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Research Article:

Evaluation of probiotic properties and the antibacterial activity of lactic acid bacteria isolated from *Rutilus kutum* intestine

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Abstract

Lactic acid bacteria are the most common bacteria which have been introduced as probiotic. This study aimed to investigate the impacts of isolated lactic acid bacteria from the *Rutilus kutum* gut on *Escherichia coli* and *Pseudomonas aeruginosa*. Lactic acid bacteria were isolated from the intestine of 100 fish which are randomly collected from the Caspian Sea and their primary probiotic properties were evaluated based on resistance to acid, bile salts and antibiotics. The inhibitory effect of the bacteria was evaluated on *Escherichia coli* and *Pseudomonas aeruginosa*, using the agar disk diffusion method. The specific band was triggered using PCR primers for 16S rRNA gene and validated via sequencing and comparing its sequence with those of gene bank databases. In this case, *Lactobacillus acidophilus* (54.79%), *Lactobacillus plantarum* (24.65%), and *Lactobacillus brevis* (20.54%) were detected. The isolated bacteria were resistant to vancomycin. The most inhibitory effect belonged to *Lactobacillus acidophilus* sp. on *E. coli* and *P. aeruginosa*; with the inhibition zone of 12 and 14 mm, respectively. *Lactobacillus plantarum* had moderate inhibitory effect on *P. aeruginosa* while *Lactobacillus brevis* had neither effect on *E. coli* nor *P. aeruginosa*.

Keywords: *Lactobacillus*, Probiotic properties, *Rutilus kutum,* PCR

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Introduction

Probiotics are live microorganisms that can improve the microbial balance of digestive tract and decrease the immoral effects of pathogens. Lactic acid bacteria are found in the intestine of most animals (Andani *et al*., 2012). They are gram-positive, non-mobile, without spore, and negative catalase. The bacteria are oxidase negative, convert sugars to the lactate and their optimum growth temperature is 30°C (Albano *et al*., 2009). Lactic acid bacteria have a good deterrent effect on *Staphylococcus aureus*, *E. coli*, and *Aeromonas hydrophila* (Sahoo *et al*., 2015). The antimicrobial effects of some bacteria have been detected; for example, *Lactobacillus* can inhibit the growth of *Vibrio cholera* and *Aeromonas* and reduce the risk of aquatic diseases (Allameh *et al*., 2017). Many strains of LAB isolated from fish can produce antibacterial agents against various pathogenic fish bacteria as well as human pathogens (Ringo *et al*., 2018). Gram-negative intestinal bacteria, especially *Salmonella*, *Shigella*, and *E. coli* are the most important causes of diarrhea in developing countries and drug resistance is a daily growing problem nowadays (Gashe *et al*., 2018; Aronowitz *et al*., 2019). Inhibition of pathogen bacteria through lactic acid bacteria (especially *Lactobacillus*) has become increasingly common (Hu *et al*., 2017). Functional properties of probiotics included balancing the immune system, decreasing serum cholesterol, gastrointestinal infections, the rate of chronic traveling diarrhea, and the rate of cancer (Arora *et al*., 2019). The most important function of probiotics in the digestion tube of fish is to improve nutritional absorbance via the production of extracellular enzymes (Adel *et al*., 2017). Studies showed that the growth, percentage of weight enhancement, specific growth rate, food consumption frequency, and protein enhancement were higher in fish that received probiotics (Jatobá *et al*., 2018). *Rutilus kutum* is found in the North of Iran (the Caspian Sea, and rivers of Guilan and Mazandaran provinces) which is considered as one of the most desirable fish in the North of Iran. The aim of the present study was to detect and evaluate the impacts of *Lactobacillus* bacteria on *E. coli* and *P.aeruginosa* pathogen bacteria.

Materials and methods

Lactobacillus isolation from the intestine of fish

One hundred *Rutilus kutum* were collected randomly from the fishermen of the Caspian Sea. Sampling was performed from the intestine of the fish. Under the sterilized condition, a part of the first section of the middle intestine was removed (1 gr), cultured on de Man, Rogosa and Sharpe (MRS) broth (Quelab, Canada), and placed in an anaerobic jar with the microaerophilic condition for 48 hours at 30°C (Azizpour, 2009). Then, the cell suspension is cultured in MRSAgar medium and placed under anaerobic conditions. The medium contains 10.0 g peptone, 20.0 g glucose, 8.0 g meat extract, 4.0 g yeast extract, 5.0 g sodium acetate, 2.0 g dipotassium hydrogen phosphate, 2.0 g triammonium citrate, 1.0 g polysorbate 80, 0.2 g magnesium sulfate, 0.05 g manganese sulfate. Gram staining as well as catalase and oxidase tests were performed on produced colonies. Bacteria that were gram-positive, and had a negative reaction for catalase and oxidase tests were maintained for further analysis (Chandran and Keerthi, 2018).

DNA purification

DNA extraction was performed using the boiling method. 1.5 ml of lactic acid bacteria was cultured for 24 hours and centrifuged in 6000 rpm for 5 min. Cellular plates was diluted in 300 microliters of TE buffer (mMTris-HCL, 0.5mM, 10 EDTA, pH8), boiled for 10 minutes in 100°C, and quickly transferred to the ice for 5 min. Tubes were centrifuged in 10000 rpm for 5 min (4°C). 200 microliter of the supernatant was collected in a new tube and kept at - 20°C for future usage (Alipour *et al*., 2018).

Validation of Lactobacillus Sp. using PCR

In order to detect different *Lactobacillus* spp., specific primers were used to amplify 16s rRNA genes. The information is shown in Table 1 (Massi *et al*., 2004). Specific primers were synthesized by Bioneer Company. The final volume of the mix was considered to be 20 microliters that included 2 μl of DNA, 4 μl of dNTPs, 0.6 μl of $MgCl_2$, 2 μl of 10x buffer, 0.2 μl of Taq polymerase enzyme, 0.5 μl of primers (20 pmol), and 13.8 μl distilled water. Thermo Cyclic device of Eppendorf Company was used to perform PCR. The used thermal program that was adjusted based on 16s rRNA gene for detection of *Lactobacillus* was as follows: primary denaturation at 95°C for 4 min, and 35 PCR cycles including denaturation at 95°C for 45 sec, annealing at 60°C (for *Lactobacillus acidophilus),* at 52°C (for *Lactobacillus plantarum),* and at 56°C (for *Lactobacillus brevis)* for 1 min, extension at 72°C for 45 sec, and final extension at 72°C for 6 min. Electrophoresis of PCR products was performed on 2% agarose. DNA bands were observed using Transilluminator UV device (Massi *et al*., 2004). PCR product was validated via sequencing (Bioneer, Korea) and comparing its sequence with the sequences of Gene Bank database. Data were analyzed using SPSS software version 20. A one-way ANOVA was used to determine significant differences. Duncan's multiple range tests (Duncan, 1955) were used to rank the treatments and mean differences which were considered significant at $p< 0.05$.

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Table 1: The sequences of primers used in this study.

species	Primer sets (target site)	Sequence (5' →3')	Amplicon (bp)
Lactobacillus	aci-ITS.F (16S)	CCTTTCTAAGGAAGCGAAGGAT	199
acidophilus	aci-ITS.R	AATTCTCTTCTCGGTCGCTCTA	
Lactobacillus	pla-ITS.F(16S)	GCCGCCTAAGGTGGGACAGAT	283
Plantarum	pla-ITS.F	TTACCTAACGGTAAATGCGA	
Lactobacillus	bre-16S(ITS)F	GTGAGATAACCTTCGGGAGT	316
brevis	bre-ITS.R	GGTCACTTCGTGATCGTCAA	

Sugar fermentation test

The sugar was dissolved by 1% in the medium phenol red broth base. A change in color from red to yellow was observed after sugar fermentation due to lactic acid production, medium acidification, and reaction with phenol red reagent indicates fermentation of sugar (Vos *et al*., 2011).

Resistance to stomach acid test

MRS broth medium was prepared, autoclaved, inoculated with individual lactic acid bacteria isolates, and incubated in an anaerobic jar (37°C for 24 h) as a pre-cultivation. MRS broth (6 ml) was poured in two Erlenmeyers per isolate, and the pH was adjusted to 3-4 by adding hydrochloric acid to each Erlenmeyer. At a post-cultivation stage, the isolated bacteria (10^8 cfu) were inoculated and incubated in each Erlenmeyer. After 24 h, the optical density (OD) was measured in the Erlenmeyers by a spectrophotometer at a wavelength of 660 nm (reduced cell density) and reported in terms of survival rates (Charernjiratragul *et al*., 2010).

Bile salt tolerance test

The purified bacteria were prepared in MRS broth medium. The bile was prepared with dilution rates of 0.4, 0.5, and 0.6%, and incubated in an anaerobic jar for 2.0 h. Control (no bile salts) and test cultures were evaluated at 2 and 24 hr for the presence or absence of growth by streaking samples onto MRS agar (Menconi *et al*., 2014).

Effects of antibiotics on the bacteria isolated from Kutum intestine

Antibiogram discs were used containing the antibiotics azithromycin, tetracycline, ampicillin, vancomycin, and streptomycin. The isolated bacteria were cultured on Muller Hinton agar medium using McFarland 0.5 standard. The antibiotic discs were placed on the medium and evaluated after 24-48 h (Wayne and Institute, 2015).

Antibacterial function against pathogenic bacteria

In order to assess activity of lactic acid bacteria, isolated bacteria were tested on *E .coli* (ATCC: 25922), and *P. aeruginosa* (ATCC: 27853) using well diffusion method. Lactic acid bacteria were kept for 24 hours in MRS broth culture medium. The supernatant of each lactic acid bacteria was prepared by centrifugation in 5000 rpm for 10 min. The supernatant was filtered with 0.22 micrometer filter. Pathogens

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entered Muller-Hinton broth culture medium and were kept for 24 hours at 37°C to reach turbidity similar to 0.5 McFarland. A sample was obtained from the culture medium using a sterilized swab and distributed on Muller-Hinton Agar culture media. After 24 hours in an anaerobic condition, the diameters of the inhibitory zone were measured and recorded using a millimeter scale (Abdel-Daim *et al*., 2013).

Results

Electrophoresis results obtained of amplification of 16s rRNA gene showed segments with 316, 283, and 199 bp in length, respectively, which showed the presence of these genes in specific *Lactobacillus* spp. (species) (Fig. 1).

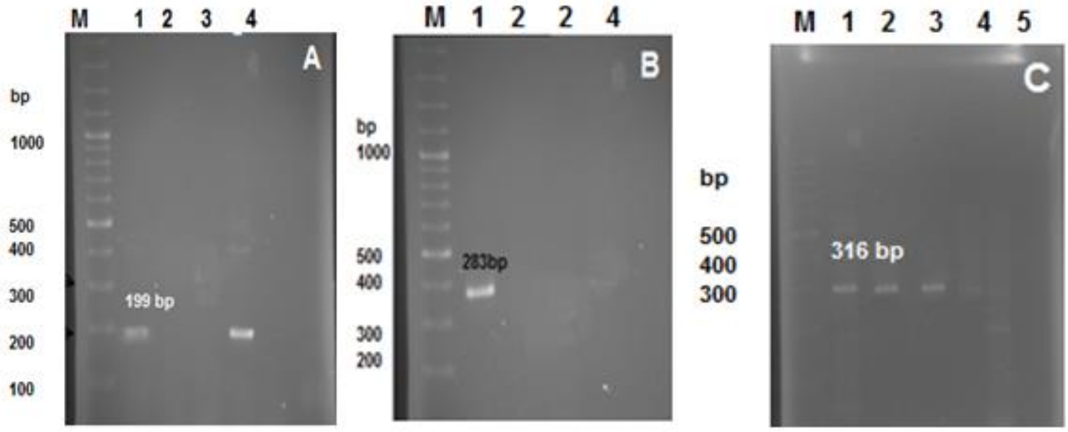

Figure 1: Results of electrophoresis from gene amplification: A: ladder (M), columns 1 and 4 show the presence of the target gene in *Lactobacillus acidophilus* (199 bp). B: columns 1 and 5 indicate the presence of target gene in *Lactobacillus plantarum* (283). C: columns 1, 3 and 4 show the presence of the target gene in *Lactobacillus brevis* (316 bp).

Seventy-three *Lactobacillus* were isolated from 100 samples of *Rutilus kutum*'s intestine, from which, *L. acidophilus* (54.79%), *L. plantarum* (24.65%), and *L. brevis* (20.54%) were detected (Table 2).

Table 2: Frequency (percentage) of isolated *Lactobacillus spp.*

species	Number (%) of isolates
Lactobacillus acidophilus	40(54.79)
L actobacillus plantarum	18(24.65)
Lactobacillus brevis	15(20.54)
Total	73

Sugar fermentation test

Table 3: Sugar fermentation in lactobacilli isolated from Kutum intestine.

Sugar	*L. acidophilus*	*L. plantarum*	*L. brevis*
Arabinose	+	+	+
Inositol	+	-	-
Sucrose	+	+	+
Rafinose	+	+	+
Rhamnose	-	-	-
Cellobiose	+	+	-
Ribose	-	+	+
Glucose	+	+	+
Fructose	+	+	+
Galactose	+	+	+
Trehalose	+	+	-
Lactose	+	+	+
Mannose	+	+	-
Mannitol	-	+	-
Melositosis	-	+	-

+: Fermented, -: Non-fermented

Resistance to stomach acid test

Table 4: Percentage of acid resistant isolates of *Lactobacilli* isolated from Kutum intestine at pH= 3-4.

Species	Percentage of acid resistant			
	<10	10 – 60	60 – 80	>80
L. acidophilus	-	-	70	-
L. plantarum	-	43	-	-
L. brevis	-	-	65	-

Bile salt tolerance test

Table 5: Evaluation of bile salt tolerance of lactobacilli bacteria isolated from fish intestine.

Strains	Bile salt tolerance test(%)		
	0.4%	0.5%	0.6%
L. acidophilus	+	+	+
L. plantarum	+	+	+
L. brevis	+	+	-

Tolerant = “-” Nontolerant = “+”

L. plantarum had the highest inhibitory zone against the azithromycin and all were resistant to the vancomycin (Table 6).

L. acidophilus had the maximum inhibitory zone on *E. coli* (12 mm) and *P.aeruginosa* (14 mm), while *L. brevis* showed no inhibitory effect and *L. plantarum* had the same effect on both *P.aeruginosa* and *E. coli* (Table 7).

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Table 6: Impacts of antibiogram antibiotics on the lactobacilli bacteria isolated from fish intestine. Diameter of inhibition zone (mm).

Species	Ampicillin	Tetracycline	Azithromycin	Streptomycin	Vancomycin
L.acidophilus	15	17	26	14	-
L. plantarum	24	23	33	15	-
L. brevis	23	16	30	10	-

*Punch diameter = 8 mm.

Table 7: Inhibitory activity of *Lactobacillus* spp. on *pseudomonas aeruginosa* and *E. coli*.

***Lactobacillus* spp.**	**Diameter of inhibition zone (mm) on *E. coli***	**Diameter of inhibition zone (mm) on *pseudomonas aeruginosa***
Lactobacillus acidophilu	12	14
Lactobacillus plantarum	11	11
Lactobacillus brevis	0	0

Discussion

Bile plays an essential role in the intestinal defense mechanism, and the intensity of its effect is evaluated by concentrations of bile salts with an average of circa 0.3-1.5%. *Lactobacillus acidophilus* and *Lactobacillus casei* were resistant within time ranges of 15-40 and 40-60 minutes, respectively, which are in agreement with the effects of bile salts in less than an hour in comparison with control samples (Mohammadian *et al*., 2014). Such resistance is attributed to enzymatic hydrolysis by lactobacilli, ultimately reducing the detergent effect of bile salts (Maragkoudakis *et al*., 2006).

The bacterial strains were challenged for tolerance to acidic conditions (Table 4). The results showed a decreasing trend of lactobacilli tolerance after an hour, which is in agreement with theprevious studiesconducted by Succi *et al*. (2005) and Mohammadian *et al*. (2014). *Lactobacillus acidophilus* and *Lactobacillus casei* can also produce lactic acid, reduce pH, and produce antimicrobial compounds such as hydrogen peroxide, bacteriosins, ethanol, antibiotics, and other compounds (Aroutcheva *et al*., 2001).

Gram-negative intestinal bacteria, especially *Salmonella*, *Shigella*, and *E. coli* are the most important causes of diarrhea in developing countries. Besides, drug resistance is a daily growing problem, competitive inhibition of pathogen bacteria by lactic acid bacteria (especially *Lactobacillus*) which are used to inhibit pathogens (Adesokan *et al*., 2008; Tajabadi *et al*., 2009). Obadina *et al*. (2006) determined the inhibitory effect of *Lactobacillus* spp. on *Pseudomonas*, *E. coli*, and *S. aureus*, which is in concordance with the results of the present study. Puttalingamma *et al*. (2006) found the effect of *L. plantarum* on *E. coli*, *Salmonella*, *Bacillus subtilis*, and *S. aureus*. Their results are in concordance with those of the present study. Results of this study demonstrated that the beneficiary effect of isolated *Lactobacillus* spp. gives it high potential to be added in fish feed.

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Jafarian *et al*. (2009) reported the effect of commercial and isolated probiotic bacteria from fish intestine on health and resistance of trout larvae, which showed increase resistance against pathogens, survival rate, ecological competence, and breeding performance. Irianto and Austin (2002) used probiotics in aquaculture as a tool for disease control and as an antimicrobial component and showed that probiotics are effective on a wide range of fish pathogens.

Le and Yang (2018), in their study on *Lactobacillus* spp. isolated from fermented salty shrimp and its antagonistic effect on *V. parahaemolyticus*, found that *L. plantarum* has a strong inhibitory effect on *Vibrio* and the mortality of animal was lower than the control. Dinev *et al*. (2018) reported that *L. plantarum* has an antibacterial effect on a wide range of Gram-positive and Gram-negative pathogens. The study of Norouzi *et al*. (2008) showed that *Lactobacillus* isolated from the oral cave has an inhibitory effect on *E. coli* that is in concordance with the present study. In 2006, Kiai *et al*. showed that 59.3% of lactobacilli and 52% of lactococci are able to prevent the growth of pathogenic bacteria. A study performed by Diaz *et al*. (2013) to identify and assess probiotic species of *lactobacillus* spp in dolphin. They have isolated the bacteria from the digestive tract of dolphin and found that there is a symbiosis between the lactobacilli bacteria and the dolphin's digestive tract to prevent other pathogens from being placed. In addition, Ghanbari *et al*. (2009) isolated lactic acid bacteria from the intestinal tract of sturgeon that also included *L. plantarum* and *L. brevis* which is in concordance with the results of the present study.

Results of the present study showed that most of the isolated bacteria had the ability to inhibit the growth of pathogenic strains. *Lactobacillus acidophilus* has a strong inhibitory effect on *E. coli*. Regular consumption of *Lactobacillus* in the fish diet may be resulted in replacing *Lactobacillus* as the dominant flora that triggers immunity, and plays an effective role in feeding of the fish.

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