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Original Article

Study on probiotic potential of *Bacillus* species isolated from the intestine of farmed rainbow trout, *Oncorhynchus mykiss*

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Abstract: The gastrointestinal tract of fishes is a complex ecosystem occupied by a large number of microorganisms, some of them could have potentially-valuable features. This research was conducted to study *Bacillus* species in the intestine of farmed rainbow trout to examine their probiotic properties, and to provide a new source of probiotics. A total of 23 farmed rainbow trout were sampled and their intestine samples were cultured. Following the morphological assay and biochemical analysis, isolated *Bacilli* were amplified by polymerase chain reaction and universal primers 27f and 1492r. *Bacillus subtilis* and *B. amyloliquefaciens* were isolated from 5 and 3 samples, respectively. *Bacillus tequilensis*, *B. cereus* and *B. licheniformis* were isolated from 1 sample. Probiotic properties of *B. subtilis* strain MSM 24, *B. amyloliquefaciens* strain TMM 25 and *B. licheniformis* strain MR 78 were confirmed. Since probiotic bacteria cause no foodborne diseases, their existence in farmed trout intestines, and their penetration into the fish tissues do not pose any risk to consumers' health.

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Introduction

The intestinal microbiota is an integral part of the gastrointestinal tract of fishes. Numerous internal and external factors regulate the microbial specifications of environment, thereby are affecting the fish intestinal microbiota (Earl et al., 2008). Bacteria are the main constituents of the intestinal microbiota of fish. The fish intestinal microbiota is characterized by high density and diversity of organisms with complex interactions (Nithya and Halami, 2013). Diverse microbial populations exist in the intestinal contents. The numbers of bacteria are higher in the intestinal contents than in the surrounding water. This shows that the intestines provide favorable environments for microorganisms. This microbial population can prevent infection by interfering with pathogens and/or limiting occupying surface for them (Huber et al., 2004). Studies have indicated that *Bacillus* species improve food consumption in fishes (Kavitha et al., 2018). Various studies reported that the intestinal microbiota of freshwater fishes encompasses of different genera such as *Acinetobacter, Aeromonas, Bacillus, Flavobacterium, Pseudomonas, Enterobacteriaceae*, and obligate anaerobic bacteria of the genera *Bacteroides*, *Fusobacterium*, and *Clostridium* (Hovda et al., 2007; Huber et al., 2004).

Bacilli bacteria are present in water and soil and they can easily reach the trout farms. Given that rainbow trout are among high-quality and marketable fishes, its farming is considered as the largest aquaculture industry in Iran (Ghorbanzadeh and Nazari, 2015). Rainbow trout are sold as non-live form in many regions of Iran, which may lead to *Bacilli* bacteria penetrating to the fish flesh; as a result, it is necessary to determine the properties of these bacteria. Since probiotics have health benefits, they may provide an alternative way to reduce the use of drugs in aquaculture and simultaneously may avoid the development of antibiotic-resistant bacteria (Chen et al., 2016).

Bacilli are being explored for the production and preservation of food for many centuries. The inherent

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ability of production of the proteins, enzymes, antimicrobial compounds, vitamins, carotenoids and other specifications are importance of study of *bacilli* in food industry. Additionally, *Bacillus* spp. are gaining interest in human health related functional foods researches coupled with their tolerance and survivability under hostile environment of gastrointestinal tract. Besides, *Bacilli* are more stable during manufacturing process, food storage and pharmaceutical industry that making them more suitable candidate for health promoting (Elshaghabee et al., 2017). Therefore, providing a new source of probiotic *Bacilli* is necessary. Hence, this research was conducted to study *Bacillus* species in the intestines of farmed rainbow trout, *Oncorhynchus mykiss*, by examining their probiotic properties, and providing a new source of probiotics.

Materials and Methods

Initial separation: A total of 23 farmed rainbow trout, 130-2230 g in weight, were collected from fish-farming ponds. The fish were sampled after euthanasia. First, the fish skin was washed using 70% alcohol. Then, their intestine was removed and washed by sterile distilled water to remove fecal matter. Next, the intestine was cut and rewashed by sterile distilled water to remove the remaining fecal matter. They were placed in sterile Petri dishes. The intestinal wall was scraped with a plastic spatula. All steps of sampling were done on ice. Undiluted mucus was used to explore the type of bacteria. Mucus was cultured on the nutrient agar (Cohen and Laux, 1995). Plates were incubated under aerobic conditions at 37°C for 5 days. After incubation, white colonies of gram-positive bacteria with 2-3 mm diameter were sampled. These bacteria were sub-cultured on nutrient agar under same conditions. Bacterial identification was carried out by morphological assay, chemical tests, PCR and probiotic tests.

Morphological assay: Gram staining and fluorescent microscopy were used for morphological study of the bacteria.

Bacterial identification by chemical methods: The bacteria were identified according to Bergey Manual of Systemic Bacteriology (Holt et al., 1994). Hence, they were identified by the mobility and catalase activity, followed by fermentation a set of carbohydrates, including arabinose, glucose, starch, mannose, mannitol, salicin, xylose, turanose and melibiose. The other biochemical tests were tween 20, o-nitrophenyl-β-d-galactopyranoside (ONPG), pH, concentration of NaCl range from 2-10%, temperature (5-65^{o}C), citrate, VP and nitrate reduction. The ability to ferment lactose was utilized to differentiate *Bacillus subtilis* from *B. amyloliquefaciens*. The sugars were sterilized by filtration (Millipore filter, 0.45 μm pore size).

Bacterial identification by PCR: In order to identify the bacteria using the PCR (Bioline, United Kingdom), they were cultured on nutrient agar. The culture medium was incubated at 37°C for 18 hours. Boiling method was used to extract DNA from bacteria (Dashti et al., 2009). The concentration and integrity of the extracted DNA was determined by ethidium bromide stained 0.8% agarose gel electrophoresis and compared against known concentrations of lambda DNA (Chandrasekara Bhagya et al., 2013).

All chemicals used were molecular grade from Sigma Company. 16S ribosomal RNA gene was used. The PCR primers were 27f (AGAGTTT GATCCTG GCTCAG) and 1492r (TACGGYTACCTTGTTA CG ACTT). Master-mix of PCR was diluted to 1×0.30 μl reaction mixture contained 15 μl master, 1 μl each PCR primer and 8 μl sterile injectable water. The PCR program was as follow: initial denaturation at 94°C for 2 min followed by denaturation at 94°C for 1 min, annealing at 55°C for 1.5 min, 30 cycles of extension at 72°C for 1 min, and the final extension at 72°C for 3 min. The PCR product was visualized on a 1% agarose gel under UV light after ethidium bromide staining (Silva et al., 2013). Gel electrophoresis was carried out at 80 V. Amplicons were purified from agarose gel using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare, United Kingdom). The PCR products were sequenced using the Sanger DNA sequencing (Teng et al., 2004). Sequences were identified using BLAST. The results were analyzed

using the BioEdit sequence editor. FASTA sequences aligned using Clustal X. PCR results aligned against bacterial DNA database (NCBI) (http://blast.ncbi.nlm.nih.gov). A homology greater than 97% was acceptable for identifying the genus. These bacteria were registered in the NCBI and DGBI database.

Probiotic assay of the isolates: The isolates were screened for hemolytic activity, lecithinase, tolerance to acidic conditions, gastric juice and bile salt, and antibiotic resistance transferability. Then, based on the results, the selected bacteria were examined for adhesiveness and invasiveness. Microbial suspension was prepared for study of theses specifications (Iranian National Standard No. 19459. 2014).

Microbial suspension preparation: The identified bacteria were cultured in nutrient broth at 37ºC for 24 hours. Culture media were centrifuged at 5000 rpm for 10 min. The supernatant was removed and pellets were washed in a phosphate-buffered saline (3 times). Then, the pellets were dissolved in the same buffer. These suspensions were adjusted to a turbidity equivalent to the 0.5 McFarland standard (Iranian National Standard No. 19459. 2014).

Acidic tolerance: In order to evaluate acidic tolerance, pH of MRS broth (10 ml) adjusted to 2.5 and 4 using hydrochloric acid. 100 µl of microbial suspensions were inoculated into MRS broth. They were incubated at 37ºC for 3 and 4 hours. After that, 1 ml of these media were inoculated into nutrient agar, followed by incubation at 37ºC for 24 hours to determine colony forming unit (Iranian National Standard No. 19459. 2014).

Gastric juice tolerance (pepsin and trypsin): In order to prepare the pepsin and trypsin media, 2 g of sodium chloride and 2.3 g of pepsin or trypsin were dissolved in 1 L of distilled water. The pH of these media were adjusted to 2-2.3. In order to evaluate gastric juice tolerance, the microbial suspension (2%) was inoculated into the pepsin and trypsin media, then, incubated at 37ºC for 24 hours. Thereafter, a 10^3 dilution was prepared from the incubated media using saline solution supplanted by 0.1% peptone water. One ml of these dilutions was cultured in nutrient agar using pour-plate method. Plates were incubated at 37ºC for 24 hours to determine colony forming unit (Iranian National Standard No. 19459. 2014).

Bile salt resistance: In order to assess bile salt resistance, 100 µl of the microbial suspension were inoculated into nutrient broth containing 0.3% of fish bile, followed by incubation at 37ºC for 8 hours. Before and after the incubation period, absorbance of the suspension was measured at a wavelength of 600 nm using UV-VIS spectrophotometer (Eppendorf, Germany). A medium without bile was used as a control sample for each bacterium (Iranian National Standard No. 19459. 2014).

Microbial adhesion and invasion: The adhesion assay was conducted according to Nithya and Halami (2013). The invasion assay was carried out as described by Rowan et al. (2001). The HepG-2 cell line was used. The cell monolayers were seeded at 5×10^4 cells per well (in RPMI 1640 medium plus 10% fetal calf serum) in 96-well plate. The cells were infected with filter-sterilized (0.2 µm) supernatants from overnight cultures (BHI, 30°C) of bacteria. 100 µl of supernatant were added to the cultured cells immediately after heat treatment (95°C, 10 min). Monolayers containing the bacterial supernatants were incubated overnight at 37°C in a 5% CO_2 atmosphere. After overnight incubation, the suspension from each well was discarded and 25 µl of fresh complete medium (RPMI+10% fetal calf serum) containing 0.004 g/mL of MTT reagent (Sigma) was added. Samples were incubated for 3 hours at 37°C in 5% CO_2 and the formazan product was solubilized by the addition of 100 ml of dimethyl sulfoxide. Optical densities of the suspensions were measured at 540 nm using an ELISA reader (Biotek, USA) and cytotoxicity was calculated (Mohkam et al. 2016).

The results of microbial adhesion and invasion tests were analyzed by SPSS Software. The samples were compared to each other by One Way ANOVA. T-test was used to compare the control with the test samples.

Antibiotic susceptibility examination: The susceptibility of the *Bacillus* isolates to erythromycin (15 µg), penicillin (10 µg), gentamicin (10 µg), amoxicillin (25 µg), azithromycin (15 µg), chloramphenicol (30 µg), cefalotin (30 µg), clindamycin (2 µg),

Table 1. The chemical identification results of *Bacillus* species isolated from the intestines of farmed rainbow trout (N= 11).

Bacteria \ Index	Weight	Motility	pH				Capability at different temperatures							
			7	8	9	10	5	10	20	30	40	50	60	65
B. tequilensis strain MS21	700	+	+	-	-	-	-	+	+	+	+	+	+	-
B. cereus MR11	355	+	+	-	-	-	-	-	+	+	+	-	-	-
B. subtilis strain MT 13	480	+	+	+	+	-	-	+	+	+	+	+	+	-
B. amyloliquefaciens MA 11	2040	+	+	+	+	-	-	+	+	+	+	+	+	-
B. subtilis strain MSM 14	135	+	+	+	+	-	-	+	+	+	+	+	+	-
B. amyloliquefaciens strain TR 15	215	+	+	+	+	-	-	+	+	+	+	+	+	-
B. subtilis strain MR 20	247	+	+	+	+	-	-	+	+	+	+	+	+	-
B. subtilis strain TAM 21	360	+	+	+	+	-	-	+	+	+	+	+	+	-
B. amyloliquefaciens strain TMM 25	2230	+	+	+	+	-	-	+	+	+	+	+	+	-
B. subtilis strain MSM 24	247	+	+	+	+	-	-	+	+	+	+	+	+	-
*B. licheni*formis strain MR 78	350	+	+	+	+	-	-	-	+	+	+	-	-	-

streptomycin (10 µg) and tetracycline (30 µg) were determined using Muller-Hinton agar. After incubation, the formation of clear zone around the discs monitored (Iranian National Standard No. 19459. 2014).

Hemolytic activity test: This test was performed on all isolates. A loopful of overnight bacteria was cultured to spot form on blood agar plate. The plates were incubated for 24 hours at 37ºC and observed for clear zones around the colonies (Iranian National Standard No. 19459. 2014).

Lecithinase activity: This test was performed using Mannitol-egg yolk-Polymyxin agar. A loopful of overnight bacteria was cultured to spot form on this medium. After incubation for 24 hr at 37ºC, the plates were checked for clear zone around the bacterial colonies (Iranian National Standard No. 19459. 2014).

Abbreviations used: GIT, Gastrointestinal tract; ONPG, O-nitrophenyl-β-d-galactopyranoside; PCR, Polymerase chain reaction.

Results

Morphological characteristics: The detected bacteria appeared to form of short or long chains of large gram positive *Bacilli*. Their spores had situations of ellipsoidal, central and sub terminal.

Chemical properties: Eleven *Bacillus* species were obtained from 23 examined fish. They showed catalase activity and could grow under both aerobic and anaerobic conditions. *Bacillus cereus* was arabinose, glucose, mannose, mannitol, salicin, xylose, and starch negative. However, it was citrate, VP, and nitrate reduction positive. This bacterium was unable to grow in the presence of salt (2-10%). Glucose, mannose, mannitol, salicin, xylose, arabinose, citrate and starch were used by *B. subtilis*, *B. amyloliquefaciens*, *B. tequilensis* and *B. licheniformis*. These bacteria were VP and nitrate reduction positive. These isolates were able to grow at the media containing 2-8% NaCl. Turanose and tween 20 were used by *B. subtilis* and *B. amyloliquefaciens*. *Bacillus amyloliquefaciens* was melibiose and lactose positive; however, it was ONPG negative. *Bacillus subtilis* was lactose negative; however, it was tween 80 positive.

According to Table 1, 11 *Bacilli* species were identified in rainbow trout. *Bacillus subtilis* and *B. amyloliquefaciens* were isolated from 5 and 3 samples, respectively. *Bacillus tequilensis*, *B. cereus* and *B. licheniformis* were isolated from one sample. All the isolated *Bacilli* were motile. These bacteria were able to growth at pH 7; however, not at pH 10. *Bacillus cereus* MR11 was not able to grow at pH at 8-9. *Bacillus cereus* MR11 and *B. licheniformis* strain MR 78, but not the other *Bacilli*, were not able to grow at temperatures 10, 50 and 60°C. The isolated *Bacilli* were able to growth at temperatures of 20, 30 and 40°C, but not 5 and 65°C. *Bacillus amyloliquefaciens* was found in the small and large fishes. *Bacillus subtilis* was found in the small and medium fishes. *Bacillus licheni* formis and *B. cereus* were observed in the medium fish. *Bacillus tequilensis* was observed in the fish with 700 g weight.

Molecular characteristics: The isolated bacteria were

Table 2. Accession numbers and similarity of *Bacillus* species isolated from the intestine of farmed rainbow trout (N=11).

Bacteria	NCBI	DGBI	Similarity (%)
B. tequilensis strain MS21	-	LC458434	99
B. cereus strain MR11	MK395545.1	-	
B. subtilis strain MT 13	-	LC458433	99
B. amyloliquefaciens strain MA 11	MK392154.1	-	99
B. subtilis strain MSM 14	MK400693.1	-	99
B. amyloliquefaciens strain TR 15	MK393391.1	-	99
B. subtilis strain MR 20	MK397791.1	-	99
B. subtilis strain TAM 21	MK397798.1	-	99
B. amyloliquefaciens strain TMM 25	MK394994.1	-	99
B. subtilis strain MSM 24	MK393445.1	-	99
B. licheniformis strain MR 78	MK395274.1	-	99

Table 3. The culture results of *Bacillus* species isolated from the intestine of the farmed rainbow trout in different conditions (N=11).

	Growth at								
Index	pH 4		pH 2.5		Bile	Gastric juice tolerance		Lecithinase	Hemolytic
Bacteria	3h	4h	3h	4h	0.3%	Pepsin	Trypsin	Activity	activity
B. tequilensis strain MS21	$1\times10^{8}\pm4.67$	$1\times10^{8}\pm2.35$	$1\times10^{8}\pm3.89$	$1\times10^{8}\pm2.67$	0.48±0.11	$1\times10^{5}\pm4.74$	$1\times10^{4}\pm1.14$	+	+
B. cereus strain MR11	$1\times10^{8}\pm4.13$	$1\times10^{8}\pm3.56$	$1\times10^{8}\pm2.87$	$1\times10^{8}\pm3.78$	0.56±0.16	$1\times10^{4}\pm4.71$	$1\times10^{6}\pm3.91$	+	+
B. subtilis strain MT 13	$1\times10^{8}\pm4.56$	$1\times10^{8}\pm3.76$	$1\times10^{8}\pm2.67$	$1\times10^{8}\pm2.98$	0.49±0.13	$1\times10^{5}\pm4.22$	$1\times10^{3}\pm4.27$	+	+
B. amyloliquefaciens strain MA 11	$1\times10^{8}\pm3.78$	$1\times10^{8}\pm3.16$	$1\times10^{8}\pm3.64$	$1\times10^{8}\pm3.15$	0.52±0.17	$1\times10^{5}\pm3.97$	$1\times10^{4}\pm4.11$	+	+
B. subtilis strain MSM 14	$1\times10^{8}\pm3.21$	$1\times10^{8}\pm3.13$	$1\times10^{8}\pm3.13$	$1\times10^{8}\pm3.24$	0.57±0.19	$1\times10^{3}\pm2.89$	$1\times10^{4}\pm3.91$	+	+
B. amyloliquefaciens strain TR 15	$1\times10^{8}\pm3.45$	$1\times10^{8}\pm2.58$	$1\times10^{8}\pm4.43$	$1\times10^{8}\pm3.74$	0.61±0.18	$1\times10^{3}\pm2.95$	$1\times10^{5}\pm3.47$	+	+
B. subtilis strain MR 20	$1\times10^{4}\pm2.19$	$1\times10^{3}\pm4.15$	$1\times10^{3}\pm3.17$	$1\times10^{2}\pm4.45$	0.91±0.15	$1\times10^{5}\pm2.31$	$1\times10^{4}\pm3.31$	+	+
B. subtilis strain TAM 21	$1\times10^{8}\pm4.15$	$1\times10^{8}\pm3.18$	$1\times10^{8}\pm3.78$	$1\times10^{8}\pm2.54$	0.06±0.02	$1\times10^{8}\pm4.17$	$1\times10^{8}\pm3.42\pm$	_	_
B. amyloliquefaciens strain TMM 25	$1\times10^{8}\pm4.98$	$1\times10^{8}\pm4.24$	$1\times10^{8}\pm3.97$	$1\times10^{8}\pm3.19$	0.02±0.01	$1\times10^{8}\pm3.16$	$1\times10^{8}\pm4.11$	_	_
B. subtilis strain MSM 24	$1\times10^{8}\pm2.78$	$1\times10^{8}\pm4.19$	$1\times10^{8}\pm3.65$	$1\times10^{8}\pm4.21$	0.4±0.14	$1\times10^{8}\pm2.23$	$1\times10^{8}\pm4.61$	_	_
B. licheniformis strain MR 78	$1\times10^{8}\pm4.19$	$1\times10^{8}\pm4.25$	$1\times10^{8}\pm2.12$	$1\times10^{7}\pm4.18$	0.4±0.12	$1\times10^{8}\pm2.59$	$1\times10^{8}\pm3.90$	_	_

99% homologous to *B. tequilensis*, *B. cereus*, *B. amyloliquefaciens* and *B. subtilis*.

Probiotic potentials: From all the *Bacilli* isolates, only *B. licheniformis* strain MR 78, *B. amyloliquefaciens* strain TMM 25, and *B. subtilis* strain MSM 24 were able to grow in the presence of bile salts and gastric juice, and different acidity levels. However, these bacteria had no hemolytic capability and lecithinase activity. Therefore, the other bacteria removed from cell toxicity and antibiotic resistance tests.

As Table 4 shows, there was no significant difference in adhesion among the samples (P<0.05), but, there was significant difference between test and positive probiotic control in this factor (P>0.05). Adhesions of the test samples were significantly lower than the adhesion of the pathogenic control (*L. monocytogenes)* (P<0.05). No invasion was observed in *B. subtilis* strain MSM 24 and *B. amyloliquefaciens* strain TMM 25, but these bacteria had no significant difference in invasion compared to negative control and the other *Bacilli*. The highest invasion was related to the pathogenic control, which were significantly different compared to the other groups (P<0.05). As the Table 5 shows, *B. subtilis* strain MSM 24, *B. amyloliquefaciens* strain TMM 25 and *B. licheniformis* strain MR 78 have no antibiotic resistance. According to the Tables 3, 4 and 5, *B. licheniformis* strain MR 78,

Table 4. Adhesion and invasion percentages of *Bacillus subtilis* strain MSM 24, *B. amyloliquefaciens* strain TMM 25 and *B. licheniformis* strain MR 78 in cell culture medium (N=3).

Index / Experiment	Adhesion (%)	Invasion (%)
Phosphate-buffered saline (as negative control)	0a	0a
Bacillus subtilis strain MSM 24	0.74±0.02b	0a
Bacillus amyloliquefaciens strain TMM 25	0.79±0.012b	0a
Bacillus licheniformis strain MR 78	0.59±0.014b	0.01±0.0001a
Bacillus coagulans (IBRC-M 10807) (as positive probiotic control)	1.1±0.09b	0.09±0.003a
L. monocytogenes (IBRC-M 10671) (as pathogen control)	2.3±0.11c	35.1±1.2b

The different letters in the same column indicate significant differences (P<0.05).

Table 5. The growth inhibition halo diameters of *Bacillus licheniformis*, *B. amyloliquefaciens* and *B. subtilis* (mm).

Bacteria / Antibiotic disk	*B. subtilis* strain MSM 24	*B. amyloliquefaciens* strain TMM 25	*B. licheniformis* strain MR 78
Erythromycin (15 µg)	32±3.19	29±1.14	32±2.28
Penicillin (10 µg)	31±2.13	28±2.25	35±3.35
Amoxicillin (25 µg)	33±3.21	32±3.23	33±2.43
Gentamycin (10 µg)	22±2.16	31±2.11	31±1.56
Azithromycin (15 µg)	32±3.24	34±3.18	33±2.53
Cefalotin (30 µg)	29±1.17	33±1.21	30±3.51
Clindamycin (2 µg)	31±2.35	35±3.39	34±3.41
Streptomycin (10 µg)	29±3.41	30±3.59	32±2.65
Tetracycline (30 µg)	34 ±2.31	29±2.42	34±2.54
Chloramphenicol (30 µg)	33±2.56	33±2.34	30±1.52

B. amyloliquefaciens strain TMM 25, and *B. subtilis* strain MSM 24 had probiotic potentiality.

Discussions

Bacilli are diverse bacterial species, found ubiquitously in nature. They are highly adaptable to different environmental conditions and capable to grow in various environments, including the gastrointestinal tract of animals, soil and plants. Different strains of *B. subtilis* were isolated form different fishes, as probiotic for foods with aquatic origin viz. *B. subtilis* AB1 in rainbow trout (Newaj-Fyzul et al., 2007), *B. subtilis* in ornamental fish (Lalloo et al., 2017), *B. subtilis* in *Labeo calbasu* (Kavitha et al., 2018), *B. subtilis* strain VITNJ1 isolated from the gastrointestinal tract of *Oreochromis niloticus* (Efendi and Usra, 2014) and *B. subtilis* strain G024 in *Paralichthys lethostigma* (Chen et al., 2016). They recommended using the *Bacillus* isolates in the aquaculture industry for their probiotic potentiality. To the best of our knowledge, the present research is the first attempt to explore the isolation of *B. subtilis* from rainbow trout in Iran. *Bacillus subtilis* is found in the upper layers (1-3 cm) of various soils (Mongkolthanaruk, 2012), therefore, this bacterium enters into trout body through water. Hence, this species was observed in more fishes compared to the other *Bacillus* species.

The present study reported the isolation of probiotic *B. amyloliquefaciens* from trout. It has been isolated from aquatic animals and other sources in different countries. Krishnan et al. (2014) isolated probiotic *Bacillus* species from the gastrointestinal tract of *Anabas testudineus* and *Labeo rohita*. Chen et al. (2016) isolated *B. amyloliquefaciens* strain N004 from the gastrointestinal tract of southern flounder and sediments of farming ponds of sea cucumber in China and recommended that it could be used as a probiotic strain. Kavitha et al. (2018) isolated probiotic *B. amyloliquefaciens* from the gastrointestinal tract of *L. calbasu*. Cao et al. (2010) isolated *B. amyloliquefaciens* from sediments in Shanghai. Sugita et al. (1998) isolated *Bacillus* species strain NM 12 from the gastrointestinal tract of Japanese coastal fishes. Nevertheless, there is no report about isolation of this bacterium from trout in Iran. Some members of *B. amyloliquefaciens* are found in plants and their roots and provide benefits for plant growth.

Bacillus amyloliquefaciens strains are widely used in various commercial formulations to boost plant growth. These bacteria are readily transmitted to aquatic animals from water flowing and through plants that harbor the bacteria (Chen et al., 2007).

Bacillus tequilensis spp. was isolated from the intestines of the farmed rainbow trout. Its isolation of aquatic animals has not been reported yet. There are a few reports of the presence of this bacterium in water. This indicates the effect of the physiochemical conditions of water or soil and/or antagonistic activity of other bacteria on the survival of this bacterium.

Bacillus licheniformis strain MR 78, *B. amyloliquefaciens* strain TMM 25, and *B. subtilis* strain MSM 24 *B. cereus* spp. was isolated from the intestine of the farmed rainbow trout. *Bacillus cereus* was isolated from other fishes by Rasool et al. (2017) and Lalloo et al. (2007). Kavitha et al. (2018) also isolated *B. cereus* from the gastrointestinal tract of *L. calbasu*. Nevertheless, its isolation of farmed rainbow trout has not been reported from Iran. *Bacillus cereus* might be as vegetative cells or spores, which can adapt to the environmental changes, thus, it resists in environment. Then, penetrates to the rivers and trout farms (Rasool et al., 2017).

Bacillus licheniformis spp. was isolated from the intestine of the farmed rainbow trout. Lalloo et al. (2007) reported the isolation of *B. licheniformis* from common carp. Pasmik et al. (2008) carried out a study on the isolation of *B. licheniformis* from Atlantic menhaden (*Brevoortia tyrannus*). *Bacillus licheniformis* may be a common flora of some fish species. It is also a part of fish feed. In addition, *B. licheniformis* is ubiquitous in the environment and used in treatment plants often enter into rivers and trout farms (Pasnik et al., 2008).

Based on the results, *B. licheniformis* strain MR 78, *B. amyloliquefaciens* strain TMM 25, and *B. subtilis* strain MSM 24 have probiotic potentiality because they were resistant to bile salt, acid and gastric juice. Furthermore, they had no antibiotic resistance, lecithinase and hemolytic activity and toxicity in cell culture. Although, *Lactobacilli* constitute the main population of probiotics; however, other bacteria such as *B. coagulans* and *B. subtilis* and so on are also among probiotics or are regarded as probiotic candidates for the development of probiotic products (Elshaghabee et al., 2017).

Conclusion

The present study demonstrated that, of the *Bacilli* isolated from the trout intestine, *B. licheniformis* strain MR 78, *B. amyloliquefaciens* strain TMM 25, and *B. subtilis* strain MSM 24 have probiotic properties. Further studies are needed to determine the bacteria anti-micrbial properties and resistance to enable one for choosing the most suitable bacteria for use in food industry as probiotic.

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