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Original Article

Isolation, characterization and molecular identification of potential probiotic lactobacilli from gastrointestinal tracts of *Capoeta razii* and *Aristichthys nobilis*

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Abstract: The aim of this study was to isolate and identify *Lactobacillus* with probiotic potential from gastrointestinal tracts (GIT) of *Capoeta razii* and bighead carp, *Aristichthys nobilis*. GIT samples of fifteen bighead carp and sixteen *C. razii* were collected and homogenized in 0.9% NaCl buffer and cultured on MRS medium. Nine isolates were obtained from *C. razii* and 18 insolates from bighead carp. The isolates were sequentially subjected to acid- and bile salt-resistance test. Three isolates from *C. razii* and one isolate from bighead carp were both acid- and bile salt-resistant. These isolates were identified by sequencing 16s rRNA and subjected to BLAST analysis to find the highest similarity in GeneBank. The isolate from bighead carp and two isolates from *C. razii* were *L. plantarum* with the highest similarity with strain DR7. One of the isolates from *C. razii* was *L. brevis* with the highest similarity with strains 100D8 and BDGP6. Further studies are encouraged to assess these probiotic effects in aquaculture species growth performance and health status.

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Introduction

Aquaculture plays an important role in supplying high quality protein for human (Khodadadi et al., 2018). However, diseases are the main threat of aquaculture industry causing significant annual economic losses (Taheri Mirghaed et al., 2019b). To counteract this problem, various disinfectant and antibiotic agents have been applied since decades ago (Hoseinifar et al., 2015). However, application of these agents has been restricted due to environmental contamination, adverse effects on host fish and rise of new drug-resistant microbes (Alderman and Hastings, 1998; Hoseini and Tarkhani, 2013; Ghelichpour et al., 2016; Hoseini and Yousefi, 2019; Zargar et al., 2020). In this regard, the fish farmers focused on strengthening the farmed fish to resist against pathogens (Yousefi et al., 2020) by using fish antioxidant and improving their innate immune system, enabling the host to kill pathogens and resist their adverse effects (Hoseini et al., 2018; Taheri Mirghaed et al., 2019a).

Among different methods, application of feed additives is a successful technique to increase fish immune and health that enables them to resist against diseases (Lee et al., 2015). There are several classes of feed additives in aquaculture industry, including herbal additives (Hoseini et al., 2019, 2020; Paray et al., 2020), organic acids (Castillo et al., 2014; Ebrahimi et al., 2017; He et al., 2017) and probiotic (Castex et al., 2010; Hoseinifar et al., 2017; Taheri Mirghaed et al., 2018). Probiotics are beneficial microbes that are isolated form the same host or others that have important health benefits in fish (Merrifield and Carnevali, 2014). They inhabit the host gut, compete for space with harmful microbes, produce compounds that kill the harmful microbes, increase digestion and absorption of nutrients and excrete compounds that increase the host health (Merrifield and Carnevali, 2014). Lactic acid bacteria (LAB) are well-known probiotics in aquaculture. They are Gram–positive, spore-free and catalase-negative that

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ferment fibers and produce lactic acid that acidifies the host gut and restricts pathogenic microbes' development (Ringo et al., 2018). Moreover, the produced lactic acid is used by butyrate-producing bacteria to produce butyrate; a necessary compound for the host gut development and health (Piazzon et al., 2017).

The first step in probiotic production is to isolation, identification and characterization of a potential microorganism (Lee et al., 2015). A probiotic bacterium must have several characteristics to be able passing the host gastrointestinal tract (GIT) and be stabilized in gut. Meanwhile, they must tolerate acidic conditions of the host stomach and be resistant against bile salts (Merrifield and Carnevali, 2014). As a result, such properties of a potential probiotic must be first determined to ensure its efficiency when used as oral supplement. There are several studies reporting isolation of LAB from gut of different fish species including *Rutilus caspicus* (Tarkhani et al., 2020), *Mugil cephalus* (Ghosh et al., 2014), and *Channa striatus* (Allameh et al., 2014), however, there is no reports about bighead carp, *Aristichthys nobilis*, as an important aquaculture species. In addition to aquaculture species, wild fish are good sources of gut bacteria, thus, are suitable for probiotic isolation. Hence, the aim of the present study was to isolate, characterize and identify probiotic LAB from gut of bighead carp and, an endemic cyprinid in Iran, *Capoeta razii*.

Materials and Methods

Fish sampling and isolation of LAB: A total of 15 bighead carps with average weight of 2000±50 g were obtained from a Simorgh fish farm, Iran, and 16 *C. razii* specimens (average weight of 15±2 g) were collected from the Lafoor River, Savadkooh, Iran. The fish were lively transported to Islamic Azad University, Babol Branch, Babol, Iran. In the laboratory, the fish were euthanized by a sharp blow on the head followed by aseptically dissection of the gastrointestinal tract (GIT). The GIT samples were homogenized in 0.9% NaCl solution. The homogenates were then inoculated on MRS broth media and incubated under microaerophilic conditions in anaerobic jars with gas pack C (37ºC; 48 h). Then, each bacterial sample was collected and cultured on MRS agar for purification (Ghanbari et al., 2009). Then, LAB were identified by Gram staining, motility, oxidase and catalase activities assessments (Ghanbari et al., 2009).

Assessment of probiotic properties of the isolated LAB: To assess probiotic properties of the isolated LAB, the bacteria resistance against acidic conditions and bile salts were determined. The bacteria samples were taken from the MRS agar medium and cultured in MRS broth medium as mentioned above. The bacteria concentrations were adjusted at 10^9 cfu/ml. After that, 1 ml off this suspension was added to 9 ml of phosphate buffered saline pH 2 (adjusted by HCl) and incubated for 3 h. Then, the suspensions were cultured on MRS agar medium and total counts with the concentration of higher than 10^6 cfu/ml were considered as acid-resistant LAB (Allameh et al., 2014).

Acid-resistant LAB were tested for bile salt resistance test. 90 µl of fresh culture of each LAB isolates in MRS broth were taken and added to two separate test tubes, one containing 0.3% oxgall and the other as control (only MRS broth). After incubation at 37ºC as mentioned above, growth inhibition rates of the isolates (both with and without oxgall) were determined by spectrophotometric method at 600 nm (Kavitha et al., 2018). The following formula was used to determine inhibition coefficient:

$$C_{inh} = \frac{(\Delta T_8 control - T_0\ control - \Delta T_8 treatment - T_0\ treatment)}{\Delta T_8 control - T_0\ control}$$

Where C_{inh} is inhibition coefficient, T_0 treatment and T_0 control are optical density (OD) of the oxgall-containing and oxgall-free culture media, respectively, before incubation. T_8 treatment and T_8 control are ODs in oxgall-containing and oxgall-free culture media, respectively, after 8 h of incubation. Based on the calculated C_{inh}, the isolates were divided into three groups, including (1) Isolates insensitive (resistant) to oxgall (C_{inh} = 0.0), (2) Isolates with decreased growth rates (0.2 < C_{inh} < 0.4) and (3) Isolates with very low resistance (C_{inh} > 0.4). Groups

Table 1. The properties of the colonies isolated form *Capoeta razii* and *Aristichthys nobilis* GIT.

Isolates	Catalase	Oxidase	Motility	Gram type
C1	-	-	-	Bacillus G+
C2	-	-	-	Bacillus G+
C3	-	-	-	Bacillus G+
C4	-	-	-	Bacillus G+
C5	-	-	-	Bacillus G+
C6	-	-	-	Bacillus G+
C7	-	-	-	Bacillus G+
C8	-	-	+	Bacillus G+
C9	-	-	-	Bacillus G+
A1	-	-	-	Bacillus G+
A2	-	-	-	Bacillus G+
A3	-	-	-	Bacillus G+
A4	-	-	-	Bacillus G+
A5	-	-	-	Bacillus G+
A6	-	-	-	Bacillus G+
A7	-	-	-	Bacillus G+
A8	-	-	-	Bacillus G+
A9	-	-	-	Bacillus G+
A10	-	-	-	Bacillus G+
A11	-	-	-	Bacillus G+
A12	-	-	-	Bacillus G+
A13	-	-	-	Bacillus G+
A14	-	-	-	Bacillus G+
A15	-	-	-	Bacillus G+
A16	+	-	-	Cocci G+
A17	+	-	-	Cocci G+
A18	-	-	-	Bacillus G+

Table 2. Acid-resistance characteristics of colonies isolated form *Capoeta razii* and *Aristichthys nobilis* GIT.

Isolates	Acid-resistant
C1	-
C2	+
C3	-
C4	-
C5	+
C6	-
C7	-
C8	-
C9	+
A1	+
A2	-
A3	+
A4	-
A5	-
A6	-
A7	+
A8	+
A9	-
A10	-
A11	-
A12	-
A13	-
A14	-
A15	+
A16	-
A17	-
A18	+

1 and 2 were considered as bile salt-resistant.

Molecular characterization of probiotic LAB: The isolates with acid and bile salt resistance characteristics were considered as probiotic and subjected to molecular identification. Genomic DNA of the samples was extracted using the ROCHE extraction kit (F. Hoffmann-La Roche AG, Basel, Switzerland) according to the provided protocol. Extracted genomic DNA was used to amplify a fragment of the 16S rRNA gene (ca. 200 bp) using specific primers (F:CTCAAAACTAAACAAAGTT TC and R:CTTGTACACACCGCCCGTCA). 50 µl of the fragments were amplified in the presence of 200 µl of dNTPs, 5.1 mM of $MgCl_2$, 200 nM of each specific primer, and 1.5 unit of Taq DNA polymerase. Thermocycler conditions for DNA replication were an initial 94°C (3 min), 35 cycles at 94°C (1 min), 53°C (45 s), 72°C (45 s), and finally 72°C (10 min). To verify the reaction accuracy, PCR products were examined on 1.5% agarose gel, and after the verification, the products were sent to Bioneer Co. (Daejeon, South Korea) for sequencing. After sequencing, the chromatograms of the sequences were analyzed using the Chromas v. 3.1 and compared with other sequences available in the GenBank using the BLAST (Basic Local Alignment Search Tool) software. The sequences were aligned by the ClstalW method using the Mega 10 software. Phylogenetic analyses were performed by the use of Maximum Parsimony method. *Staphylococcus aureus* strain ATCC 12600 (NR_118997) and *Escherichia coli* (NR_024570) were used as the outgroup.

Results

In this study, 9 and 18 LAB were isolated from GIT of *C. razii* and bighead carp, respectively. The isolates gram staining, motility, catalase, oxidase activities are presented in Table 1. C1-C9 and A1-A18 bacteria were isolated from *C. razii* and bighead carp, respectively. Among them, catalase-positive and catalase-negative, oxidase-negative, motility-positive, and gram-negative comprised 7.4 and 92.6, 100, 3.7,

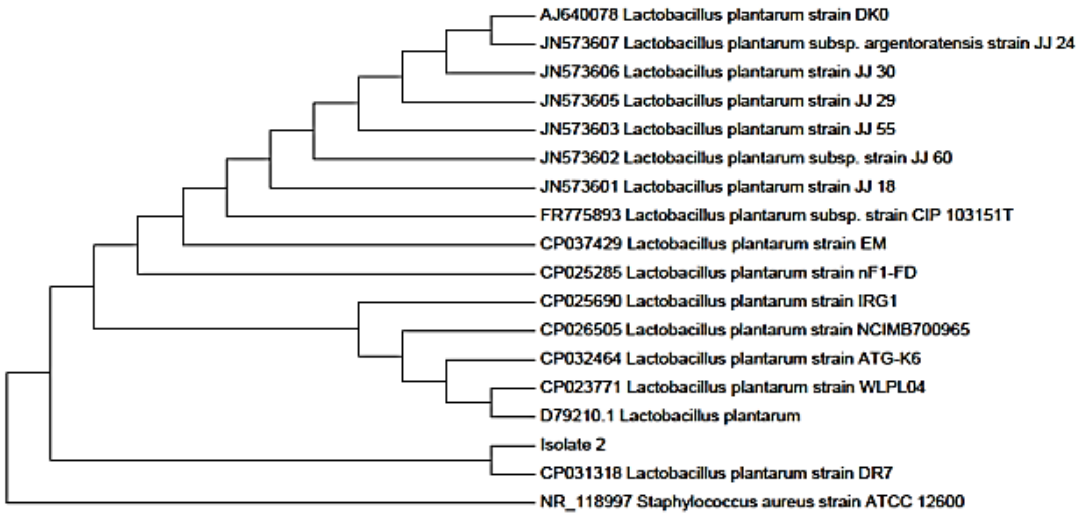

Figure 1. Phylogenetic relationships among C2 isolated sequences and some members of *Lactobacillus plantarum* by MP method based on 16S rRNA gene.

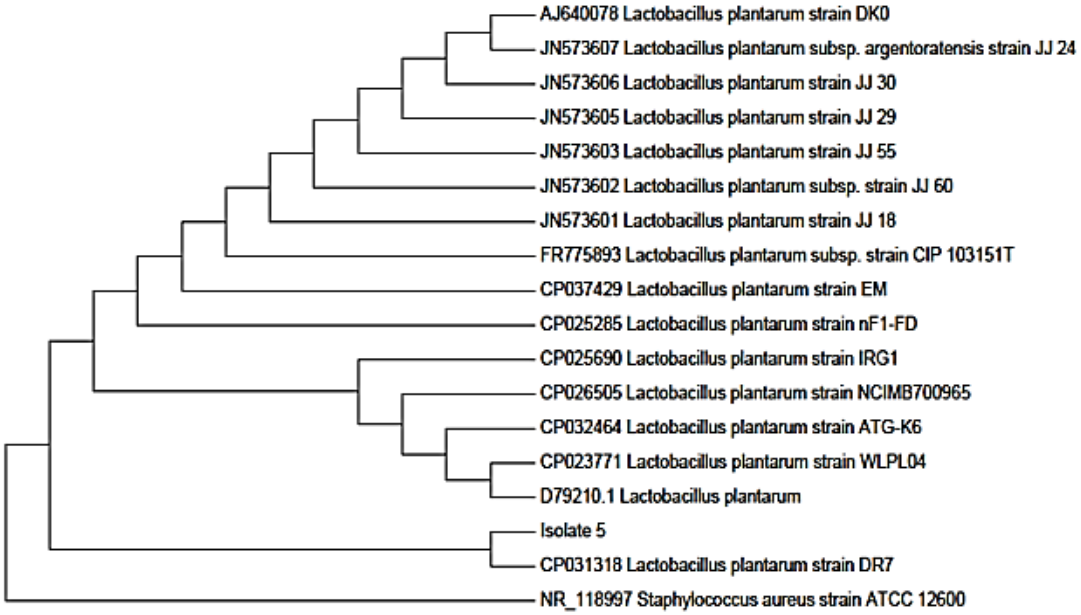

Figure 2. Phylogenetic relationships among C5 isolated sequences and some members of *Lactobacillus plantarum* by MP method based on 16S rRNA gene.

Table 3. Bile salt-resistance characteristics of the acid-resistant isolated form *Capoeta razii* and *Aristichthys nobilis* GIT.

Isolates	C_{inh}
C2	0.4
C5	0.012
C9	0.14
A1	0.54
A3	0.31
A7	0.51
A8	0.58
A15	0.76
A18	0.14

and 96.3 of the isolates, respectively. Gram-positive cocci and bacilli accounted for 3.7% and 96.3%, respectively.

The results of acid resistance test of the isolates are presented in Table 2. Accordingly, C9, C5, C2, A18, A15, A8, A7, A3, and A1 tolerated the acidic conditions, accounting for 29.6% of the total isolates. According to bile salt-resistance tests (Table 3), C9, C5, C2 and A18 isolates were resistant to bile salts. Resistance to bile salts was observed in 44.4% of acid-

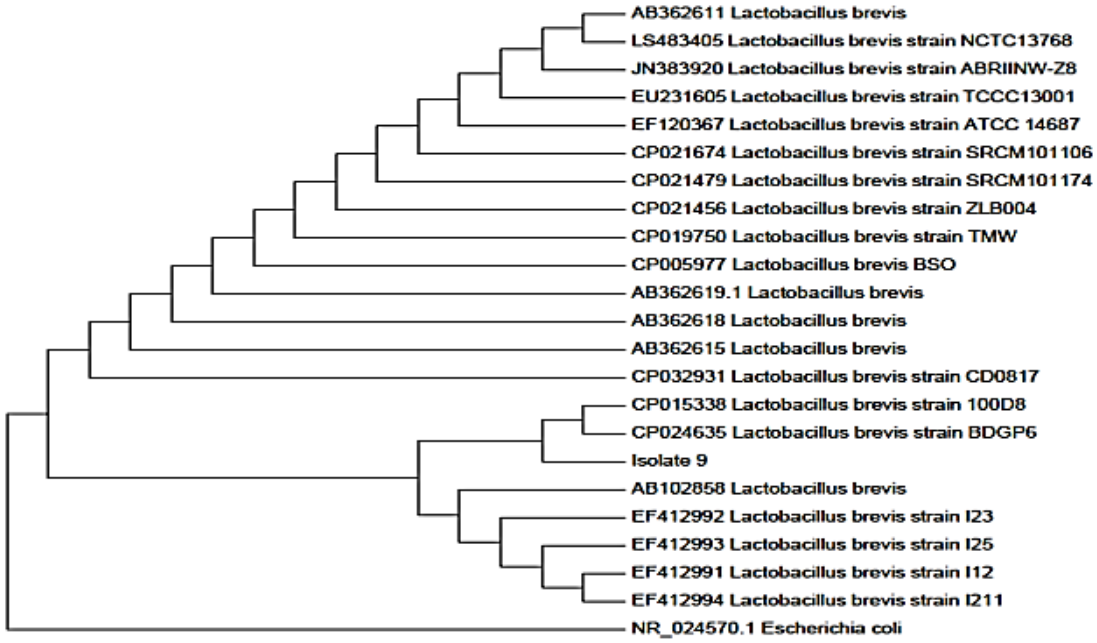

Figure 3. Phylogenetic relationships among C9 isolated sequences and some members of *Lactobacillus brevis* by MP method based on 16S rRNA gene.

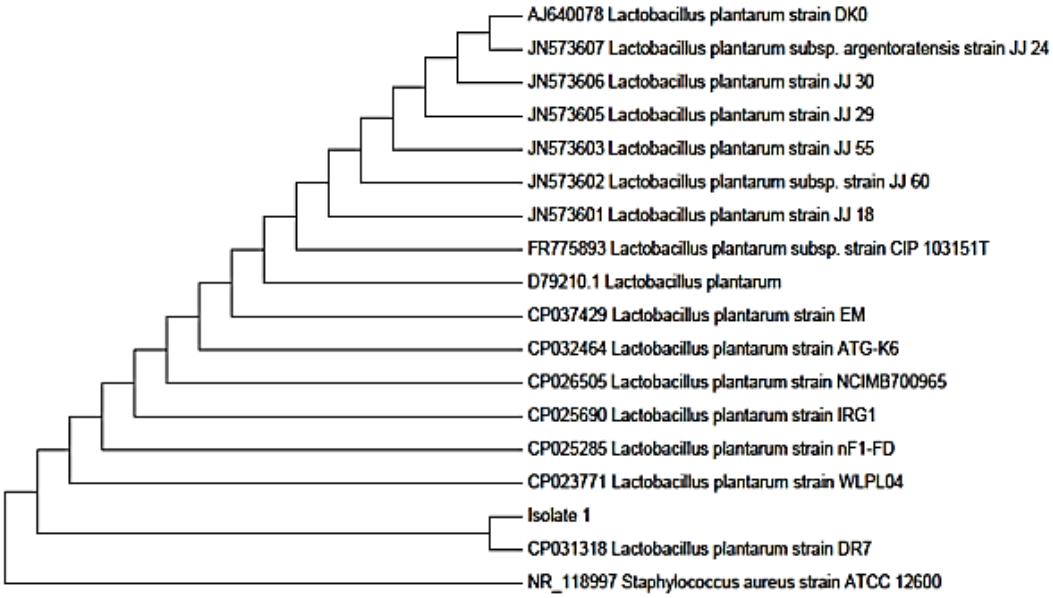

Figure 4. Phylogenetic relationships among A18 isolated sequences and some members of *Lactobacillus plantarum* by MP method based on 16S rRNA gene.

resistant isolates.

BLAST analysis of the sequences demonstrated that A18, C2, and C5 isolates were highly similar to *Lactobacillus plantarum,* and C9 isolate exhibited high similarity to *L. brevis*. Figures 1-4 depict the phylogenetic relationships among A18, C2, C5, and C9 isolates and different genotypes of lactobacilli based on the 16S rRNA gene. According to the results, A18, C2, and C5 isolates presented the most similarity with *L. plantarum* strain DR7; whereas, isolate C9 exhibited the most similarity with *L. brevis* strains 100D8 and BDGP6.

Discussions

Introduction of new probiotics is important for developing new supplemented aquafeed that improve

the host health (Ahmadifar et al., 2019). In the present study, two most important properties of a probiotic bacterium were investigated, resistance to acid and bile salts (Allameh et al., 2014). A successful probiotic must be able to tolerate acidic conditions of host stomach and bile salts in the host intestine; otherwise, it fails to stabilized in the host intestine. Four out 27 isolates were acid- and bile salt-resistant in the present study, which were very similar to *L. plantarum* DR7 and *L. bveris* 100D8 and BDGP6.

There are several studies about the isolation of *L. plantarum* from different fish; however, no one reported the strain DR7. For example, Fečkaninová et al. (2019) isolated *L. plantarum* CCM 8674 from rainbow trout, *Oncorhynchus mykiss*, from Slovakia and found it a potential probiotic. Balcázar et al. (2008) isolated strain CLFP 238 from rainbow trout and found the strain reduced adhesion of pathogenic bacteria to the fish intestinal mucus. Giri et al. (2013) isolated strain VSG3 from rohu carp, *Labeo rohita*, gut and administered it to the same species via dietary route. They found the probiotic improved the host growth, immunity and disease resistance. According to the present study, further studies are needed to illustrate benefits of *L. plantarum* DR7 isolated from *C. razii* and bighead carp on fish growth and health. The probiotic may have significant effects on fish growth and health as studies on human have demonstrated that strain DR7 was beneficial in reducing stress and anxiety, immune function and disease curing (Chong et al., 2019a, b).

Besides, *L. bveris* has been isolated form different fish species. For example, strain FPTLB3 was isolated from gut of Mrigal carp, *Cirrhinus mrigala*, with capacity to inhibit different bacterial species growth (Banerjee et al., 2013). Strain CGMCC 1.2028 was isolated from zebrafish, *Danio rerio* (Zhou et al., 2012). Moreover, previous studies have shown different strains of *L. brevis* had positive effects in fish. For example, strain JCM 1170 induced changes in gut microbial community and inflammatory responses, and subsidized mortality during bacterial infection in hybrid tilapia, *Oreochromis niloticus* ♀ × *O. aureus* ♂ (Liu et al., 2013). Likewise, dietary administration of strain JCM 1559 significantly increased bactericidal activity and resistance against bacterial infection in Nile Tilapia, *Oreochromis niloticus* (Pimentel and Katagiri, 2008).

In conclusion, three isolates from *C. razii* and one isolate from bighead carp exhibited probiotic properties based on acid- and bile salt-resistance tests. Sequencing of the isolates demonstrated they are very similar to *L. plantarum* DR7 and *L. bveris* 100D8 and BDGP6. Further studies are needed to investigate their growth-promoting and health-boosting effects in aquaculture species.

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