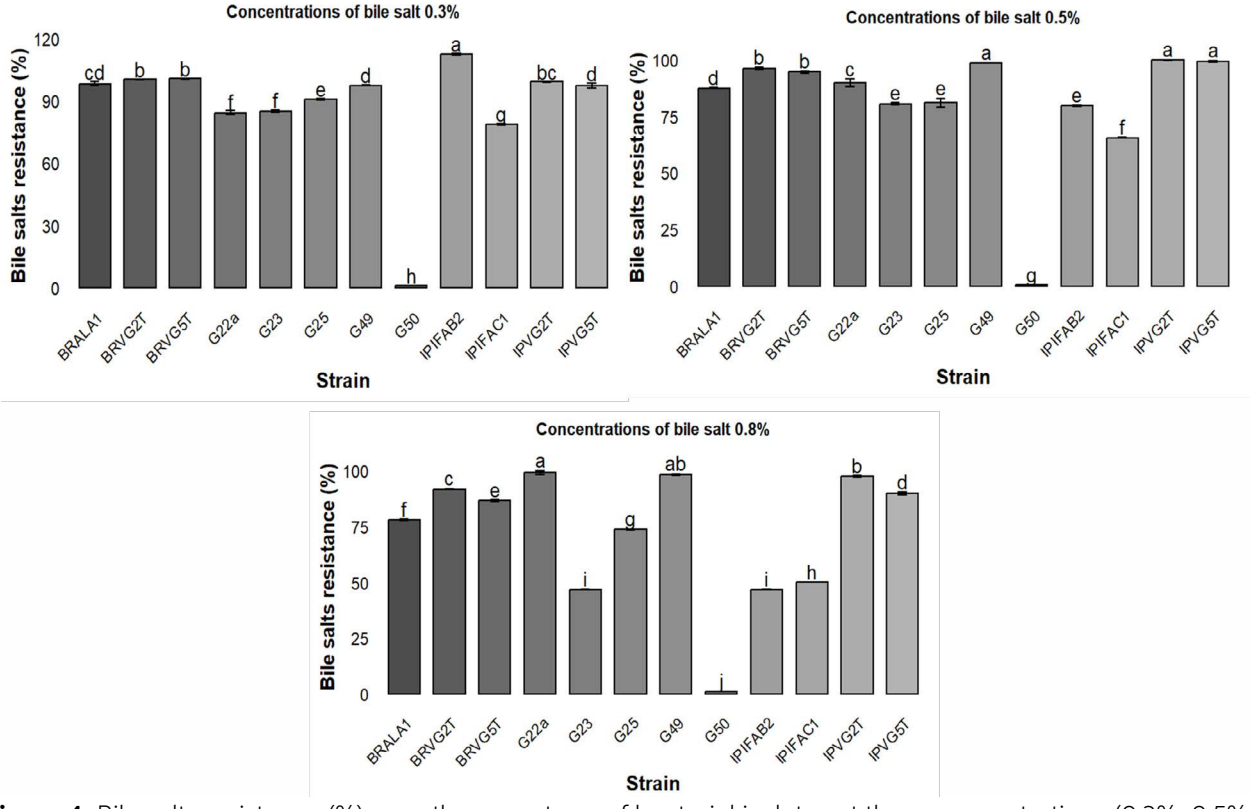

Figure 4. Bile salts resistance (%) growth percentage of bacterial isolates at three concentrations (0.3%, 0.5% y 0.8%). Bars show mean ± SD (n=3). Different letters indicate significant differences among strains within each bile salt concentration (Tukey's HSD, p< 0.05).

indicate that several isolates can withstand conditions mimicking the harsh, bile-rich and acidic gastrointestinal environment, a key requirement for probiotic functionality (Begley *et al.*, 2006)

Antibiotic susceptibility testing revealed varied resistance patterns among isolates; however, five isolates were susceptible to all six antibiotics tested, whereas seven exhibited resistance to at least one antibiotic (Table 2). Isolation of antibiotic-susceptible strains is particularly relevant in Peruvian aquaculture. The antibiotic resistance patterns observed here differ from those reported by Kotzent *et al.* (2021), who identified *Pediococcus, Lactococcus, Enterococcus,* and *Staphylococcus* isolates from gamitana with resistance to multiple antibiotics including ceftriaxone, doxycycline, streptomycin, gentamicin, oxytetracycline, penicillin, sulfamethoxazole/trimethoprim, and vancomycin. Similar findings were reported by do Vale Pereira *et al.* (2017) and Castañeda *et al.* (2018), who isolated multidrug-resistant strains from *Arapaima gigas* and *Oreochromis niloticus*, respectively. It is essential to highlight that antibiotic-resistant bacteria are undesirable as probiotic candidates because of the potential transfer of antibiotic resistance genes to other bacteria (Gueimonde *et al.*, 2013; Li *et al.*, 2020).

Biofilm-forming ability, an important trait that promotes colonization and persistence within the host, was observed in six isolates, evidenced by black colonies on Congo red agar (Figure 2; Table 3). Biofilms are structured microbial communities embedded in a self-produced extracellular matrix that confer enhanced resistance to environmental stressors (Mgomi *et al.*, 2023; Salas-Jara *et al.*, 2016).

Antagonistic activity is another key probiotic characteristic, as probiotic bacteria can inhibit or suppress pathogen growth through the production of antimicrobial compounds such as hydrogen peroxide, nitric oxide, and bacteriocins (Dobson *et al.*, 2012). Furthermore, probiotics have been used in aquaculture production against both Gram-positive and Gram-negative pathogens (Pereira *et al.*, 2022). In this study, five isolates displayed antagonistic activity against *L. garvieae* (Table 3). Among them, isolates IPIFAB2, IPIFAC1

Table 2. Antibiotic susceptibility in potentially probiotic bacteria isolated from gamitana

Isolates code	CL (30 µg)		EM (15 µg)		GEN (10 µg)		OT (30 µg)		PEN (10 µg)		TE (30 µg)	
	Halo (mm)	Result	Halo (mm)	Result	Halo (mm)	Result	Halo (mm)	Result	Halo (mm)	Result	Halo (mm)	Result
G22a	28.0	S	34.0	S	25.7	S	24.3	S	14.7	R	18.3	I
G25	23.7	S	24.7	S	14.7	R	22.3	S	22.0	S	23.7	S
BRVG2T	31.0	S	17.3	I	20.0	S	27.0	S	15.3	I	16.7	I
BRVG5T	27.3	S	17.0	I	18.7	I	22.7	S	15.0	I	21.7	S
IPVG5T	25.7	S	14.3	R	18.3	I	24.3	S	0.0	R	21.0	S
IPVG4T	30.7	S	16.7	I	17.7	I	25.7	S	15.0	I	24.7	S
G50	13.7	R	0.0	R	16.7	I	8.3	R	0.0	R	0.0	R
G23	21.3	S	25.0	S	15.7	I	23.0	S	17.7	I	22.3	S
IPIFAB2	23.7	S	30.0	S	25.0	S	16.0	I	15.0	I	15.0	I
IPIFAC1	16.7	I	25.7	S	27.0	S	16.0	I	13.0	R	14.0	R
BRALA1	27.0	S	24.0	S	23.0	S	13.0	R	15.0	I	16.0	I
G49	22.7	S	10.0	R	15.3	I	18.7	I	0.0	R	17.0	I

CL= Chloramphenicol, EM= Erythromycin, GEN= Gentamicin, OT= Oxytetracycline, PEN= Penicillin, TE= Tetracycline. S= Sensitive, I= Intermediary, R= Resistant

Table 3. Antagonistic activity of pathogenic bacteria, biofilm and enzymatic production in potentially probiotic bacteria isolated from gamitana

Isolates code	Species	Diameter of inhibition zones (mm)			Biofilm	Amylase	Protease
		Streptococcus agalactiae	*Lactococcus garvieae*	*Aeromonas hydrophila*			
IPIFAB2	*Lactobacillus brevis*	0.0 ± 0.00	3.2 ± 0.15^{b}	0.0 ± 0.00	POS	NEG	POS
IPIFAC1	*Lactobacillus brevis*	0.0 ± 0.00	3.9 ± 0.1^{a}	0.0 ± 0.00	POS	NEG	POS
BRALA1	*Lactobacillus brevis*	0.0 ± 0.00	2.3 ± 0.1^{c}	0.0 ± 0.00	POS	NEG	POS
G25	*Lactococcus garvieae*	0.0 ± 0.00	1.9 ± 0.12^{d}	0.0 ± 0.00	POS	NEG	NEG
G49	*Klebsiella variicola*	0.0 ± 0.00	0.0 ± 0.00^{f}	0.0 ± 0.00	POS	POS	NEG
G23	*Lactococcus gravieae*	0.0 ± 0.00	1.2 ± 0.21^{c}	0.0 ± 0.00	POS	NEG	NEG

Abbreviation: NEG= Negative result; POS= Positive result. Data are means of three replicate values ± standard error of the mean. Values with different letters differ significantly ($p< 0.05$) according to Tukey´s test and one-way ANOVA.

and BRALA1 showed significantly stronger inhibition (3.2 ± 0.15b to 3.9 ± 0.1a mm) compared to G25 and G23 (1.2 ± 0.21c to 1.9 ± 0.12d mm), whereas G49 exhibited no inhibition. This antagonistic activity is likely mediated by bacteriocins such as brevican, a heat-stable peptide previously reported to inhibit *L. garvieae* (Fhoula *et al.*, 2013). Similar antimicrobial compounds have been characterized in fish associated probiotic candidates using MALDI-TOF/TOF and bacteriocin-encoding gene detection (Castañeda *et al.*, 2024; Feria *et al.*, 2019). Kotzent *et al.* (2021) similarly reported antagonistic activity of autochthonous isolates against *L. garviae*, although their isolates also inhibited *A. hydrophila*, which was not observed in our study. Because pathogens such as *Aeromonas* spp., *Streptococcus* spp., and *L. garvieae* are frequently encountered in gamitana aculture system and represent emerging threats in South America (Ferrante *et al.*, 2020; Gallani *et al.*, 2020, Egger, 2023), identifying probiotic strains capable of inhibiting these pathogens is of high importance.

Enzymatic activity is another desirable probiotic trait, as microbial enzymes contribute to digestion and nutrient utilization by breaking down complex macromolecules. Enzymes such as lipases, phytases, amylases, cellulases, and proteases play major roles in this process, and probiotics may also stimulate endogenous enzyme production (Wuertz *et al.*, 2021). In this study, three isolates (IPIFAB2, IPIFAC1, and BRALA1) exhibited proteolytic activity, while G49 displayed amylolytic activity (Table 3). Similar results were reported by Wulaningtyas & Agustitni (2023), who isolated *Enterococcus* spp. with high protease activity from gamitana.

Molecular identification showed that the isolates shared high similarity with *Lactobacillus brevis, Lactococcus garvieae*, and *Klebsiella variicola* (Table 4). *L. brevis,* like other lactobacilli, has Qualified Presumption of Safety (QPS) status and is widely used in the food fermentation industry (Feyereisen *et al.*, 2019). Dietary supplementation with *L. brevis* has demonstrated improvements in growth performance and immune function in species such as *Oncorhynchus mykiss* (Niazi *et al.*,

Table 4. Identification of potentially probiotic bacteria isolated from intestine and gill of gamitana

Location	Stage	Source	Code	Specie	NCBI Accession number	Similarity %
Nieva	Adult	Intestine	IPIFAB2	*Lactobacillus brevis*	MT640328.1	99.75
Nieva	Adult	Intestine	IPIFAC1	*Lactobacillus brevis*	MT640328.1	99.83
Villa Gonzalo	Fingerling	Gill	BRALA1	*Lactobacillus brevis*	MT640328.1	99.92
Villa Gonzalo	Juvenile	Intestine	G25	*Lactococcus garvieae*	MT611574.1	99.49
Villa Gonzalo	Fingerling	Gill	G49	*Klebsiella variicola*	MN428217.1	99.66
Villa Gonzalo	Juvenile	Intestine	G23	*Lactococcus garvieae*	MT611574.1	100.00

2023) and Sander lucioperca (Faeed *et al.*, 2022), highlighting its probiotic potential. Although *L. garvieae* is a recognized pathogen in aquaculture, it has also been isolated from the intestinal microbiota of healthy fish (Patel *et al.*, 2020; Mohideen *et al.*, 2023). Additionally, Abdelfatah & Mahboub (2019) demonstrated that dairy-origin *L. garvieae* could inhibit *S. aureus* in *O. niloticus*, suggesting that some strains may have probiotic applications although safety must be rigorously assessed.

Integrated quantitative and functional analyses indicate that *L. brevis* isolates possess superior probiotic potential compared to *L. garvieae* and *Klebsiella variicola.* Specially, *L. brevis* showed high bile salt tolerance (≥ 78% survival at 0.8%), moderate acid tolerance (31–36% at pH 4), strong antagonistic activity against *L. garvieae* (2.3–3.9 mm inhibition zones; $p<0.05$), biofilm forming ability, and proteolytic activity traits essential for gastrointestinal colonization and contribution to protein digestion. In contrast, *L. garvieae* and *Klebsiella variicola* displayed limited probiotic attributes, including weak or absent antagonism, poor acid tolerance, and lack of proteolytic activity. Overall, *L. brevis* consistently outperformed the other isolates, highlighting its potential as an effective autochthonous probiotic for *C. macropomum* aquaculture, with implications for improved health status, disease prevention, reduced antibiotics use, and enhanced sustainability (Begley at al., 2006).

This study is limited by its exclusive use of in vitro assays; therefore, in vivo colonization, persistence, and immunomodulatory effects remain unverified. Protective efficacy was not assessed through pathogen-challenge trials, and optimal probiotic dosage or delivery strategies were not evaluated.

Future research should include in vivo feeding trials to validate probiotic effects, whole-genome sequencing to confirm antimicrobial peptide genes and absence of virulence factors, microbiome analyses to evaluate gut colonization, and scale-up studies for commercial formulation development.

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