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Identification and probiotic properties of lactic acid bacterial isolated from freshwater fish

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Abstract

Since chemotherapeutic agents used to control microbial fish diseases have led to resistance in bacteria, alternative methods have emerged in recent years. In this regard, identification of lactic acid bacteria with good probiotic properties and determination of their probiotic properties are extremely important. In this study, 25 lactic acid bacteria were isolated from freshwater fish and identified at species level using phenotypic, biochemical and molecular tests. The pH tolerance, antagonistic activity and antibiotic sensitivity of these 25 strains were examined, and they were considered as bacteria displaying the best activity in the potential probiotic treatment of fish diseases. *Lactococcus lactis* species numbered F2, F4, F9 and F10 were determined to have a potential probiotic capacity.

Keywords: Probiotic, Molecular identification, Lactic acid bacteria, ARDRA technique

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Introduction

Nowadays, fish diseases emerging due to the increase in commercial-scale aquaculture operations have become a major limiting factor in aquaculture (Bondad-Reantaso *et al*., 2005; Kesarcodi-Watson *et al*., 2008; Subasinghe *et al*., 2009). Major bacterial pathogens leading to fish diseases are *Aeromonas salmonicida* (Furunculosis), *Yersinia ruckeri* (enteric red mouth disease), *Vibrio anguillarum* (vibriosis), *Aeromonas hydrophila* (minor surface lesions, septicemia, internal bleeding) and *Lactococcus garvieae*' (hemorrhagic septicemia and meningoencephalitis) (Balcazar *et al*., 2007 ab; Öztürk and Altınok, 2014). These diseases are quite common all over the world and cause serious economic losses in salmon and trout aquaculture (Austin and Austin, 2012). Vaccination and antibiotics are extensively used to combat fish diseases. When administered to immunologically immature fish, vaccination is ineffective. Antibiotics cause an increase in resistance among pathogenic bacteria and when these resistant bacteria enter the intestinal tract of humans, they pose a threat to the treatment of diseases (Denev *et al*., 2009). Therefore, during the last 10 years, there has been an increased interest in using probiotics to prevent the growth of pathogenic microorganisms and to decrease the rate of fish diseases (Ringo and Gatesoupe, 1998; Ringo, 2004; Perez-Sanchez *et al*., 2011). Good probiotic stomach acids should generate resistance to damage caused by bile salts and proteases; reduce the intestinal pH by producing lactic acid and thereby prevent the growth of pathogenic bacteria, reduce the production of several toxic and carcinogenic metabolites, help the absorption of minerals such as calcium due to the increased intestinal acidity, and be able to produce compounds such as bacteriocins, organic acids, and hydrogen peroxide which inhibit the growth of pathogenic microorganisms, and vitamin B and K. Lactic acid bacteria (LAB) have the above-mentioned properties and they are abundantly found in nature (Perez-Sanchez *et al*., 2011; Corcionivoschi *et al*., 2010). In studies conducted so far, lactic acid bacteria have been demonstrated to greatly differ from one species of fish to another and from one geographic region to another (Buntin *et al*., 2008; Bushell and Burns, 2012). Therefore, isolation and identification of bacteria in the gastrointestinal system of fish have gained importance. Since phenotypic and biochemical tests used for the identification of LABs are inadequate, molecular biological methods have been used recently. Through molecular biological methods, it has been possible to understand the characterization of the microbiota of the stomach and gut, and interactions of bacteria with bacteria and bacteria with host in sickness and in health (Brunvold *et al*., 2007; Liu *et al*., 2008).

In this study, the aim was to determine phenotypic and molecular characterization, antagonistic effects, pH tolerance and antimicrobial activities of LAB isolated from

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freshwater fish due to the need for new probiotics to fight diseases.

Materials and methods

Isolation of bacteria

Gut samples obtained from healthy rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), pool fish (*Carassius gibelio*) and zander (*Sander lucioperca*) living in Egirdir Lake were diluted for the isolation of LAB. The LAB isolates were inoculated in the selective media and in TSA to enhance the chances of isolation. All the fish were treated in 1 liter of water containing 150 mg triacine methanesulphonate (MS-222) for 15 min and killed (the ethic committee with 68385072-604/0224 number in 01.03.2014 date obtained from Mediterranean Aquaculture Research Directorate-TURKEY) before the start of the study. Then one gram of the sample obtained from the fish gut was placed in 10 ml PBS (phosphate-buffered saline) and diluted 10^{-7} times. 0.1 ml of dilutions were seeded on TSA (Tryptic soy agar: Merck KGaA Darmstadt Germany) (this medium was used to increase the chances of isolation), MRS (de Man Rogosa and Sharpe agar (Merck KGaA Darmstadt Germany) and M17 (Conda Pronadisa) agar and incubated at 22 °C under aerobic and anaerobic conditions (The MRS petri was incubated in anaerobic jar-Anaerocult A to produce anaerobic conditions) for 24-48 hours (Balcazar *et al*., 2007a). Colonies isolated from TSA, MRS and M17 agars were stored at -80°C in TSB (Tryptic Soy Broth: Merck KGaA Darmstadt Germany) containing 15% glycerol (Balcazar *et al*., 2007 b; Perez-Sanchez *et al*., 2011).

Phenotypic and biochemical characteristics of the 25 isolates obtained from the colonies grown on MRS, TSA and M17 agar were identified with phenotypic and biochemical tests using the Bergeys Manual of Systematic Bacteriology (Hammes and Hertel, 2009; Teuber, 2009).

DNA extraction and molecular identification of the genus Lactobacillus

DNA isolation was carried out with the rapid phylogenetic analysis (Liu *et al*., 2000). For the molecular biological identification, Lacto 16S-F (GGA ATC TTC CAC AAT GGA CG) and Lacto 16S-R (CGC TTT ACG CCC AAT AAA TCC GG) primers specific to the genus Lactobacillus were used and a partial region (216 bp) of 16 S rDNA was amplified using PCR (Polymerase Chain Reaction) (Abdulamir *et al*., 2010).

Amplified ribosomal DNA restriction analysis (ARDRA) and sequencing

Then, after the 16S rDNA about 1.5 kb regions of the 25 isolates were amplified with 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGYTACCTTGTTACGACTT-3′) primers using PCR through ARDRA (Amplified Ribosomal DNA Restriction Analysis). They were cut with the Msp I (Promega), Hae III (Promega), Hinf I (Promega) enzymes and the fragments were formed through electrophoresis

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(Soto *et al*., 2010). The images were grouped as follows. After the 16srDNA regions isolated from the organisms were cut with 3 different restriction enzymes, electrophoresis was carried out. As clearly described in the electrophoresis images, samples showing the same band patterns were collected in the same group and one sample from each group was sent to the sequence. Since there were 2 groups in our study, obtaining the accession numbers of the sequence results of the two samples representing these 2 groups was sufficient. However, as noted in the text, to avoid errors due to slight differences and to be sure, not one sample but 6 samples from one of the groups (F2, F4, F40, F53, F66, F69), and two samples from the other group (F70, F36) were sent the sequence. Three strains selected to represent each group were sequenced with the same primers used in PCR. The sequence data obtained were compared with the sequences in the GenBank database using the BLAST algorithm and then sent to NCBI (National Center for Biotechnology Information) to receive an access number.

Detection of antagonistic activity

Antagonistic activities of these 25 strains against the test pathogen organisms of *Salmonella typhimirium* (ATCC 14028), *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* ATCC 27853, *Yersinia ruckerii*, *L. garviae* ATCC 49156 were determined with the disc diffusion technique and was performed as triplicates. After the pathogenic bacteria were incubated on the TSA agar at 22 °C for 24 hours, their inoculum density was adjusted to 0.5 to Mac Farland (50-100 µl), and they were seeded on the general medium through swab cultivation. At the same time, fresh cultures of lactic acid bacteria prepared by incubating LAB on the MRS broth for 24 hours were centrifuged at 8000 rpm, at 4 °C for 5 min, and cell-free solutions were obtained. After the sterile discs placed in petri dishes containing pathogenic bacteria were soaked in the obtained supernatant, the petri dishes were incubated at 22 °C for 24 hours. The inhibition zones which formed after incubation were measured and the results were recorded (Allameh *et al*., 2012; Chemlal-Kherraz *et al*., 2012).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of isolates was performed with the disc diffusion method in accordance with guidelines for the Clinical and Laboratory Standards Institute (CLSI), and the following antibiotics were tested with the disk diffusion method: Doxcycline (30 µg), Enoxacine (10 µg), Erythromycin (15µg), Florfenicol (30 µg), Trimethoprim/sulfamethoxazole (1.25/23.75 µg), Enrofloxacin (5µg), Oxytetracycline (30µg), Chloramphenicol (30 µg) (CLSI, 2008).

pH tolerance

The isolates' tolerance towards different pH conditions (pH 1 and pH 3) was determined using the modified method implemented by Perez-Sanchez *et al*. (2011).

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Experimental infection

For experimental infection the method used by N. Haines *et al*. (2013) was used with a minor modification. To determine if F4 isolate which has the most proper probiotic potential from fish would produce infection in rainbow trout, a group of 20 trout (9-10g) were inoculated intraperitoneally with 1 $x10^5$ bacteria by optical density in a final volume of 10 ul sterile PBS. Animals were held in 2 (PVC π 150cm=880L) at 16°C and monitored daily for clinical signs of streptococcosis, including evidence of external hemorrhage, exophthalmia, lethargy, and loss of appetite. Two weeks after inoculation all fish were euthanized and the spleens were aseptically removed, macerated and suspended in liquid media for re-isolation as described above.

Results

Upon the completion of the phenotypic tests, it was found that 22 of the 25 isolates belonged to the genus Lactobacillus whereas 3 of them belonged to the genus *Lactococcus*. After being amplified with primers specific to the genus Lactobacillus, all of these 25 isolates yielded 216 bp band specific to the genus Lactobacillus (Fig. 1). Definitive identification was made after the ARDRA method was implemented and sequence analysis of the selected strains exhibiting identic restriction pattern were performed (Figs. 2, 3, 4). However, after the sequence analysis, while 23 of these 25 strains were identified, 2 were not (F17 and F48). The 23 identified strains were divided into two groups. One of the groups included 19 *L. lactis* strains, and the other included 4 *Carnobacterium maltoramaticum* strains. Due to the very small visually detectable differences within these two groups, accession numbers of these 8 strains were obtained in order to avoid possible errors. They are as follows: *Lactococcoccus lactis* strain F2, KM017400; *L. lactis* strain F4, KM017401; *L. lactis* strain F40, F17402; *L. lactis* strain F53, F17403; *L. lactis* strain F66, 17404; *L. lactis* strain F69, 17405; *Carnobacterium maltoramaticum* strain F36, KM017406; *C. maltoramaticum* strain F70, KM017407.

Results of antagonistic effects of the 25 isolates are given in Table 1. Of the strains, those numbered F2, F4, F9 and F38 showed the highest antagonistic effects against four of the five test bacteria. Strains numbered F7, F37 and F38 showed the highest antagonistic activity against *L. garviae* ATCC 49156; F4, F10, F30 and F38 against *E. coli* 0157: H7; F2, F4, F7, F8, F9, F33, F38, F53 and F54 against *S. typhimirium*; F2, F4, F6, F8, F9, F10, F30, F38, F39, F40, F48, F53, F54, F70 and F75 against *P. aeriginosa* ATCC 27853; and F2, F6, F9, F38, F39, F68, F69 and F75 against *Yersinia ruckerii*.

The results of the antibiotic susceptibility test are given in Table 2. Of the samples studied, only those numbered F8 and F48 were sensitive to all antibiotics tested. The highest antibiotic resistance was to Enoxacin, Oxytetracycline and Chloramphenicol. All the strains were sensitive to Vancomycin. Except for those

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numbered F70 and F72, all the strains were sensitive to penicillin.

pH tolerance results are given in Table 3. While no growth was observed in the majority of the samples at pH 1, most of the samples proliferated at pH 3 after a three-hour incubation. The strains which displayed the highest survival rate at pH 3 were those numbered F2, F4, F8, F9, F10, F48, F53 and F69.

Table 1. Diameter of inhibition zone (mm).

Numbers of isolates	***E. coli***	***S.typhimirium***	***P.aeruginosa* ATCC 27853**	***Y. ruckerii***	***L.garviae* ATCC 49156**
F2	9	12	14	16	10
F4	12	10	14	2	14
F6	11	9	10	12	16
F7	11	11	4	6	18
F8	8	11	10	8	2
F9	10	10	11	10	8
F10	13	9	11	4	6
F17	8	7	-	2	6
F30	14	11	10	2	2
F33	11	10	-	-	-
F36	8	4	-	-	2
F37	9	5	-	6	16
F38	10	10	12	10	18
F39	-	8	11	10	10
F40	-	6	10	-	2
F48	2	7	12	-	-
F53	4	13	12	4	2
F54	3	12	16	4	4
F66	-	8	8	6	6
F68	4	6	8	12	6
F69	5	7	8	12	6
F70	-	-	10	4	6
F71	4	9	-	2	2
F72	6	4	-	2	2
F75	8	9	10	10	6

Table 2: Results of antibiotic susceptibility testing.

İsolates	**Doxycyline**	**Enoxacin**	**Erithromycin**	**Florfenicol**	**Trimethoprim**	**Oxytetracycline**	**Enrofloxacine**	**Chloramphenicol**
F2	S	R	S	S	S	R	R	R
F4	S	R	R	S	S	R	S	R
F6	S	R	S	S	S	R	S	S
F7	S	R	S	S	S	R	S	S
F8	S	S	S	S	S	S	S	S
F9	S	S	S	S	S	R	S	S
F10	S	S	S	S	S	R	S	R
F17	S	I	S	S	S	S	S	R
F30	S	I	S	S	S	I	S	R
F33	S	S	S	S	S	R	S	R
F36	S	S	S	S	S	S	S	R
F37	S	S	S	S	S	S	S	R
F38	S	R	S	S	S	R	I	S
F39	S	R	S	S	S	R	S	S
F40	S	I	S	S	S	R	S	R
F48	S	S	S	S	S	S	S	S
F53	S	R	S	S	S	R	S	R
F54	S	S	S	S	R	R	S	I
F66	S	R	S	S	S	R	S	R
F68	S	R	S	S	S	R	I	S
F69	S	R	S	S	S	R	S	S
F70	S	S	S	S	S	S	S	R
F71	S	S	S	S	S	S	S	R
F72	S	S	S	S	S	S	S	R
F75	S	S	S	S	S	R	S	R

***S:** sensitive**, *R:** resistant**, *I:**Intermediate sensitive

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Table 3: Results of pH resistance.

Number of isolates	pH 1			pH 3		
	0. h	3.h	%alive number	0. h	3. h	%alive number
F2	$1.3x\ 10^9$	0	0	$1.3x10^9$	$1.0x10^9$	82
F4	$1.0x\ 10^7$	$4.0x10^5$	4.1	$1.0x10^7$	$5.0x10^6$	50
F6	$2.5x10^9$	0	0	$2.5x10^9$	$7.4x10^8$	29
F7	$1.0x10^8$	0	0	$1.0x10^8$	$1.4x10^7$	15
F8	$1.3x10^9$	0	0	$1.3x10^9$	$5.3x10^8$	40
F9	$8.1x10^7$	0	0	$8.1x10^7$	$6.1x10^7$	74
F10	$2.4x10^8$	0	0	$2.4x10^8$	$1.5x10^8$	61
F17	$2.1x\ 10^9$	0	0	$2.1x10^9$	0	0
F30	$2.7x\ 10^7$	0	0	$3.0x10^7$	$5.2x10^5$	1.2
F33	$3.6x\ 10^8$	0	0	$3.6x10^9$	0	0
F36	$8.1x10^8$	$3.4x10^6$	1.7	$9.1x10^8$	$6.7x10^8$	29
F37	$9.0x10^8$	$4.4x10^6$	1.9	$9.0x10^8$	$6.7x10^7$	7.4
F38	$1.8x10^7$	$3.4x10^4$	0.2	$1.8x10^7$	$8.2x10^5$	4.4
F39	$2.1x10^8$	$1.2x10^6$	0.5	$2.1x10^8$	$1.8x10^7$	7.3
F40	$2.5x10^9$	$3.6x10^7$	1.4	$2.5x10^9$	$5.8x10^8$	22
F48	$8.4x10^8$	$2.6x10^8$	28	$8.4x10^8$	$8.4x10^8$	48
F53	$2.6x10^9$	0	0	$2.6x10^9$	$1.5x10^9$	51
F54	$3.2x10^9$	0	0	$3.2x10^9$	$2.1x10^9$	46
F66	$3.9x10^9$	$5.2x10^3$	0	$3.8x10^9$	$8.5x10^8$	21
F68	$3.8x\ 10^9$	$3.8x10^9$	0.42	$3.8x10^9$	$3.0x10^9$	39
F69	$3.8x10^9$	$4.0x10^7$	0.4	$3.8x10^9$	$2.7x10^9$	65
F70	$1.5x10^8$	$2.8x10^3$	0	$1.5x10^8$	$5.8x10^7$	37
F71	$3.4x10^9$	$1.1x10^6$	0	$3.4x10^9$	$1.3x10^9$	40
F72	$2.1x10^8$	0	0	$2.1x10^8$	0	0
F75	$7.6x10^9$	$1.0x10^5$	0	$7.6x10^9$	$2.0x10^9$	28

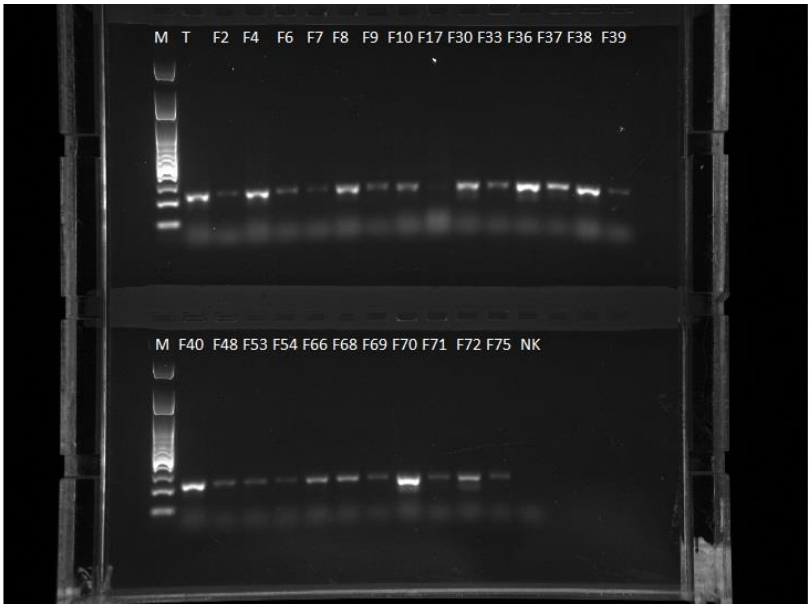

Figure 1: T; Reference strain, *L. acidaphilus* ATCC 4356, F 2-F75: LAB , NK: negative control

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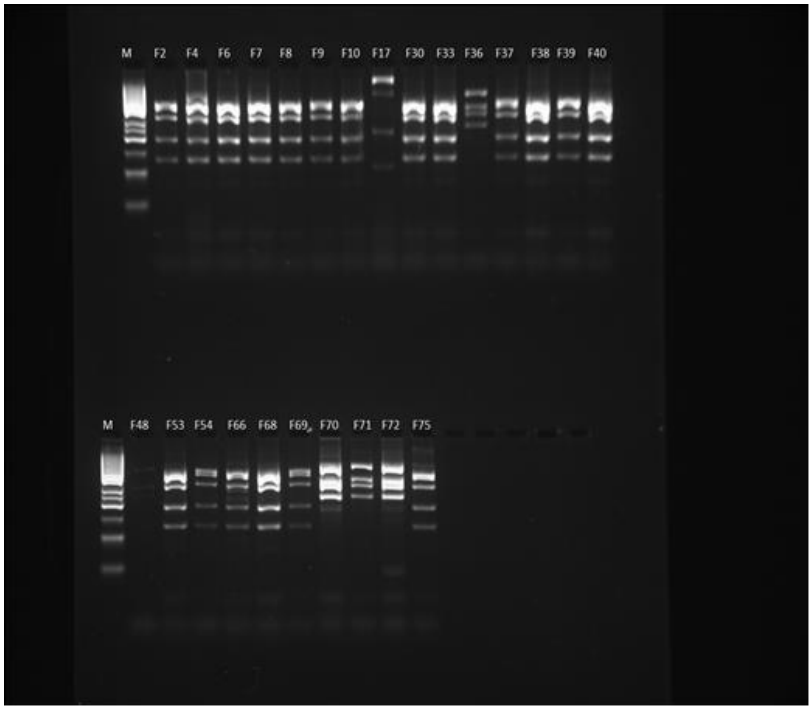

Figure 2: Restriction fragment profiles of the 16 S rDNA regions of the strains cut by *Hae*III enzyme. *Lactococcus lactis* group; F2, F4, F6, F7, F8 , F9 , F10, F30, F33, F37, F38, F39, F40, F53, F54, F66, F68, F69, F75 *Carnobacterium maltoramaticum* group; F36, F70, F71, F72, Uncharacterized bacterium: F17, F48.

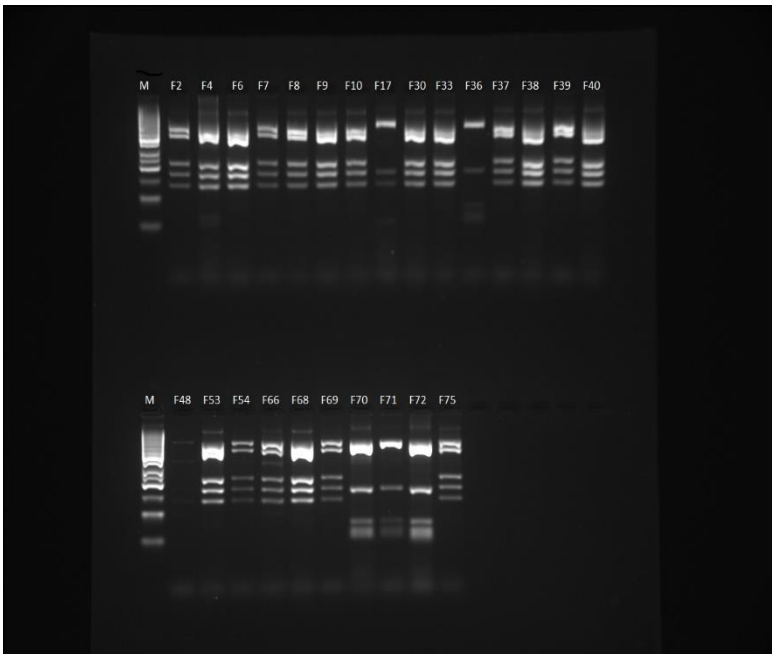

Figure 3: Restriction fragment profiles of the 16 S rDNA regions of the strains cut by *Msp*I enzyme. *Lactococcus lactis* group; F2, F4, F6, F7, F8, F9 , F10, F30, F33, F37, F38, F39, F40, F53, F54, F66, F68, F69, F75 *Carnobacterium maltoramaticum* group; F36, F70, F71, F72, Uncharacterized bacterium: F17, F48.

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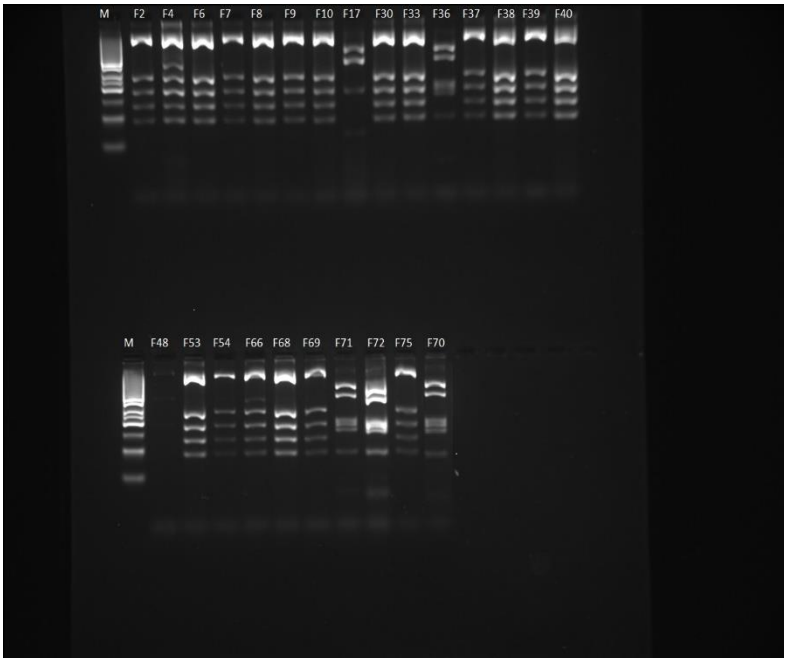

Figure 4: Restriction fragment profiles of the 16 S rDNA regions of the strains cut by *Hinf*I enzyme. *Lactococcus lactis* group; F2, F4, F6, F7, F8, F9 , F10, F30, F33, F37, F38, F39, F40, F53, F54, F66, F68, F69, F75 *Carnobacterium maltoramaticum* group; F36, F70, F71, F72, Uncharacterized bacterium: F17, F48.

Discussion

The results of PCR performed using primers specific to the genus *Lactobacillus* confirmed the phenotypic test results. Three isolates identified as *Lactococcoccus* through phenotypic methods were identified as *Lactobacillus* with these primers. However, the sequence results of the selected samples obtained through ARDRA did not support the results of phenotypic tests and the PCR. This result indicated that the selected primers were not specific to the genus *Lactobacillus*. Indeed, the results determined in the NCBI showed that 16S rDNA regions of the genera *Lactobacillus* and *Lactococcus* were similar at 84%, and that the selected primers could amplify the same size region in the genus *Lactococcus*. Antibiotic resistance and sensitivity in lactic acid bacteria vary depending on the strains and the source of isolation (Salminen *et al*., 1998). In some studies conducted so far (Coppola *et al*., 2005; Kim and Austin, 2008; Chemlal-Kherraz *et al*., 2012), it was reported that most of the antibiotic-resistant probiotics might be helpful when fish are administered antibiotics and that beneficial microorganisms could stay in the gut of the fish for a long time; therefore, their resistivity could be an advantage. Since LAB which have the potential for being used as probiotics should generally be sensitive to antibiotics (Karapetkov *et al*., 2011), their survival rate at pH 1 and pH 3 should be high (Chemlal-Kherraz *et al*., 2012), and they should display a good antagonistic activity over pathogenic bacteria (Balcazar *et al*., 2006; Jini *et*

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al., 2011; Zapata and Lara-Flores, 2013; Chemlal-Kherraz *et al*., 2012),

Bacterial isolates were obtained from healthy fish and experimentally inoculated rainbow trout didn't show any signs of disease. *L. lactis* species (F2, F4, F9 and F10) identified in this study were thought to have a potential probiotic capacity. In further studies, experiments with these potential probiotic strains will be continued. The species with probiotic features were determined to be sensitive to antibiotics used in the treatment of fish diseases in the present study because Lake Egirdir is a natural freshwater lake. There is a need for low-cost, broad-spectrum potential probiotics which have no adverse effects on humans and the environment, which are effective against various fish diseases and which can be used in all kinds of environmental conditions in aquaculture. The study is expected to provide information on the reduction of the use of antibiotics and chemotherapeutic agents and raise the awareness of the manufacturers. It is also expected to help manufacturers to better understand eco-friendly, sustainable, organic production systems. The potential of LABs to be used as probiotics will be investigated in the further studies.

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