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Research Article

Isolation and identification of autochthonous potential probiotic candidates from the gastrointestinal tract of Caspian striped mullet (*Mugil cephalus*)

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Keywords

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Gene sequencing,
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Probiotic

Abstract

This study aimed to screen and identify potential probiotic strains from the gastrointestinal tract of Caspian striped mullet (*Mugil cephalus*). Eight out of forty isolates showed antibacterial activities against fish-associated pathogens: *Yersinia ruckeri*, *Salmonella enterica*, *Escherichia coli* O157:H7, and *Staphylococcus aureus*. Biochemical tests showed that 50% of the isolates were capable of glucose fermentation, 75% fermented arabinose, 37.5% and 62.5% were able to metabolize raffinose and mannitol, respectively. None of the eight isolates demonstrated the ability to ferment all tested sugars. The isolates demonstrated tolerance to a high bile concentration (4000 mg/L) as well as to mildly acidic and alkaline pH conditions. Three isolates exhibited100% sequence identity with *Carnobacterium maltaromaticum.* One of the isolates exhibited 99% sequence identity with *Carnobacterium divergens*. Two isolates exhibited 71% sequence identity with Weissella koreensis and other Weissella species. Sequencing analysis revealed that the fish gut isolates are closely related to lactic acid bacteria, particularly *C. maltaromaticum* and *C. divergens*. The results indicate that the isolates have potential as probiotics in aquaculture due to ability to enhance gut health and suppress pathogenic bacteria.

Article info

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Introduction

Fish paly a vital role in the aquaculture industry and progress in the productivity largely depends on the effective control of emerging diseases. Emerging diseases in fish have resulted in the widespread use of antimicrobials in recent years, which will lead to the spread of antimicrobial-resistant pathogens in the future (Miller and Harbottle, 2018, Marijani, 2022). Today, the aquaculture industry uses traditional methods and antibiotics as well as synthetic chemicals to combat diseases and it is in urgent need of developing more suitable alternative methods to inhibit fish pathogens and reduce the accumulation of antibiotic residues in fish tissue (Yilmaz *et al.,* 2022). These methods, mainly, include phage therapy (Culot *et al.,* 2019, Pereira *et al.,* 2022, Mazlumi *et al.,* 2022) and probiotic therapy (Pepoyan *et al.,* 2017, Balayan *et al.,* 2019). Among the bacteria used for probiotic therapy lactic acid bacteria (LAB) are the most commonly used and well-studied. LAB are considered effective probiotics due to their ability to produce lactic acid, which helps maintain an acidic environment that inhibits the growth of harmful pathogens in the gut. They are also known for their beneficial effects on gut health, immune function, and overall well-being. LAB include various strains of Lactobacillus, Bifidobacterium, and Streptococcus species. LAB are the inhabitants of the digestive tract of aquatic animals (Feliatra *et al.,* 2018), most of which having the necessary properties to be used as probiotics in the aquaculture industry worldwide (Soltani *et al.,* 2019). Another advantage of using probiotics in the aquatic animal diet is that probiotics improve the health of aquatic animals without causing negative side effects (Pérez-Sánchez *et al.,* 2018). Proper nourishing of aquatic animals directly affects growth adequacy and the development of active immunity. Studies have shown that the gut microflora plays an important role in the proper growth and active defense against pathogens in humans and animals. Probiotics isolated from aquatic animals are transported through water and other living organisms and exert their vital function once they reach the host's intestine. Suitable places for the growth of microorganisms in the aquatic host body include the skin, gills, and digestive tract (Dawood *et al.,* 2019). Therefore, the content of the intestine is a valuable source of microorganisms with probiotic properties that can naturally be used as a food supplement. The gut microbiome plays an important role in the growth, behavior, digestion, health, and immunity of fish (Ringø *et al.,* 2020).

Approximately, 90% of the different microorganisms in the gut of fish belong to *Proteobacteria*, *Firmicutes,* and *Bacteroidetes* class (Johny *et al.,* 2021). Probiotic potential in various bacterial strains has been shown to be associated with cell surface properties such as auto-aggregation capacity, cell surface hydrophobicity, and biofilm formation ability. Cell surface properties are widely used to characterize and select probiotic strains in vitro (Dlamini *et al.,* 2019, Pepoyan *et al.,* 2020). Adhesion to the intestinal epithelium is one of the main criteria for selecting probiotics. This ability may increase the survival of probiotics in the gastrointestinal tract, thereby allowing the bacteria to exert their beneficial effects on host health (Okochi *et al.,* 2017). The first step in adhesion is likely to be auto-aggregation, which forms a barrier that prevents other bacteria from adhering to the intestinal epithelium (Saito *et al.,* 2019). Another characteristic that affects the ability of bacteria to adhere to intestinal epithelial cells is cell surface hydrophobicity. Evidence suggests that bacteria with higher hydrophobicity have a greater ability to attach to intestinal epithelial cells (de Souza *et al.,* 2019). Therefore, auto-aggregation, cell surface hydrophobicity, and coagulation are important and significant characteristics that provide potential advantages for microorganisms during colonization in the intestine.

Since modern aquaculture faces two major challenges: 1) reducing the amount of water used for cultivation 2) increasing the production efficiency per production unit, it is likely that producers will increase the density of the production unit to meet market demand, which creates great stress for the aquaculture animals (Hoseinifar *et al.,* 2020, Van Doan *et al.,* 2020). Based on studies conducted, the benefits of probiotics and their essential role in improving health, enhancing resistance against disease, higher immunity, better growth performance, intestinal epithelial barrier integrity, gut microbiome and even quality of water have been demonstrated. Also, the practical use of probiotics in aquaculture diets can help minimize the adverse effects associated with the presence of living organisms (Khademzade *et al.,* 2020, El-Saadony *et al.,* 2021). In this regard, this study investigated the biochemical properties and antimicrobial activity of potential probiotics isolated from the digestive tract of Caspian striped mullet (*Mugil cephalus*) caught from Caspian Sea (Mazandaran, Iran).

Materials and methods

Chemicals and materials

All the chemicals and culture media were obtained from Scharlau Chemical Co. (Barcelona, Spain). DNA extraction kit was purchased from Sinacolon Co. (Tehran, Iran)

Sampling

Forty striped Caspian Sea grey mullet fish (*Mugil cephalus*) were purchased from the fish market of Fereydunkenar (Mazandaran, Iran). The freshly caught, fully healthy fish, with an average weight of 800–1000 grams, were immediately transferred to the laboratory in ice boxes.

Isolation and cultivation of potential probiotics

The gastrointestinal tract of the fish was separated carefully, homogenized, and diluted to 1:10 using phosphate-buffered saline (PBS, pH=7.5). The prepared dilutions were cultured onto MRS (DE MAN, ROGOSA and SHARPE) agar plates in triplicate and incubated at 25°C for 3 to 5 days under anaerobic condition. After this period, oxidase negative and catalase negative colonies with distinct shapes and appearances were selected and cultured in MRS broth.

Carbohydrate fermentation test by potential probiotics

The solutions of 2% glucose, mannitol, arabinose, and raffinose were separately prepared in MRS broth, sterilized using 0.22 µm syringe filters, and adjusted to a final volume of 10 ml. For each isolate, all carbohydrate solutions were prepared followed by incubation for 48 hours at 25±1°C. Carbohydrate fermentation was evaluated using bromothymol blue that turns yellow and blue in weakly acidic solutions and weakly basic solutions, respectively (Karami *et al.,* 2017).

pH tolerance of potential probiotics

To assess pH tolerance, bacterial suspensions equivalent to 4 McFarland unit were prepared for each strain. Then, 10 µL of each suspension was added to 990 µL of phosphate-buffered saline at different pH levels (1.5, 3.0, 7.5, and 9), and incubated at 28°C for 1.5 h. To evaluate survival rate after incubation period, the suspensions were cultured on MRS agar and cell enumeration was conducted after 48 h at 25°C (Reda *et al.,* 2018).

Bile salt tolerance of potential probiotics

To evaluate bile salt tolerance in isolated potential probiotics, a suspension of each isolate equivalent to 4 McFarland units was prepared. Then, 10 µL of each suspension was transferred to 990 µL of phosphate-buffered saline with different bile salt concentrations (2000, 3000, and 4000 ppm) followed by incubation at 37°C for 1h. After incubation period in the presence of bile salts, 10 µL of the mixture was cultured on MRS agar, incubated at 25±1°C for 48 h (Reda *et al.,* 2018).

Acid tolerance of potential probiotic isolates

Potential probiotic isolates were grown in MRS broth under anaerobic conditions at 37°C for 48 h and the absorbance of each isolate was s measured at 620 nm using a spectrophotometer (Nova Spec II, Pharmacia). Then, 1 ml aliquots (7.05 log cfu/mL) of each culture were transferred to MRS broth tube adjusted to pH 3.0 with 5 N HCl. After 24h of anaerobic incubation at 37°C, any tubes showing turbidity, indicating bacterial growth, will be considered acid-resistant. The bacterial count will be measured using a spectrophotometer at 620 nm (Reda *et al.,* 2018).

Antimicrobial activity of isolated potential probiotics against food pathogens

The inhibitory activity of potential probiotic isolates on selected food and fish pathogens was determined by the well-diffusion method. For the agar well diffusion assay, an overnight culture of the pathogen strains (*Escherichia coli, Staphylococcus aureus, Salmonella entrica* and *Yersinia ruckeri*) was used to inoculate (10^6 cfu/ml) to BHI (Brain Heart Infusion) agar at 37 °C. Wells of 5 mm diameter were made in agar plates and 50 µL of probiotic culture supernatant that expected containing antibacterial effects was transferred to each well. Inhibitory zone of potential probiotics was measured upon 24 h incubation at 37°C. Enrofloxacin was used as the control antibiotic (Gueimonde *et al.,* 2013).

Molecular test and 16S rRNA region sequencing for accurate strain identification

Bacterial DNA was extracted using a DNA extraction kit. The PCR (polymerase chain reaction) reaction mixture included the master mix (containing DNA polymerase), $MgCl_2$ enzyme buffer, and 4 nucleotides (dNTPs) at 12.5 µL, along with 5 µL of template DNA and 1 µL each of specific forward (AGA GTT TGA TCC TGG CTC AG) and reverse universal primers (GGT TAC CTT GTT ACG ACT T) targeting the bacterial 16S rDNA region. The final volume was adjusted to 25 µL using distilled water. The PCR process involves an initial denaturation step at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and a final extension step at 72°C for 2 minutes Electrophoresis on an agarose gel was conducted to confirm the accuracy of the PCR product and determine the amplicon length (Valcheva *et al.,* 2007).

Statistical analysis

SPSS v22.00 software was used for data analysis and comparison of means. The data obtained from bacterial tolerance against bile salt, acid, different pH values and the diameter of inhibition zones were analyzed using Tukey's test and Two-Way ANOVA. The significance levels of $p<0.01$ and $p<0.05$ were considered in statistical analyses.

Results

Morphological feature

Having been catalase and oxidase negative, Gram-stained colonies were observed for morphological assessments using a light microscope. The colonies showed the form of bacilli with purple color that represents Gram-positive bacteria.

Carbohydrates fermentation

The results of biochemical tests using sugar-based media are presented in Table 1. According to the data, 50% of the isolates were capable of glucose fermentation, 75% fermented arabinose, 37.5% and 62.5% of them were able to metabolize raffinose and mannitol, respectively. Among 8 tested isolates, none were capable of fermenting all sugars.

Table 1: The results of biochemical evaluation of isolated potential probiotics from gastrointestinal tract of Caspian Sea grey mullet fish using sugar-based media.

Sample*	Glucose	Arabinose	Raffinose	Mannitol
A	+	-	-	-
B	-	-	-	+
C	-	+	+	+
D	-	+	-	+
E	+	+	-	+
F	+	+	+	-
G	+	+	-	+
H	-	+	+	-

*Represents the isolated found in gastrointestinal tract of Caspian Sea grey mullet fish.

pH resistance

The survival rate of potential probiotics isolated from the digestive tract of Caspian mullet is listed in Table 2. A significant higher survival rate was observed in strain A at different pHs, and the highest survival rate in this strain was at pH 7.5, no survival was observed rate was at pH 1.5 (p<0.05). The survival rate of isolate B was affected at different pHs, and the highest survival rate was observed at pH 7.5 and 9. Also, no growth was observed at pH 1.5 (p<0.05). In isolates C, D, E and H, the lowest survival rate (no growth) was at pH 1.5, but there was no significant difference in the survival rate at other pHs (3.0, 7.5, and 9.0). In isolate F, the highest survival rate was found at pH 3.0 (p<0.05). In isolate G, the highest survival rate was obtained at pHs 9.0.

Table 2: pH resistance of isolated potential probiotics from gastrointestinal tract of Caspian Sea grey mullet fish.

Sample*	pH=1.5	pH=3.0	pH=7.5	pH=9
A	No growth	$2.86±0.05^{aα}$	$2.96±0.00^{bα}$	$2.74±0.00^{aα}$
B	No growth	$2.60±0.00^{aα}$	$2.77±0.05^{bα}$	$2.75±0.00^{bα}$
C	No growth	$2.63±0.01^{aα}$	$2.74±0.00^{aα}$	$2.65±0.01^{aα}$
D	No growth	$2.68±0.05^{aα}$	$2.71±0.00^{aα}$	$2.65±0.00^{aα}$
E	No growth	$2.77±0.01^{aα}$	$2.77±0.05^{aα}$	$2.69±0.01^{aα}$
F	No growth	$2.35±0.02^{aβ}$	$2.19±0.00^{bβ}$	No growth
G	No growth	$2.33±0.00^{aβ}$	$2.56±0.05^{bα}$	$2.87±0.00^{bβ}$
H	No growth	$2.69±0.09^{aα}$	$2.76±0.00^{aα}$	$2.74±0.00^{aα}$

*Represents the isolated found in gastrointestinal tract of Caspian Sea grey mullet fish.
[a] Lowercase letters indicate significant differences (p<0.01) in each row.
[α] Latin letters indicate significant differences (p<0.01) in each column.

Survival in bile salt

The survival rate of potential probiotics isolated from the gut of Caspian mullet is presented in Table 3. A significant higher survival rate was observed in isolates B, C, and D compared to other strains at different concentrations of bile salt (p<0.05) and the highest survival rate was found in C strain (p<0.05). The survival rate of isolates A and H was affected dramatically at different concentrations of bile salt, and the lowest survival rate was obtained at the concentration of 4000 ppm in strain A (p<0.05). Notably, isolate F failed to grow in the presence of bile salts, further highlighting the varying bile salt tolerance among probiotic strains.

Table 3: The results of bile salt resistance of isolated potential probiotics from gastrointestinal tract of Caspian Sea grey mullet fish.

Sample*	Concentrations of bile salt (ppm)			
	Control	2000	3000	4000
A	$3.56±0.01^{aα}$	$2.98±0.05^{bα}$	$1.47±0.00^{cα}$	$0.689±0.005^{dα}$
B	$3.69±0.08^{aα}$	$2.10±0.00^{bα}$	$1.85±0.05^{cα}$	$1.19±0.00^{dβ}$
C	$3.50±0.05^{aα}$	$3.03±0.01^{aβ}$	$2.81±0.00^{bβ}$	$1.34±0.01^{cβ}$
D	$3.44±0.05^{aα}$	$2.8±0.05^{bα}$	$2.01±0.00^{cβ}$	$1.15±0.0^{dβ}$
E	$3.81±0.00^{aα}$	$2.52±0.01^{bα}$	$1.70±0.05^{cα}$	$0.921±0.04^{dα}$
F	$3.25±0.05^{aα}$	No growth	No growth	No growth
G	$3.49±0.05^{aα}$	$2.33±0.00^{bα}$	$1.57±0.05^{cα}$	$1.0±0.02^{dβ}$
H	$3.16±0.05^{aα}$	$2.33±0.09^{bα}$	$1.29±0.00^{cα}$	$0.771±0.15^{dα}$

*Represents the isolated found in gastrointestinal tract of Caspian Sea grey mullet fish.
[a] Lowercase letters indicate significant differences (p<0.05) in each row.
[α] Latin letters indicate significant differences (p<0.05) in each column.

Acid resistance

Acid tolerance (pH=3) of potential probiotics isolated from the gastrointestinal tract of Caspian mullet is presented in Table 4. A significant higher survival and growth rate was observed in isolates B, C, G, and H compared to other ones, with the highest growth rate being that of isolate B ($p<0.01$). The highest growth rate was found in B isolate ($p<0.01$) and isolate E showed the lowest rate ($p<0.01$).

Table 4: The results of acid resistance of isolated potential probiotics from gastrointestinal tract of Caspian Sea grey mullet fish.

Sample*	0 h	24 h
A	7.25±0.01 $^{a\alpha}$	7.98±0.05 $^{b\beta}$
B	7.11±0.08 $^{a\alpha}$	8.10±0.00 $^{b\beta}$
C	7.50±0.05 $^{a\alpha}$	8.03±0.01 $^{b\beta}$
D	7.44±0.05 $^{a\alpha}$	7.80±0.05 $^{b\beta}$
E	7.81±0.00 $^{a\alpha}$	7.52±0.01 $^{a\alpha}$
F	7.25±0.05 $^{a\alpha}$	7.38±0.01 $^{a\alpha}$
G	7.49±0.05 $^{a\alpha}$	8.33±0.00 $^{b\gamma}$
H	7.16±0.05 $^{a\alpha}$	8.25±0.09 $^{b\gamma}$

*Represents the isolated found in gastrointestinal tract of Caspian Sea grey mullet fish.

^{a} Lowercase letters indicate significant differences ($p<0.01$) in each row.

$^{\alpha}$ Latin letters indicate significant differences ($p<0.01$) in each column.

Antibacterial activity

The antibacterial properties of 8 different isolated potential probiotics from gastrointestinal tract of Caspian Sea grey mullet fish are presented in Tables 5 and 6. In brief, the isolates indicated stronger antagonistic properties against *Salmonella entrica* and *Staphylococcus aureus* compared to enrofloxacin employed in the study pointing to their broad spectrum of activity against both Gram-negative and Gram-positive pathogens ($p<0.01$). In case of *E.coli* and *Yersinia ruckeri*, *the isolates showed lower antimicrobial activity in comparison to* enrofloxacin ($p<0.01$). Conversely, however, the potential probiotic isolates in this study had comparatively lower activity than enrofloxacin against *E.coli* and *Y. ruckeri*, suggesting some specificity in their antimicrobial activity ($p<0.01$).

Table 5: The results antibacterial activity of isolated potential probiotics from gastrointestinal tract of Caspian Sea grey mullet fish against *Yersinia ruckeri* and *Salmonella entrica*.

Sample*	Growth inhibitory zone by LAB isolates against *Yersinia ruckeri*	Growth inhibitory zone by enrofloxacin against *Yersinia ruckeri*	Growth inhibitory zone by LAB isolates against *Salmonella entrica*	Growth inhibitory zone by enrofloxacin against *Salmonella entrica*
A	No growth$^{a\beta}$	2.00±0.05 $^{a\alpha}$	4.50±0.05 $^{d\beta}$	4.00±0.05 $^{a\alpha}$
B	No growth$^{a\beta}$	2.00±0.00 $^{a\alpha}$	3.50±0.06 $^{c\beta}$	4.00±0.05 $^{a\alpha}$
C	1.50±0.01 $^{c\beta}$	2.00±0.01 $^{a\alpha}$	No growth$^{a\beta}$	4.20±0.01 $^{a\alpha}$
D	0.50±0.01 $^{b\beta}$	2.50±0.05 $^{a\alpha}$	5.50±0.02 $^{f\beta}$	5.00±0.05 $^{b\alpha}$
E	2.30±0.01 $^{e\alpha}$	2.50±0.01 $^{b\alpha}$	7.50±0.01 $^{g\beta}$	4.50±0.04 $^{c\alpha}$
F	1.50±0.00 $^{c\beta}$	2.00±0.01 $^{a\alpha}$	5.50±0.01 $^{f\beta}$	4.05±0.01 $^{a\alpha}$
G	2.00±0.00 $^{d\beta}$	2.5±0.01 $^{b\alpha}$	5.00±0.01 $^{e\beta}$	4.00±0.0 $^{a\alpha}$
H	1.75±0.07 $^{c\beta}$	2.50±0.09 $^{b\alpha}$	1.50±0.09 $^{b\beta}$	4.00±0.03 $^{a\alpha}$

*Represents the isolated found in gastrointestinal tract of Caspian Sea grey mullet fish.

^{a} Lowercase letters indicate significant differences ($p<0.01$) in each row for each microorganism, separately.

$^{\alpha}$ Latin letters indicate significant differences ($p<0.01$) in each column for each microorganism, separately.

Table 6: The results antibacterial activity of isolated potential probiotics from gastrointestinal tract of Caspian Sea grey mullet fish against *E. coli* and *Staphylococcus aureus*.

Sample*	Growth inhibitory zone by LAB isolates against *E.coli*	Growth inhibitory zone by enrofloxacin against *E.coli*	Growth inhibitory zone by LAB isolates against *Staphylococcus aureus*	Growth inhibitory zone by enrofloxacin against *Staphylococcus aureus*
A	$4.00\pm0.01^{d\beta}$	$3.00\pm0.01^{a\alpha}$	$6.00\pm0.00^{g\beta}$	$4.50\pm0.03^{c\alpha}$
B	$4.50\pm0.00^{c\beta}$	$5.00\pm0.00^{e\alpha}$	$5.00\pm0.02^{e\beta}$	$4.00\pm0.00^{b\alpha}$
C	$3.00\pm0.00^{b\beta}$	$4.00\pm0.04^{c\alpha}$	No growth$^{a\beta}$	$4.70\pm0.00^{c\alpha}$
D	$4.50\pm0.01^{e\alpha}$	$4.50\pm0.05^{d\alpha}$	$4.50\pm0.00^{d\alpha}$	$4.50\pm0.07^{c\alpha}$
E	$3.50\pm0.01^{c\beta}$	$4.50\pm0.0^{d\alpha}$	$6.50\pm0.01^{h\beta}$	$5.00\pm0.01^{d\alpha}$
F	$2.50\pm0.07^{a\beta}$	$3.50\pm0.01^{b\alpha}$	$3.50\pm0.01^{c\beta}$	$4.20\pm0.01^{b\alpha}$
G	$2.50\pm0.02^{a\beta}$	$4.00\pm0.01^{c\alpha}$	$1.00\pm0.00^{b\beta}$	$4.20\pm0.0^{b\alpha}$
H	$3.00\pm0.01^{b\beta}$	$4.00\pm0.06^{c\alpha}$	$5.50\pm0.00^{f\beta}$	$3.00\pm0.00^{a\alpha}$

*Represents the isolated found in gastrointestinal tract of Caspian Sea grey mullet fish.
[a] Lowercase letters indicate significant differences (p<0.01) in each row for each microorganism, separately.
[α] Latin letters indicate significant differences (p<0.01) in each column for each microorganism, separately.

Taxonomic identification of the isolated probiotic bacteria

Carnobacterium maltaromaticum showed 100% sequence similarities with three isolates (A, G, and H) and 99% similarity with one isolate (F). Ninety-nine percentage similarities were recorded between B isolate and *Carnobacterium divergens*. Two isolates (B and C) showed 71% sequence similarity with *Weisella koreensis* and other *Weisella* species. The phylogenetic tree constructed with the strains showing high similarity percentages is shown in Figure 1. The fish gut isolates, through the sequencing results, have a high genetic relatedness to lactic acid bacteria, especially *Carnobacterium maltaromaticum* and *Carnobacterium divergens*. Isolates A, G, and H showed 100% sequence similarities with C. *maltaromaticum.*

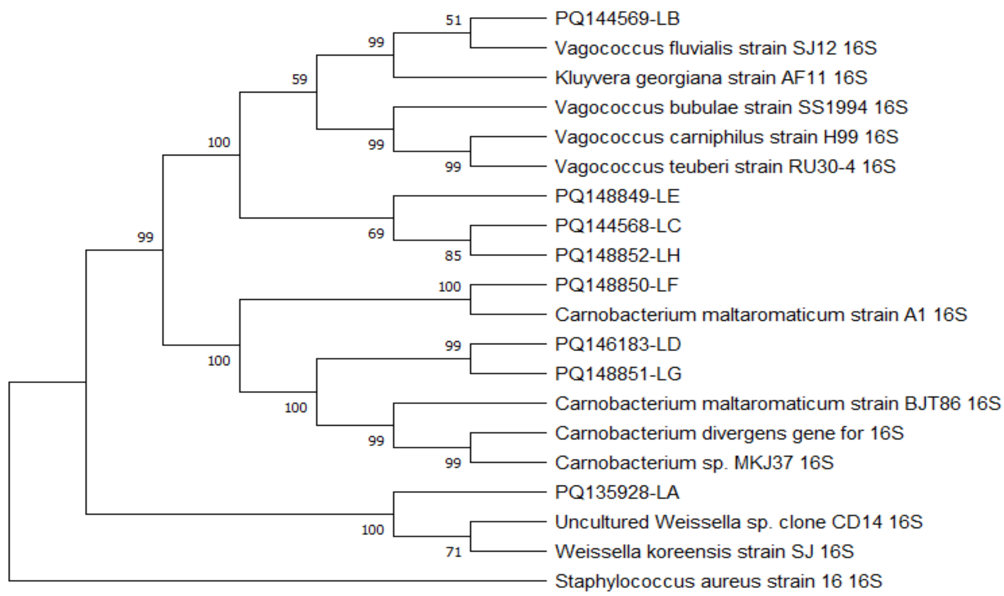

Figure 1: Phylogenetic tree showing species relatedness of isolated potential probiotics from gastrointestinal tract of Caspian Sea grey mullet fish.

Discussion

Identifying probiotics in fish gut involves analyzing the microbial communities present in the gut using techniques like DNA sequencing or biochemical tests beside biochemical tests. Specific strains of beneficial bacteria, such as *Lactobacillus*, *Bifidobacterium* or *Carnobacterium* be isolated and identified based on their biochemical and genetic markers. According to the results of biochemical tests using sugar-based media, half of the isolates fermented glucose, and more than half could ferment arabinose and mannitol. Patterns of fermentation of sugars in the gastrointestinal potential probiotics isolates of Caspian Sea grey mullet fish reflect the heterogeneous metabolic capacity of the strains. Most common was the fermentation of glucose, corresponding to the glucose utilization potential of probiotics as a preferred carbon source, which has been known for a long time (Adesulu-Dahunsi *et al.,* 2017). Higher fermentation abilities of arabinose and mannitol are reflective of the ability of the species to work with different carbohydrate substrates in the gut microenvironment of the fish (Kumar *et al.,* 2013). Inability of any of the isolates to ferment all the sugars checked is in accordance with findings in other studies that show heterogeneous metabolic activities of probiotic species (Gómez-Sala *et al.,* 2019). The presence of raffinose fermenters also reflects the discriminatory carbohydrate metabolism of these isolates, which could be responsible for their ecological fitness in the gut environment (Shang *et al.,* 2018). The diversity suggests that these potential probiotics could have crucial functions in the balance of gut microbiota by metabolizing different carbohydrates in the fish diet.

The result of the pH tolerance test of the potential probiotics isolates from the gut of Caspian mullet shows remarkable variation in survival among different strains at different pH levels. Strain A had much greater survival in pH 7.5, suggesting that it might be well adapted to the neutral environment, which coincides with the optimal pH for most lactic acid bacteria (König and Fröhlich, 2017). Strain B was tolerant at pH 9 and 7.5, indicative of its resistance to slightly alkaline environments, a feature typically prevalent in probiotic strains (De Vuyst and Vandamme, 2012). All the isolates failed to grow at pH 1.5, in accordance with the general sensitivity of most lactic acid bacteria to extremely acidic environments (Minich *et al.,* 2020). Strain F also showed a survival peak at pH 3.0, which is an indication of adaptation to acidic environments, as reported for certain probiotic strains (Wang *et al.,* 2018). Isolates' survival at pH 3.0 and pH 9.0 suggests that they have an effective mechanism of pH tolerance, which would prove to be useful in surviving the gut's variable environment of fish. These findings demonstrate the diversity of acid resistance systems in potential probiotics, which could influence their ecological niches among fish microbiota.

A significant high survival rate of some isolates in the presence of bile salt was observed. Same findings have been reported by Govindaraj *et al*. (2021), who found that probiotics isolated from fish guts exhibited notable bile salt tolerance, suggesting that fish-associated probiotics are often well-adapted to withstand gastrointestinal stresses (Govindaraj *et al.,* 2021). Additionally, studies by Ayyash *et al*. (2021) on probiotic isolates from marine environments also highlighted high bile salt tolerance, underscoring the adaptation of these isolates to harsh gut conditions, including the presence of bile salts (Ayyash *et al.,* 2021). This suggests that these isolates possess robust mechanisms to withstand the stressful conditions imposed by bile salts, which is critical for their survival in the gastrointestinal tract, where bile salts are naturally present (Shehata *et al.,* 2016). The sensitivity of some isolates to bile salts indicates these strains may not be as resilient in the host's gut environment, potentially limiting their probiotic potential (Kusada *et al.,* 2021). This is consistent with findings from a study by Hanol Bektas (2020), where Lactobacillus strains isolated from freshwater fish showed reduced survival in the presence of bile salts, particularly at high concentrations, highlighting strain-specific variations in bile salt resistance (Hanol Bektas, 2020). Additionally, a study by Wanka *et al*. (2018) found that many probiotic isolates, particularly those from terrestrial environments, exhibited decreased viability in high bile salt concentrations, further supporting the notion that not all probiotic strains are naturally equipped to survive in the gastrointestinal tract (Wanka *et al.,* 2018). Notably, isolate F failed to grow in the presence of bile salts, further highlighting the varying bile salt tolerance among probiotic strains. Such findings are essential for identifying the most suitable strains for probiotic applications, especially in aquaculture and fish gut health management (Xu *et al.,* 2016).

The acid tolerance of the potential probiotic isolates from the gut of Caspian Sea grey mullet varies with strain performance. This outcome reveals that resistant isolates possess more effective defense systems to overcome unfavorable gastrointestinal situations because of, for example, increased stability of the cell membrane or proton pump activity, as indicated in corresponding studies (Quinto *et al.,* 2014). Isolate E's reduced growth rate may reflect its weaker adaptation to acidic conditions, as with the other probiotic strains of lower acid tolerance. These findings are in line with previous reports that probiotic strains exhibit strain-specific resistance to acidic pH and bile salt, which are significant factors for their potential use as probiotics in fish or human food (Muthukumar and Kandeepan, 2015; Alonso *et al.,* 2019). Strain-specific variations evidenced in the current study suggest the need for further research for the selection of the most suitable strains of Lactobacillus to be used as probiotics in aquaculture.

The antibacterial properties of 8 different isolated potential probiotics from gastrointestinal tract of Caspian Sea grey mullet fish indicated high inhibitory activity against *Salmonella entrica* and *Staphylococcus aureus* compared to enrofloxacin employed. In case of *E.coli* and *Yersinia ruckeri*, *the isolates showed lower antagonistic activity in comparison to* enrofloxacin. This finding concurs with previous reports that indicated the antimicrobial capacity of LAB strains isolated from various fish species, including *Lactobacillus* and *Enterococcus* strains that were also effective against food-borne pathogens (Zhang *et al.,* 2022). Also, this is in agreement with findings by Amarantini *et al*. (2019) and Nurhikmayani *et al*. (2019), where LAB strains were differentially effective depending on the pathogen (Amarantini *et al.,* 2019, Nurhikmayani *et al.,* 2019). The greater antibacterial activity against *S. enterica* and *S. aureus* may be attributed to the secretion of antimicrobial compounds, which have been reported to inhibit the growth of such pathogens (Yang *et al.,* 2012). The lower activity against *E.coli* and *Y. ruckeri* may, however, be attributed to the pathogen's resistance mechanisms or the characteristics of the LAB strains themselves. The antibacterial potential of the isolates that were screened in this research can be attributed on the basis of several possible mechanisms involving production of organic acids, bacteriocins, and hydrogen peroxide. The LAB were observed to lower the pH of their environment by production of lactic acid and thus suppress the growth of pathogenic microorganisms by interfering with their cellular mechanism (Bintsis, 2018). In addition, many LAB strains have been found to produce bacteriocins, antimicrobial peptides with targeted activity against closely related bacteria and hence confer a competitive advantage in the intestinal environment (Zapaśnik *et al.,* 2022). The strong inhibition of *S. enterica* and *S. aureus* can be attributed to such bacteriocins, which preferentially target Gram-positive bacteria, while the reduced activity against *E. coli* and *Y. ruckeri* may be a sign of the natural resistance of these pathogens to these compounds. In addition, the capacity of certain LAB strains to generate hydrogen peroxide may be responsible for the antimicrobial activity observed against *S. aureus*, in particular, since this compound is capable of degrading cellular structures (Admassie, 2018). These mechanisms all indicate the broad-spectrum potential of probiotics as natural biocontrol agents.

The fish gut isolates, through the sequencing results, have a high genetic relatedness LAB, especially *Carnobacterium maltaromaticum* and *Carnobacterium divergens*, the antimicrobial competent species with some probiotic value as reported by Arbulu et al. in 2022 (Arbulu *et al.,* 2022). Three isoltes showed a 99% similarity with *C. divergens*, a species that has also been studied for its role in the inhibition of pathogenic microorganisms in food systems (Lorenzo *et al.,* 2018). A phylogenetic tree constructed with these isolates revealed distinct taxonomic groupings and indicated that they may serve as potential candidates for probiotic purposes in aquaculture due to their beneficial roles in gut health and inhibition of pathogens.

In conclusion, screening of the bacterial isolates from the gut of Caspian striped mullet (*Mugil cephalus*), to identify potential probiotics using 16S rRNA revealed the isolation of eight strains which had bacilli morphology. All the isolates showed satisfactory acidic and bile tolerance and pH resistance. Comparing with probiotic candidates, *Carnobacterium maltaromaticum* and *Carnobacterium divergens* had the highest antagonistic effects against pathogens guiding us to further studies with focus on investigating their functional role in fish microbiomes and their applicability as probiotics.

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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