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Probiotic properties of some lactic acid bacteria isolated from intestine of cultured common carp, *Cyprinus carpio*, in Khuzestan province

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Abstract

In recent years, using suitable probiotic bacteria is one of the big concerns in aquaculture industry. For each species presence of lactic acid bacteria in the gut of that species, makes them a suitable probiotic candidate as food additive. In this study, lactic acid bacteria of intestine of common carp from captured fish ponds of Khuzestan province were examined. For this purpose 30 fish from several ponds were sampled. Lactic acid bacteria were isolated using specific culture media and identified by biochemical and molecular tests. Definite identification was performed by the PCR method and sequencing. The isolated probiotic bacteria identified as *Enterococcus gallinarum*, *Enterococcus casseliflavus*, *Lactococcus lactis, and Staphylococcus hominis*. The bacteria isolated in this study were able to grow in the temperature range of 25-45 ° C, pH renge of 3 to 9, media salinity containing 1-4% NaCl, and most of them in different bile doses of 7.5-2.5%. Assessing the antagonistic activity of these isolates showed that they have a relative ability when compared with a model bacteria (*Aeromonas hydrophila)*. However, the results showed that the isolated bacteria were sensitive to the commonly used antibiotics in aquatic animals. The bacteria were appeared genetically similar to the bacteria that isolated from commercial products and aquatic organisms of Indonesia, China, Japan, The United States and India. This study is the first report of isolation and identification of Enterococcus spp with probiotic capabilities in common carp in Iran as Iranian strain.

Key words: *Cyprinus carpio*, Probiotic, Lactic acid bacteria, Khuzestan

Introduction

Probiotic bacteria isolated from the aquatic microbial flora of each region have a major role in the health and growth of fish in that area. Despite the progress in development at industrial aquaculture, there are some problems such as deterioration of water quality, increasing incidence and prevalence of diseases that might lead to

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excessive use of antibiotics which it's side-effect is the occurrence of antibiotic resistance in the other aquatics bacteria (Alcaide, *et al*, 2010, Baquero, *et al*. 2008). The accumulation of residual antibiotics in aquaculture products may affect microbial biodiversity (Cabello, 2006).

Probiotics are bacterial food additives that balance the beneficial microbial biomass of the intestine and improve the immune system through increasing the population of the flora in the intestinal tract. The functions of probiotics are based on the competitive exclusion of pathogenic bacteria, antagonistic compounds synthesis, and producing of antibiotics substances. Adhesion to the intestinal mucosa, immunity modulation, tolerance to bile acid and low pH, and non-resistance to antibiotics are the essential characteristics of probiotics which have been isolated from human, dairy products and animals. These beneficial bacteria can be applied in aquaculture too (Hagi and Hoshino, 2009).

In recent years, many studies have been done on isolation and study of different factors affect the properties (habitat, stress, sex, and phylogeny) of probiotics bacteria from cyprinid fishes in Iran and other parts of the worlds. Most of these reports were about other fish species such as Shirbout, *Barbus grypus*. (Mohamadian *et al*, 2014, Mohamadian *et al*, 2016).

The natural presence of Lactic acid bacteria (LAB) in the fish gut makes them a suitable probiotic candidate for aquaculture. The most common genera of the LAB which are isolated from fish include Lactobacillus, Lactococcus, Enterococcus, Leuconostoc, Carnobacterium, and Vagococcus. Lactic acid bacteria are often gram-positive, catalase-negative, none motile and can be seen in cocci, coccobacilli, bacilli or polymorph shape (Vlková, *et al*, 2012).

There are a lot of morphological variations among the gastrointestinal tract of fishes. The presence or absence of the stomach is the main criteria for the classification of different types of them. According to the fact that Cyprinids don't have true stomach and it makes their gastrointestinal tract alkaline, LAB which is feed for them must be adapted to alkaline pH (Svanberg and Locker, 2020). Although many experiments have been done on probiotics in different aquatic species, a few comprehensive studies are available about probiotic bacteria in the gastrointestinal tract of *Cyprinus carpio*. This study aimed to identify and determine some LAB isolated from the intestine of common carp in Khuzestan province, the Southwest of Iran. Then the isolated bacteria were assessed in vitro, for the characterization of probiotic properties.

Material and Methods

Collection and processing of fish samples

A total of 30 common carp, *Cyprinus carpio*, weighing between 20-200 g were collected in three batches from 3 fish farms in Khuzestan province, the southwest of Iran during spring and summer seasons. The fish were human killed by anesthetic drug overdose (phenoxy ethanol, 0.1 ml^{-1}) and a 1 cm piece of the intestine (the end quarter of intestine) was cut and put into sterile tubes which contain 10 mL sterile normal saline. The samples were shaken, and serial dilution of 10^{-1}- 10^{-4} was prepared. The rest of the procedures were different from isolating Enterococcus, Lactococcus, and Staphylococcus as below: for Enterococcus, 100 µl of the suspension from each dilution were cultured on Kanamycin Aesculin Azide (KAA) agar and then were incubated at 37 ºC for 24 hours. For Lactococcus and Staphylococcus, the same amounts of the suspension were cultured on MRS Agar (DE MAN, ROGOSA, and SHARPE Agar) and were incubated at 30 ºC for 48 hours.

Enterococcus colonies with the typical characteristic of pure black and white which were surrounded by a dark zone, Lactococcus, and Staphylococcus with rough and convex colonies respectively were selected. Each colony from the pure cultures was harvested and sub cultured to

the three other plates using the quadrant streak method. Randomly, 30 colonies were isolated from 84 plates. Selected colonies were investigated using morphological methods, gram staining, and finally the biochemical, physiological and molecular tests.

Isolated bacteria species for probiotics properties

Temperatures tolerance test

Enterococcus isolates were cultured in nutrient agar; however, Lactococcus and Staphylococcus were cultured in MRS agar by quadrant streak method to isolate the single colonies. For evaluation of the temperature tolerance, the 100 µl of the fresh bacterial suspension (108 CFU ml^{-1}) was added into the tubes containing 5 ml nutrient broth media for Enterococcus and MRS broth for Lactococcus and Staphylococcus. Tubes were incubated at 25ºC, 30ºC, 37ºC, 40ºC, and 45ºC for 24 hours in triplicate. Each plate was incubated at their temperature for 24 hours and afterward bacterial growth was monitored by comparison with sterile serum, and the colonies were counted by the spread plate method.

Salinity tolerance test

Salinity tolerance was assayed by adding 100 µl of the fresh bacterial suspension (10^8 CFU ml^{-1}) of bacterial suspension to 5 ml nutrient broth media for Enterococcus and MRS broth for Lactococcus and Staphylococcus; including 1, 2, 3 and 4% (W/V) sodium chloride salt. All the cultures were incubated at 37 ºC for 24 hours. The growth of bacteria was investigated by dilution with sterile serum spread plate method, and these diluted were counted after the plates were incubated for 24 hours at 37 ºC.

Bile salt tolerance test

Bile salt tolerance was tested by adding 100 µl of the fresh bacterial suspension (10^8 CFU ml^{-1}) to 5 ml nutrient broth, and MRS broth media contained 0.0, 2.5, 5, and 7.5% (w/v) bile salt. All the cultures were incubated at 37 ºC for 1.5 h. The growth rate was assessed by dilution with sterile serum, and spread plate method and these diluted were counted, after the plates incubation at 37 ºC for 24 hours (Nikoskelainen, *et al*, 2003).

pH tolerance test

Tolerance to medium pH was measured by adding 100 µl of the fresh bacterial suspension (10^8 CFU ml^{-1}) to 5 ml nutrient broth and MRS broth media which their pH adjusted to 3, 4, 5, 6, 7, 8 and 9 pH using HCl 1% and NaOH 3N. All cultures were incubated at 37 ºC for 1 hour. Dilution with sterile serum and spread plate method as the growth rate of bacteria was measured after the plates were incubated at 37 ºC for 24 hours.

Antibiotic sensitivity test

The antibiotic sensitivity test was carried out for the selected strains on the most common antibiotics in aquaculture by the disc diffusion technique. Antibiotic resistance of each isolate was used to determine the dose of MAST (Mast Group Ltd) antibiotics. For Enterococcus, antibiotics contained Chloramphenicol 10 µg/disc, Penicillin 2 µg/disc, Ampicillin 2 µg/disc, Erythromycin 5 µg/disc, Gentamicin 10µg/disc, Streptomycin 5µg/disc, Enrofloxacine 5 µg/disc, and Ciprofloxacin 5 µg/disc. For Lactococcus and Staphylococcus, the antibiotics included Penicillin G 10 Unit/disk, Enrofloxacin 5 µg/disc, Amoxicillin 25 µg/disk, Streptomycin 5 µg/disc, Erythromycin 15 µg/disc, and Gentamicin 120 µg/disc. 50 µl of the strain cultured in broth for 24 hours was spread on Muller-Hinton agar, and antibiotic Bio-discs were subsequently placed on plates. Finally, the plates were incubated at 37 °C for 24 hours to determining and measuring the inhibition zone. The interpretations and zone sizes were illustrated based on the Cantón method (Cantón, 2010).

Antibacterial activity of the isolated bacterial strain

A virulent strain of *Aeromonas hydrophila* (Ahangarzadeh, *et al*, 2015), was used to determine the antibacterial effect of the candidate strain. The pathogenic bacteria were cultured in nutrient broth and incubated at 25 ºC for 24 hours. Simultaneously, the selected strains were cultured in nutrient broth and MRS broth at 37 ºC for 24 hours. Selected strain culture with 10^8 spread on LB agar by the swap. Five wells were created on LB agar. 20µl of culture with 108 CFU/ml shed to each well of LB agar and was incubated at 37 ºC for 24 hours to measuring the inhibition zone.

PCR, *16S rRNA* gene amplification

DNA extraction for molecular analysis was done from the bacterial cells according to the standard procedure for Gram-positive bacteria (DNG-plusTM kit, Sinaclon, IRAN). The 16S rRNA gene was amplified using related primers (27F, 5'-AGAGTTTGATCMTGGCTCAG-3', and 1492R, 5'-TACGGYTACC TTGTTA CGA CT T-3'). PCR amplification was performed in 25µL reaction volumes containing 12.5 µL Master Mix (PCR Master Mix 2x, 80Test/25ul-MM2011, Sinaclon, IRAN), 1µl of each primer and 1µl of DNA template. The PCR profile carried out on the MJ MiniTM thermocycler (Bio-Rad, USA) started with 3 min initial denaturation at 95 ºC, followed by 1 cycle, each consisting of a denaturation step at 95 ºC for 30sec, annealing step at 56.7 ºC for 30 seconds and the elongation step at 72 ºC for 90 sec. PCR was terminated by a final elongation step at 72 ºC for 7 minutes. The amplified products were approximately 1500 bp.

***16S rRNA* gene sequencing identification**

The 16S rRNA sequence coding region of strains was amplified by polymerase chain reaction (PCR). The sequences from 16S rRNA gene PCR products that were generated using universal bacterial primers 27 F and 1492 R were used to determine the identities of strains. Strains were sequenced by Macrogene Company, Korea. Sanger dideoxy sequencing methods were used to obtain these sequences. The sequences derived from the isolates were analyzed using the sequences analysis by Bioedit version7 and BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and their sequences were submitted to the Bankit. The accession numbers are shown in Table 6. Neighbor-joining phylogenetic trees were calculated using ClustalW in combination with the MEGA 7 package. Aligned sequences were edited to a uniform length, and a sequence identity matrix was generated for the alignments. The sequences of the strains Intended were aligned with that of the strain types to determine the identity of isolates. Bootstrap 1000 was performed to evaluate the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and were in the units of the number of base substitutions per site (Kimura, 1980).

Statistical Analyses

Data are presented as Mean ± SD. Parameters were analyzed by one-way analysis of variance (ANOVA) and Tukey post hoc test. All statistical analyses were tested at the 0.05 level of probability, using SPSS version 24.0 for PC and GraphPad prism version-7. There were three repetitions for all of the probiotic tests.

Results

Isolated bacteria identification

Morphology and Biochemical tests: All of the isolated bacteria in this study were Gram-positive. Enterococcus appeared as cocci in pairs or short-chain, Staphylococcus appeared in single, pairs and tetrads, and Lactococcus was ovoid shape in pairs or short chains. All of the necessary biochemical tests were done. The isolates were catalase and oxidase-negative, sorbitol-negative and mannitol-positive.

Lactococcus and Enterococcus were able to hydrolyze esculin.

PCR test, *16S rRNA* gene amplification and sequencing identification: In the present study, the *16S rRNA gene* of the total genomic DNA from LAB isolates was amplified and sequenced for identification. Amplification using universal primer produced a PCR product of approximately 1500bp (Figure 1). The purified 16S rRNA sequencing data of isolates were employed for bacterial identification. *16S rRNA gene* Sequencing was done by Sanger method for *Enterococcus Galinarum* and *Casseliflavus*, *Lactococcus lactis*, *Staphylococcus Hominis*, and then were compared and evaluated in Gene Bank. These sequences have been annotated in Genebank and the Accession numbers are as follow: *Enterococcus galinarum* (isolate 1, MF925494), *Enterococcus casseliflavus* (isolate 2, MF925490), *Enterococcus galinarum* (isolate 3, MF925493), *Enterococccus casseliflavus* (isolate 4, MF925492), *Enterococcus casseliflavus* (isolate 5, MF925491), *Lactococcus lactis* (isolate 6, MG098806), *Lactococcus lactis* (isolate 7, MG098804), *Staphylococcus hominis* (isolate 8, MG098803) and *Staphylococcus hominis* (isolate 9, MG098805). The sequences of the selected isolates were aligned with the 16S rRNA sequences from the GeneBank database to identify the studied microorganism. 16S rRNA sequencing data of the selected isolates clearly showed 99% homology to *Enterococcus gallinarum*, *Enterococcus casseliflavus*, *Lactococcus lactis*, and *Staphylococcus hominis*. The evolutionary history was inferred using the Neighbor-Joining method by Kimura 2-parameter model. The evolutionary distances were computed using the Kimura 2-parameter model and are in the units of the number of base substitutions per site. There were a total of 1500 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The *Enterococcus gallinarum*, *Enterococcus casseliflavus, Staphylococcus hominis* and, *Lactococcus lactis* displayed the highest nucleotide homology Chinese isolates (LC119138.1, KJ803876.1, MF327693.1, KU922413.1, and MG551237.1, 99%).

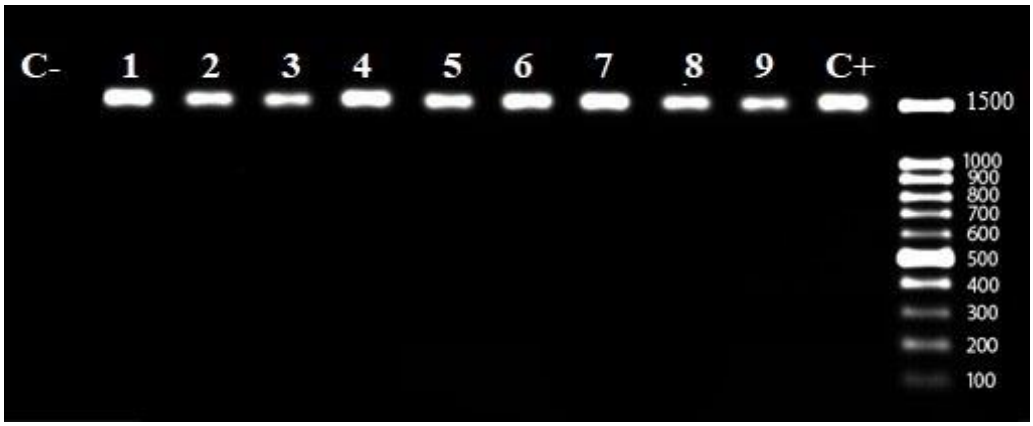

Figure 1. Amplification *16S rRNA* gene using universal primer produced a PCR product of approximately 1500bp. Positive band at 1500bp, C+ positive control, C− *negative control, M100bp DNA ladder marker. Enterococcus galinarum (isolate 1, MF925494), Enterococcus casseliflavus* (isolate 2, MF925490), *Enterococcus galinarum* (isolate 3, MF925493), *Enterococcccus casseliflavus* (isolate 4, MF925492), *Enterococcus casseliflavus* (isolate 5, MF925491), *Lactococcus lactis* (isolate 6, MG098806), *Lactococcus lactis* (isolate 7, MG098804), *Staphylococcus hominis* (isolate 8, MG098803) and *Staphylococcus hominis* (isolate 9, MG098805).

Growth temperature

All of the isolates were grown over a broad range of temperatures 25-45 ºC for 24 hours. Their optimum growth was seen in the temperature range of 25-30 ° C.

Statistical analysis of the different temperatures about the three genera of bacteria showed that there was a significant difference between the growth of isolated bacteria at different temperatures (P<0.05), But the results of the comparison between the three genus bacteria showed that there is no significant difference between the growth of bacteria at a specific temperature (P>0.05). The result of various temperature growth is mentioned in Figure 2.

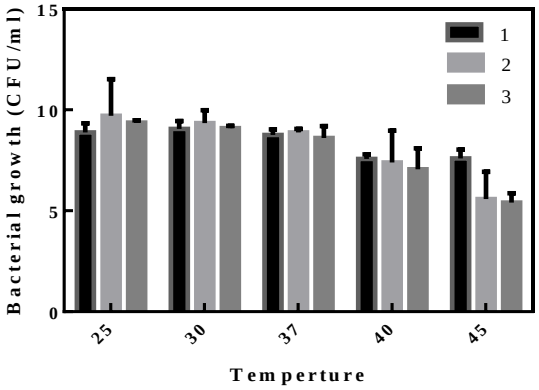

Figure 2. *Growth temperature.* Lactic Acid Growth Pattern of isolated bacteria from the common carp of Khuzestan province under different temperature conditions over a period of 24 hours (the numbers are logarithmically mean ± standard deviation). 1: Enterococcus, 2: *Lactococcus lactis*, 3: *staphylococcus hominis*.

Salinity tolerance

The isolation rate was evaluated in different levels of salinity for 24-hour incubation at 37 ºC in term of CFU ml^{-1}. By increasing the concentrations of salt, the growths of isolates gradually were reduced. Optimum of bacterial growth was determined in salinities of 1-3%. The statistical analysis of salinity between 3 genera of bacteria showed that there was a significant difference between the growth of bacteria in different salinity (P <0.05). Also, the results of the comparison between the three genera showed that the difference between the growth of bacteria in the specified salinity (P>0.05). There was a significant difference between the isolation of Enterococcus and Lactococcus bacteria at salinity 2% (P <0.05) (Figure 3).

pH tolerance

The selected strains showed relatively strong resistance to high pH levels. The durability of the isolations at degrees of different pH for 1 hour incubation at 37 ºC was demonstrated in figure 4. The survivals of the isolates were decreased in low pH whereas their resistances were increased gradually on high pH. Optimum growth of these bacteria was at pH 7 to 8, and the growth pattern of them revealed a significant difference in acidic pH and alkaline pH (P<0.05) but there were no significant differences between the bacterial growth at a specified pH (P>0.05).

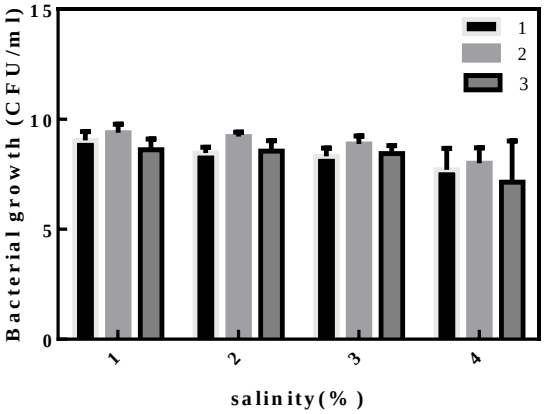

Figure 3. *Salinity tolerance*. Lactic Acid Growth Pattern of isolated bacteria from the common carp in Khuzestan province at different concentrations of NaCl in 24 hours (numbers are logarithmic mean ± standard deviation). 1: Enterococcus, 2: *Lactococcus lactis*, 3: *staphylococcus hominis*.

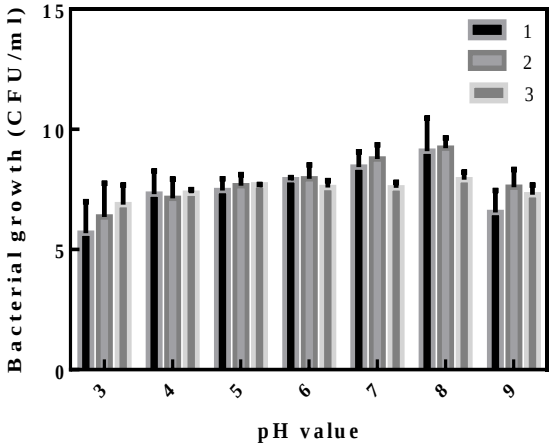

Figure 4. *pH tolerance*. The lactic acid growth pattern of isolated bacteria from the common carp in Khuzestan province under different pH conditions over a period of one hour (the numbers are logarithmic mean ± standard deviation).1: Enterococcus, 2: *Lactococcus lactis*, 3: *staphylococcus hominis*.

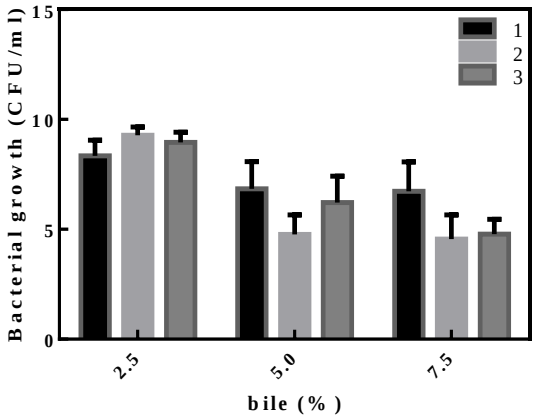

Figure 5. *Bile salt tolerance.* Lactic Acid Growth Pattern of bacteria isolated from common carp of Khuzestan province in different concentrations of bile salts over a period of one and half hours (numbers are logarithmic mean ± standard deviation). 1: Enterococcus, 2: *Lactococcus lactis*, 3: *staphylococcus hominis*.

Bile salt tolerance

The result of tolerating bile salt of isolations was shown with three different concentrations for 1/5-hour incubation at 37 ^{o}C in term of CFU ml^{-1} (figure 5). Growth medium with 0% bile served as control and survivability of the isolate was represented in percentage. The maximum growth was observed in all of the isolations in control without bile salt and medium with 2.5% bile salt. Reduction of tolerance was seen 5 and 7.5% bile salt. Among *Enterococcus*, isolate 5 had less bile tolerance than the other Enterococcus isolates. Lactococcus and Staphylococcus were able to grow in a narrower range of bile salts. Statistical analysis of bile tolerance between the three genera bacteria provided that there was a significant difference between bacterial growth in different percentages of bile ($P<0.05$), also among the three genera bacteria, there was a difference between bacterial growth in the particular of bile percentage ($P<0.05$).

Antibiotic sensitivity test

The susceptibility and resistance pattern of all of the isolates against nine antibiotics was done. Annotations indicated that five isolates of Enterococcus were susceptible to Enrofloxacin, Ampicillin, Penicillin, Chloramphenicol, tetracycline, Erythromycin, Ciprofloxacin, Streptomycin, and Gentamycin and only one isolate had relative resistance to ampicillin. Antibiotic susceptibility results showed that Lactococcus and Staphylococcus isolates in this study were susceptible to Enrofloxacin, Amoxicillin, Streptomycin, Penicillin, Erythromycin and Gentamicin bacteria. Susceptibility to antibiotics can be considered a positive characteristic of bacteria employed in probiotics. The interpretations of the inhibition zone were determined according to the zone size of the chart of CLSI.

Antibacterial activity of the isolated bacterial strain

All isolates with antimicrobial activity against *Aeromonas hydrophilia* were used for the quantitative determination of their antimicrobial activities. Approximately, all isolates had the same antimicrobial activity against this pathogen. In this study,

Enterococcus casillifulus was the least inhibited, and *Enterococcus galinarium* 1 was the most inhibited isolate.

Discussion

The most substantial amount of world fish production, as well as in Iran, belong to carps (Merrifield, *et al*, 2014). Even though this kind of fish production in Iran, in comparison with other fishes is approximately more than 52%, significant economic losses often occur in this species culture due to infectious diseases. However, using antibiotics may results in antibiotic resistance in the bacterial community. According to previous researches, probiotics are used as an alternative for antibiotics. Probably the best way to improve fish health conditions is to use their lactic acid bacteria, because these kinds of bacteria are well adapted to the physiological conditions of the host and could play a significant role in the ecology of the gastrointestinal tract, and should, therefore, be more competitive than lactic acid bacteria from other sources (Balcázar, *et al*, 2008). Zones and period diversities can influence the isolated species. Despite several studies on aquatic probiotics, a few reports on isolated bacteria from *Cyprinus carpio* are available.

In this study, many probiotic bacteria were isolated from distal intestinal samples and then identified as *Enterococcus gallinarum*, *Enterococcus casseliflavus*, *Lactococcus lactis*, and *Staphylococcus homonis*. All three genus isolated from common carp is the first report as probiotic bacteria from Iran. According to many reports, from upper parts of the gastrointestinal tract to hindguts, the population of microflora increase gradually but their variation decreases. Lactic acid bacteria were isolated from the intestinal tract of common carp and freshwater shrimp and identified them by biochemical and molecular methods. In their investigation they identified *Enterococcus faecium*, *Lactococcus garveia*, and *Pediococcus acidilactici* (Cai, *et al*, 1999). Among them, *Lactobacillus gvareia* was the dominant bacterial population. Also, the effect of *Enterococcus gallinarium* L1 as a probiotic on the immune system of four species of fish has been investigated. Phagocytic activity and peroxidase production of leukocytes were observed in these four species 30 minutes after incubation with *Enterococcus gallinarium*. The highest levels of peroxidase in red porgy fish and most phagocytic activity have been observed in sea breams (Román, *et al*, 2015).

In our study we investigate the lactic acid bacteria species in freshwater fish species, and seasonal variations of their populations. It has been shown, *Lactococcus lactis* was dominant in summer in common carp. In the screening and evaluation of probiotic properties of lactic acid bacteria in the common carp in Japan, the dominant lactic acid bacteria population was *Lactococcus lactis* in the summer and winter, *Lactococcus raphinolacticus* (Hagi and Hoshino, 2009).

Staphylococcus hominis was isolated in this study. After the manual feeding of the common carp for 30 days with a sweet potato probiotic, bacteria containing *Bacillus pumilus, Staphylococcus aureus, Staphylococcus hominis*, and *Aeromonas veronii* were isolated. The presence of these bacteria in the common carp improved its growth and health. Also, these bacteria do not have pathogenicity for this species of fish (Djauhari, *et al*, 2017).

Determination of salinity tolerance of fish is essential for fish production in different waters, as well as for the determination of stocking density. Common carp is a Freshwater fish species and can tolerate an only narrow range of salinity usually less than 5 ppt. Our study has shown; Enterococcus, Lactococcus, and Staphylococcus isolated from *Cyprinus carpio* were able to grow by 1% to 4% salinity. All three isolated species had the same ability to grow in different salinity (1-

3%) however, *Staphylococcus hominis's* growth, decreased by 4% salinity compared to the other species. There was a significant difference between the isolation of Enterococcus and Lactococcus bacteria isolates in salinity 2% (P <0.05) (Mangat and Hundal, 2014). A study on salinity tolerance of certain freshwater fish conducted among carps species resulted that higher salinity tolerance was observed in *Cyprinus carpio* and the lowest one showed in mrigal and fringe-lipped carp (Mohamadkasim, 1982).

In this study, pH tolerance was observed in all of the isolated species. The range of the pH examination was 3 to 9. pH is the principal factor that affects water quality. The homeostasis can be influenced by pH and fish growth, and consequently, the reproduction may change. The optimal pH range is 6.5-9.0 for *Cyprinus carpio*. The highest tolerance was recorded in alkaline pH 8, and at lower pH, moderate tolerance was observed. Two Enterococcus isolates had the same tolerance in pH 5 to 8. LAB isolated from freshwater had not available growth in pH 3.5. However, some of them grew in pH 4, and most of the isolates grew in pH 4.5 to 9 (Muthukumar and Kandeepan, 2015).

The digestion time may be influenced by several factors such as diet, age, size, species, and stress, previous nutrition history effect, so all of these factors can affect secreted enzymes, bile salt, and gut microbiome community (Hagey, *et al*, 2010). Cypriniformes have unique bile salt composition (C27 bile alcohol (5α-cyprinol) among all species. Bile salt diversity can occur as a result of its production in hepatocytes and gut microbiome activities. The liver synthesizes primary bile salt, but secondary bile salt is produced by extrahepatic changes, especially as a result of host bacteria activity in the distal part of the gut. In this study, all of the isolated species could tolerate a wide range of bile salt. The highest tolerance was observed in 2.5% bile salt, also the same tolerance was recorded at about 5% and 7.5%. The growth of Enterococcus bacteria in bile (2.5% and 5%) was significantly higher than Lactococcus (P <0.05), and the growth of Enterococcus bacteria in 7.5% bile was also higher in comparison with the Lactococcus and Staphylococcus (P <0.05). One of the most important aspects of the LAB is its high resistance to the toxicity of bile salt and other steroids. The main goals of using LAB in probiotics is its ability to modify the bacterial population by decreasing the concentration of secondary bile salt which can cause colon cancer (Philipp, 2011, Bourouni, *et al*, 2015). It has been shown to have an inhibitory effect on *Aeromonas hydrophila*, *Aeromonas salmoninosida*, *Vibrio anguillarum*, *Staphylococcus aureus* and *Carnobacterium sp.*. Phospholipid Biosurfactants produced by *Staphylococcus hominis* isolated from tilapia has an inhibitory effect on the growth of *Aeromonas hydrophilia*. In the present study, all three genera isolates had an inhibitory effect on *Aeromonas hydrophila* growth. The sequences of isolated bacteria in this study were compared with sequences in the Genebank, especially sequences in neighboring countries of Iran. Despite this fact, Iran has maritime borders with southern countries like Iraq, but the sequence results in this study showed the high similarities with sequences from Chinese probiotic bacteria; probably it can due to importing the common carp eggs from China.

In this study, many probiotic bacteria were isolated from distal intestinal samples and four species were identified (i.e. *Enterococcus gallinarum*, *Enterococcus casseliflavus*, *Lactococcus lactis*, and *Staphylococcus homonis)*. All these three genera and four species isolated from common carp had the good probiotic capability and they are the first report as probiotic bacteria in common carp from Iran. Because of the good probiotic properties of current study isolates, these

strains could be potentially used in aquaculture and as healthy food products. However further in vivo study is necessary to prove the good effects of these isolates. Probiotics are considered as feed additives or dietary supplements providing more healthy nutrition without the need for antibiotic-type growth promoters. Hence, the aquaculture industry can produce superior quality products for human consumption by using such additives.

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Conflict of interest

The authors of this article declare no conflict of interest.

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References

Ahangarzadeh, M., Ghorbanpour, M., Peyghan. R. (2015). Role of *Aeromonas hydrophila* in bacterial septicemia of cultured carps in khouzestan province. *Iranian Journal of Veterinary Research*, 11(3): 5-16.

Alcaide, E, Blasco, M.D. & Esteve, C. (2010). Mechanisms of quinolone resistance in *Aeromonas species* isolated from humans, water and eels. *Research in microbiology*, 161(1): 40-45.

Balcázar, J.L., Vendrell, D. & de Blas, I. (2008). Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. *Aquaculture*, 278(1-4): 188-191.

Baquero, F, Martínez, J.L., Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Currunt Opinion in Biotechnology*; 19(3): 260-265.

Bourouni, O.C., Barros-Velazquez, J. & Calo-Mata, P. (2015). Lactic acid bacteria associated with the digestive tract and skin of Sea bream (*Sparus aurata*) cultured in Tunisia. African Journal of Microbiology Research, 9(31): 1853-1865.

Cabello, F.C. (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental microbiology*, 8(7): 1137-1144.

Cai, Y., Suyanandana, P. & Saman, P. (1999). Classification and characterization of lactic acid bacteria isolated from the intestines of common carp and freshwater prawns. *Journal* of *General* and *Applied Microbiology*, 45(4): 177-184.

Cantón R. (2010). Interpretive reading of the antibiogram: a clinical necessity. *Enfermedades Infecciosas y Microbiología Clínica*. 28(6): 375-385.

Djauhari, R., Sukenda, A. & Suprayudi, M.A, (2017). Application of microencapsulated synbiotic to improve the growth performance and health status of common carp (*Cyprinus carpio*) cultured in the ponds. *Pakistan Journal of Biotechnolpgy*, 14(1): 43- 47.

Hagey, L.R., Møller, P.R., & Hofmann, A.F. (2010). Diversity of bile salts in fish and amphibians: evolution of a complex biochemical pathway. *Physiological* and *Biochemical Zoology*, 83(2): 308-321.

Hagi, T. & Hoshino, T. (2009). Screening and characterization of potential probiotic lactic acid bacteria from cultured common carp intestine. *Bioscience, Biotechnology and Biochemistry*, 73(7): 1479-1483.

Hanol Bektas, Z., Ucar, F.B. & Giray, B. (2020). Identification and probiotic properties of lactic acid bacterial isolated from freshwater fish. *Iranian Journal of Fisheries Sciences*. 19(4): 1795 – 1807.

Kimura, M. A. (1980). simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal* of *Molecular Evolution*. 16(2): 111-120.

Mangat, H. & Hundal, S. (2014). Salinity tolerance of laboratory reared fingerlings of common carp, *Cyprinus carpio* (Linn.) during different seasons. *International Journal* of *Advanced* Research, 2(11): 491-496.

Merrifield, D.L., Balcázar, J.L., Daniels, C. (2014). Indigenous lactic acid bacteria in fish and crustaceans. In: Daniel, M and Einar, R. (eds). Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics. (1th Edn.), Hoboken, New Jersey, John Wiley & Sons, Ltd : PP: 128-156.

Mohammadian, T., Alishahi, M., Tabandeh, M.R., Ghorbanpoor, M. & Gharibi, M. (2016) Probiotic effects of *Lactobacillus plantarum* and *L. delbrueckii sp. bulguricus* on some immune-related parameters in *Barbus grypus*. *Aquaculture International*. 24:225-242

Mohammadian, T., Ghorbanpour, M., Alishahi, M. and Tabandeh, M.R. (2014) Isolation and biochemical identification of potentially probiotic bacteria from *Barbus grypus* intestine. *Iranian Journal of Veterinary medicine*. 10: 89-102.

Mohamadkasim, H. (1982). Effect of temperature on respiration of *Labeo fimbriatus* (Bloch). Indian Journal of Fisheries, 29(1-2): 85-93.

Muthukumar, P. & Kandeepan, C. (2015). Isolation, identification and characterization of probiotic organisms from intestine of fresh water fishes. *International Journal* of *Current Microbiology* and *Applied* Sciences, 4: 607-616.

Nikoskelainen, S., Ouwehand, A.C. & Bylund, G. (2003). Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Fish and shellfish immunology*, 15(5): 443-452.

Philipp, B. (2011). Bacterial degradation of bile salts. *Applied Microbiology* and *Microbial Biotechnology*, 89(4): 903-915.

Román, L., Padilla, D. & Acosta, F. (2015). The effect of probiotic *Enterococcus gallinarum* L-1 on the innate immune parameters of outstanding species to marine aquaculture. *Journal of Applied Animal Research*, 43(2): 177-183.

Svanberg, I. & Locker, A. (2020). Ethnoichthyology of freshwater fish in Europe: a review of vanishing traditional fisheries and their cultural significance in changing landscapes from the later medieval period with a focus on northern Europe. *Journal of Ethnobiology and Ethnomedicine*, 16(68): 1-29.

Vlková, E., Kalous, L. & Bunešová, V. (2012). Occurrence of bifidobacteria and lactobacilli in digestive tract of some freshwater fishes. Biologia, 67(2): 411-416.

Wuertz, S., Schroeder, A. & Wanka, K.M. (2021). Probiotics in Fish Nutrition Long-Standing Household Remedy or Native Nutraceuticals? *Water*, 13(1348): 1-19.

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بررسی خصوصیات پروبیوتیکی باکتری‌های اسید لاکتیکی جدا شده از روده ماهی کپور معمولی خوزستان

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چکیده

در سال‌های اخیر تهیه و معرفی باکتری‌های با خاصیت پروبیوتیکی مناسب، از اهمیت به سزایی در آبزی پروری برخوردار شده است. برای هر گونه از ماهیان، باکتری‌هایی که از روده‌ی همان گونه ماهی جدا می‌شود، احتمال موفقیت بیشتری از نظر معرفی به عنوان گونه‌ی پروبیوتیکی دارد. در این مطالعه باکتری‌های اسید لاکتیک متنوعی از روده‌ی ماهی کپور معمولی استان خوزستان جداسازی و به روش‌های بیوشیمیایی و مولکولی (روش PCR و سپس توالی یابی) شناسایی شدند. برای این منظور تعداد ۳۰ قطعه ماهی کپور معمولی از مزارع مختلف استان صید و از روده‌ی آن‌ها بر روی محیط‌های اختصاصی کشت داده شد. باکتری‌های با خواص پروبیوتیکی جدا شده شامل: انتروکوکوس گالیناروم، انتروکوکوس کازیلوفلاووس، لاکتوکوکوس لاکتیس و استافیلوکوکوس هومینیس بودند. این باکتری‌ها قابلیت رشد در دمای ۲۵ تا ۴۵ درجه‌ی سانتی‌گراد، پ-اچ بین ۳ تا ۹، محیط حاوی ۱ تا ۴ درصد نمک و محیط حاوی نمک‌های صفراوی ۲/۵ تا ۷/۵ درصد بوده‌اند. از نظر مقاومت در مقابل آنتی‌بیوتیک‌ها، مقاومت قابل توجهی در مقایسه با باکتری مدل (آئروموناس هیدروفیلا) داشته‌اند. اگرچه نتایج نشان داد این باکتری‌ها نسبت به بسیاری از آنتی‌بیوتیک‌های متداول، حساس بودند. در مقایسه فیلوژنی این باکتری‌ها، مشخص گردید این گونه‌ها از نظر ژنتیکی با گونه‌های جدا شده از کشورهای اندونزی، چین، ژاپن، امریکا و هند مشابهت دارند. این مطالعه اولین مورد شناسایی باکتری‌های جنس انتروکوکوس با خواص پروبیوتیکی از ماهی کپور معمولی خوزستان می‌باشد.

کلمات کلیدی: کپور معمولی، پروبیوتیک، لاکتیک اسید، خوزستان

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