

# Some *Lactobacillus*, *Leuconostoc* and *Acetobacter* strains in traditional Turkish yoghurt, cheese, kefir samples as a probiotic candidate

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## Abstract

Lactic acid bacteria which are important for production of fermented milk products contain many strains called *Lactobacillus*, *Streptococcus*, *Lactococcus* and *Leuconostococcus*. As a result, lactic acid bacteria are called 'milk-souring (fermenting)' organisms. In addition to the fermentation abilities of *Lactobacillus* spp., it is important for aroma, texture and acid formation and comprises the most important group of lactic acid bacteria. Their critical importance comes from their metabolic capacity and probiotic features. In this research, yogurt, cheese and kefir samples were collected from cities in Turkey and used to isolate. Isolates were identified phenotypically and genotypically characterized. The probiotic features antibacterial activity against *Staphylococcus aureus* ATCC6538, *Listeria monocytogenes* DSM12464, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC51299, and *Salmonella* Enteritidis ATCC 130762; bile and acid salt tolerance, susceptibility to chloramphenicol, erythromycin, penicillin G, gentamicin, vancomycin, streptomycin, kanamycin, and tetracycline of isolates were determined. Isolates, were identified as *Lactobacillus paracasei* subspecies (subsp.) *paracasei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Acetobacter ghanensis*, *Acetobacter fabarum*, *Acetobacter* subsp., *Leuconostoc pseudomesenteroides*, and *Leuconostoc mesenteroides* subsp. *mesenteroides*. Some isolates were tolerant of acid and bile salt, some strains were resistant to antibiotics, and some could inhibit pathogens. In this study, isolates were determined to have probiotic features. As a result of the study, it was determined that some isolates showed probiotic properties and had strong antibacterial activity. Isolates can be used as a natural alternative in infections.

**Keywords:** *Lactobacillus*, *Leuconostoc*, *Acetobacter*, Probiotic properties

## INTRODUCTION

Probiotic bacteria are defined as live microorganisms. When taken into the body in certain amounts, they are microorganisms with health benefits beyond basic nutrition (Coeuret et al., 2004). Probiotic, should meet some requirements, adherence to human enteric epithelial cells, like resistance to the bile and gastric acids, bile salt hydrolase activity, ability to reduce pathogen adhesion to the gastrointestinal tract, and antimicrobial activity against pathogenic bacteria (Kolacek, 2017). The use of probiotics is more generally accepted, milder, safer of foods than commonly used natural and chemical preservatives (Vieco-Saiz et al., 2019).

Dairy products have been consumed for centuries and yogurt, cheese, kefir are most popular worldwide (Buttriss, 1997). All dairy products are unique because of fermentation type, environmental conditions and they have similar or different microflora with each other. The determination of microflora of fermented dairy

products is important for determining characteristics features and further improving of fermented dairy products. There are studies investigating the microbial content of fermented dairy products (Simova et al., 2002, Manolopoulou et al. 2003, Zamfir et al., 2006, Waldherr et al., 2010, Aldrete-Tapia et al. 2014, Garofalo et al., 2015, Sarikkha et al., 2015, Xue et al. 2018).

The main aim of this research was to use genotypic and phenotypic methods to identify *Lactobacillus*, *Leuconostoc*, and *Acetobacter* strains. Then, the evaluation of tolerance to acidity and bile salts, antimicrobial activity, hydrophobicity, and antibiotic susceptibility were investigated.

## MATERIAL AND METHOD

The study used naturally fermented milk products. These included 19 yogurts, 3 cheeses, and 1 kefir sample (Tekirdag, Antalya, Hatay, Van, Edirne cities in Turkey). The samples were stored in sterile sample containers, brought to the laboratory and stored in a refrigerator until study.

### Phenotypic Characterization

Samples were seeded on MRS agar. With the aim of phenotypic characterization of those with different dimensions, shapes or colors, gram staining, catalase test, ability to create gas from glucose and ammonium from arginine, and developmental ability with different temperatures and different salt concentrations were investigated.

### Genotypic Characterization of Isolates

DNA isolation comprised the stages of lysis of bacteria, removal of proteins, precipitation of DNA and purification. The Genomic DNA Purification KIT (Fermentas, FINLAND) was used to complete isolation.

For identification of bacteria with the 16S rDNA method, general bacterial primers were used benefiting from the homology of the 16S rDNA region proliferated with polymerase chain reaction (PCR). In studies, forward primer 5' AGAGTTTGATCCCTGGCTCAG- 3' and reverse primer 5'- CCGTCAATTCCTTTGAGTTT - 3' were used (Beasley and Saris 2004). In the study, 500 µl PCR tubes were completed to total volume 50 µl with 17.5 µl sterile water produced for molecular studies, 2.5 µl buffer (not containing MgCl<sub>2</sub>), 0.5 µl (deoxynucleotidetriphosphate) dNTPmix (mixture prepared with dATP, dCTP, dGTP, dTTP, each concentration 200 µM), 0.5 µl primers, 0.5 µl Taq DNA polymerase enzyme and 2 µl MgCl<sub>2</sub> and finally 1 µl DNA addition. After the tubes were inserted in the PCR chamber, PCR reaction parameters were programmed as 94 °C 5-min initial denaturation, 94 °C 45 s denaturation (opening of double chains), 53 °C 1 min annealing (adhering of primers), and 72 °C 1 min extension (chain extension). This procedure was repeated 30 times. Tubes removed from the PCR were stored at -40 °C. Later PCR products were purified and then DNA array analysis.

Sequence was directed ABI 3130 genetic analyzer in the BLASTN program, then NCBI web site (<http://www.ncbi.nlm.nih.gov>) was used.

### Determination of acid tolerance of isolates

After *Lactobacillus* spp. and *Leuconostoc* spp. isolates were incubated overnight at 37 °C and *Acetobacter* spp. isolates incubated at 32°C for 72h in MRS fluid medium, seeding was performed on 10 mL fresh MRS fluid media with pH set to 3 with HCl (3M) and initial counts were identified with cultural methods. The prepared bacterial cultures were incubated for 3 hours (180 min) at 37 °C. 1 mL was taken from each of the pH 3 cultures and serial dilutions with 9 mL sterile physiologic saline up to 10<sup>-6</sup> were prepared. Seeding was performed on MRS media with these dilutions. Analyses were performed in triplicate. The colonies developing on the MRS media were counted and the viability rates were identified compared to initial counts (Charteris et al. 1998).

### Detection of antibiotic resistance of isolates

Eight different antibiotic disks (erythromycin, streptomycin, vancomycin, penicillin G, kanamycin, gentamycin, chloramphenicol, and tetracycline,) were used to investigate the resistance of the isolates to antibiotics.

Sterile MRS agar medium was cooled to 45-50 °C and active cultures of isolates on MRS fluid media were mixed at rates of 100 µL. Antibiotic disks were placed on the petri dishes, after incubation the diameter of the inhibition zones forming around the disks was measured. Analyses were completed with three replications (Sadrani et al. 2014).

### Determination of bile salt resistance of isolates

Tolerance of isolates to bile salt was identified according to the method of Kotsou et al. (2008). Active cultures (2236 g) were centrifuged for 5 min and pellets were diluted with MRS broth. 0.3% bile salt or MRS broth for the control group, were added and 50 µL of the inoculum mentioned above was added and left for incubation at 37 °C for 24 hours. Analyses were performed with three repeats and bile salt resistance of isolates seeded on MRS agar at 0 and 24 hours were determined.

### Determination of hydrophobicity of isolates

The hydrophobicity ability of isolate was determined according to the method reported by Perez et al. (1998). From fresh bacteria cultures, 2 mL was taken, vortexed with 0.4 mL xylene for 120 s and then absorbance measured at 600 nm with a spectrophotometer (Shimadzu 1208). The analyses had three repetitions. The cell surface hydrophobicity was calculated with the aid of the following formula.

$$\text{Hydrophobicity (\%)} = [(A_0 - A) / A_0] \times 100$$

The A<sub>0</sub> and A values are the absorbance values before

and after extraction with xylene.

### Determination of antibacterial activities of isolates

Fresh cultures were prepared from isolates in MRS fluid media, cultures were centrifuged and after obtaining cell-free solution, the supernatant was passed through a cellulose acetate filter with 0.2 µm pore size. For antibacterial activity, 18-hour cultures of the chosen test bacteria [*Enterococcus faecalis* ATCC51299, *Listeria monocytogenes* DSM12464, *Salmonella* Enteritidis ATCC 13076, *Staphylococcus aureus* ATCC6538, and *Escherichia coli* ATCC 25922] were poured onto petri dishes containing nutrient agar and wells with 6 mm diameter were opened. Supernatant from the isolate to be tested was pipetted into each well and the diameters of the inhibition zones forming around the wells were measured and recorded after incubation. Analyses were performed in triplicate (Arıcı et al. 2004).

### Statistical analysis

Acid tolerance and bile tolerance of isolates calculated with two-way ANOVA using the Graph Prism 7.0 program. The % hydrophobicity values of the isolates were calculated with one-way ANOVA using the Graphprism 7.0 program. Differences were considered significant at p value <0.01.

## RESULTS AND DISCUSSION

### Identification of Isolates

A total of 105 isolates were obtained from 23 samples. With the aim of genotypic identification of gram (+) and catalase (-) samples among isolates assessed in terms of morphology after phenotypic identification analyses.

While the study continued, permission was granted and samples were stored in the laboratory. With the aim of isolation, general bacterial primers were used for identification of bacteria with the 16S rDNA method using homology proliferated in the 16S rDNA region with polymerase chain reaction (PCR).

After determining the basal sequence, this sequence was compared with the database using a program on the internet (<http://www.ncbi.nlm.nih.gov/BLAST/>). Screening results determined which microorganism the researched array sequence may belong to and the percentage similarity. Among the isolates, 95-99% similarity was identified with reference strains for 1 isolates with *Leuconostoc pseudomesenteroides* (TDP 71), 2 isolates with *Leuconostoc mesenteroides* subsp. *mesenteroides* (TDP 22, TDP 50), 2 isolates with *Acetobacter fabarum* (TDP 54, TDP 90), 1 isolate with *Acetobacter* spp. (TDP 69), 3 isolates with *Acetobacter ghanensis* (TDP 21, TDP 38, TDP 40), 4 isolates with *Lactobacillus paracasei* subsp. *paracasei* (TDP 1, TDP 2, TDP 3, TDP 28), and 19 isolates with *Lactobacillus delbrueckii* subsp. *bulgaricus* TDP (37, 41, 56, 57, 58, 59, 63, 66, 70, 72, 88, 89, 92, 93, 95, 97, 98, 100, 103).

### Antibacterial activities of isolates

The antagonism ability of the bacterial isolates was ordered according to the size of the zones of inhibition against *Salmonella* Enteritidis ATCC 13076, *S.aureus* ATCC6538, *L.monocytogenes* DSM 12464, *E.faecalis* ATCC 51299, *E.coli* ATCC 25922 (Table 1). When the antibacterial properties of isolates are investigated, most inhibition activity was identified against *Salmonella* Enteritidis ATCC 13076 and *Enterococcus faecalis* ATCC 51299. All isolates were effective against *Enterococcus faecalis* ATCC 51299 (8.7-15.9 mm). The largest inhibition zone against *Salmonella* Enteritidis ATCC 13076 was 10.3 mm with isolate TDP 63. Only 9 isolates created zones against *Staphylococcus aureus* ATCC 6538. TDP 50 and TDP 90 isolates had the largest zones against *Staphylococcus aureus* ATCC 6538 (8.5 mm). The antibacterial effect of *Lactobacillus paracasei* subsp. *paracasei* was stronger compared to *Lactobacillus delbrueckii* subsp. *bulgaricus*.

*Acetobacter* isolates were more effective against *Escherichia coli* ATCC 25922 (14.5-15.9 mm) than the others. *Leuconostoc mesenteroides* subsp. *mesenteroides* exhibited inhibitory ability against all test bacteria, albeit with small zones.

Studies of *Lactobacillus* spp. isolated from milk products showed they were effective against, *Escherichia coli*, *Salmonella typhimurium*, and *S.aureus* (Patra et al., 2011, Tambekar and Bhutada, 2010; Abosereh et al. 2016). Akpınar and Yerlikaya, (2021) reported that; many of the *Lactobacillus paracasei* strains from kefir and raw milk showed higher antagonistic effects than *Leu. mesenteroides* strains. Pisano et al., (2022) reported that *L. plantarum* from cheese reduced by 3–4 log<sub>10</sub> CFU/g *L.monocytogenes* ATCC 7644. Some researchers reported that *Leuconostoc mesenteroides* strains had antimicrobial activity against *S. aureus* and *E. coli* (inhibition zones ranging from 7.42 to 16.00 mm) using the agar well diffusion method. (Rani and Agrawal, 2008; Ryu and Chang, 2013). Haghshenas et al. (2015) found that strains of *Acetobacter syzygii* 38Lac, *A. indonesiensis* 10HN L., and *A. cibirongensis* 34L were able to exhibit antimicrobial activity to important human pathogens.

The data obtained in the study were higher compared to data stated by Abosereh et al. (2016), but similar to data from studies by Etöz (2006), Rani and Agrawal, 2008, Patra et al. (2011), Tambekar and Bhutada (2010), and Ryu and Chang, 2013.

### Acid tolerance of isolates

Among the isolates, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Lactobacillus paracasei* subsp. *paracasei* were identified to be more susceptible to low pH (Figure 1). The acid tolerance of isolates was assessed with two-way ANOVA and results differed according to time and bacteria (p<0.0001).

**Table 1.** Antibacterial activity of isolates determined by agar spot assay (mm)

Isolate Number	<i>S. Enteritidis</i> ATCC 13076	<i>S.aureus</i> ATCC6538	<i>L.monocytogenes</i> DSM12464	<i>E. faecalis</i> ATCC51299	<i>E. coli</i> ATCC 25922
TDP1	9.1±0.1	8.2±0.3	9.7±0.7	10.7±0.8	6.5±0.5
TDP 2	9.3±0.6	7.9±0.2	9.8±0.9	10.7±0.7	6.4±0.2
TDP 3	9.0±0.7	7.3±0.5	9.4±0.4	10.5±0.5	6.4±0.1
TDP 21	-	-	-	15.9±1	9.5±0.7
TDP 22	8.5±0.4	8.2±0.2	8.9±0.3	10.8±0.4	6.4±0.6
TDP 28	9.9±0.5	8.4±0.4	10.1±1	10.5±1.2	6.7±0.3
TDP 37	8.9±0.2	-	-	9.4±0.5	-
TDP 38	8.1±0.5	-	9.5±0.2	15.3±1.3	9.1±0.8
TDP 40	-	-	9.7±0.7	14.5±0.9	9.3±0.6
TDP 41	8.7±0.3	-	-	9.4±0.5	-
TDP 50	8.5±0.3	8.5±0.2	8.5±0.4	9.6±0.9	7.1±0.3
TDP 54	8.3±0.2	-	9.3±0.8	15.6±1.1	9.1±0.9
TDP 56	8.9±0.3	-	-	9.7±1	-
TDP 57	8.5±0.1	-	-	8.8±0.6	-
TDP 58	8.1±0.7	-	-	9.6±0.8	-
TDP 59	8.9±0.4	8.2±0.3	8.4±0.6	9.5±0.6	-
TDP 63	10.3±0.9	-	-	9.1±0.5	-
TDP 66	8.2±0.8	-	-	9.7±0.7	-
TDP 69	-	-	9.5±0.4	15.3±0.6	9.1±0.4
TDP 70	8.3±0.6	-	-	9.8±1.2	-
TDP 71	-	-	-	14.3±0.4	8.8±0.2
TDP 72	8.5±0.3	-	-	8.9±0.9	-
TDP 88	8.9±0.6	-	-	9.0±0.5	-
TDP 89	9.2±0.9	8.1±0.2	8.1±0.3	9.4±0.2	-
TDP 90	8.2±0.3	-	8.9±0.4	15.0±0.8	9.4±0.3
TDP 92	8.0±0.8	-	-	9.2±0.7	-
TDP 93	8.9±0.5	-	-	9.6±0.5	-
TDP 95	8.9±0.2	8.5±0.3	-	9.4±0.2	-
TDP 97	9.0±1	-	8.1±0.3	8.7±0.6	-
TDP 98	8.9±0.4	-	-	9.4±0.9	-
TDP 100	9.3±0.4	-	-	10.8±0.7	-
TDP 103	8.1±0.5	-	-	9.9±0.2	-

When the properties of *Lactobacillus* spp. are investigated in studies, isolates were identified to preserve their viability at pH 3 (Prasad et al., 1999; Maragkoudakis et al. 2006; Minelli et al. 2004). *Lactobacillus paracasei* were inhibited at pH 2, while they were reported to be resistant at pH 3 (Schillinger et al. 2005; Abosereh et al. 2016). Some studies have reported that *Leuconostoc mesenteroides* spp. from natural yogurt and whey have demonstrated the ability to survive at low pH (Perea et al., 2007; Rani and Agrawal, 2008) Haghshenas et al. (2015), found that *Acetobacter* strains had high survival rate (> 44–78%) in traditional dairy products, after conditions (pH 2.5 for 3 hour).

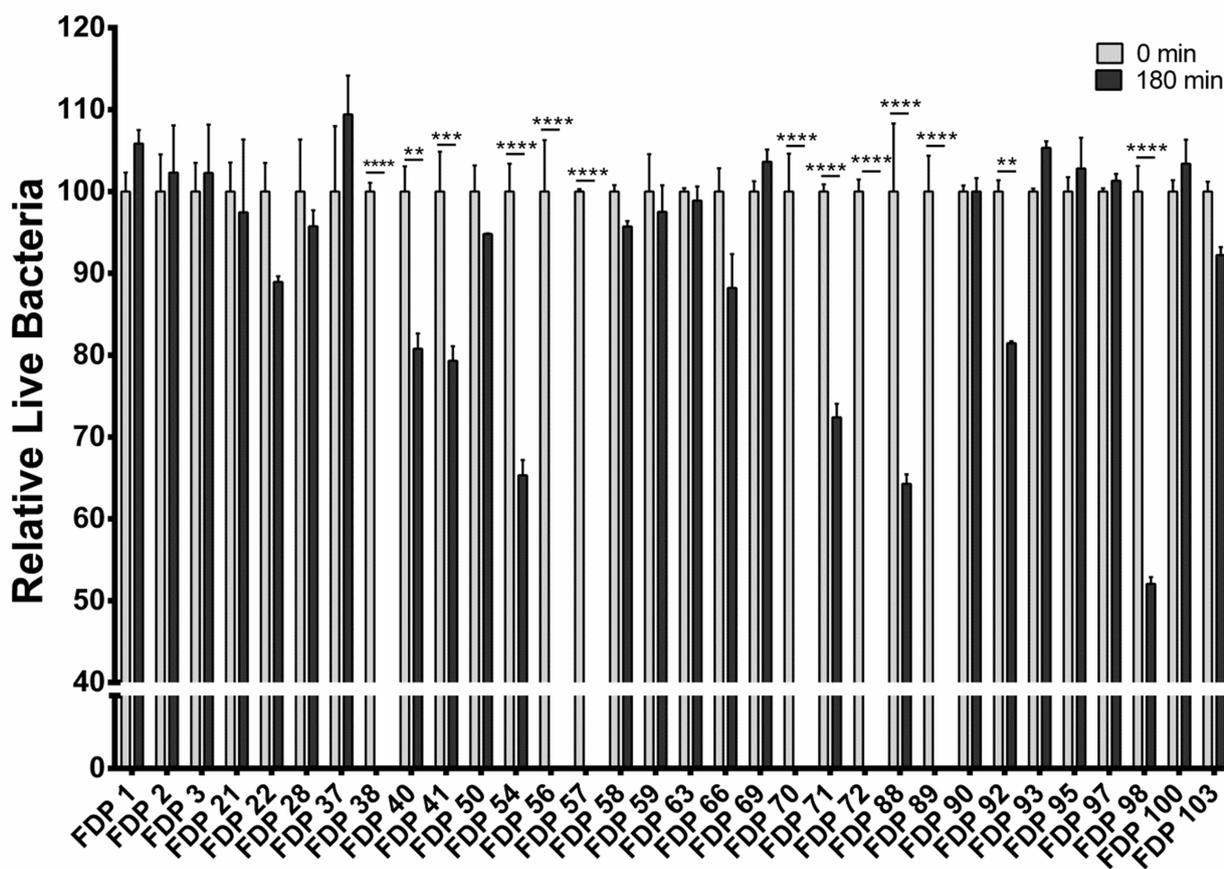
Data analyzed by two-way ANOVA using the Graph Prism

7.0 program. Statistical differences between bacteria groups are depicted on the tops of bars as follows: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, and \*\*\*\*p < 0.0001.

These results confirmed by many other studies Maragkoudakis et al. (2006), Prasad et al. (1999), and Haghshenas et al. (2015) while they are different to the data obtained by Schillinger et al. (2005) due to that study taking pH 2 as reference.

#### Antibiotic Resistance of Isolates

Isolates were resistant to Vancomycin except *Acetobacter* strains. All isolates were susceptible to tetracycline and chloramphenicol. Majority of the lactobacilli, *Leuconostoc* and *Acetobacter* strains were resistant to erythromycin



**Figure 1.** Acid tolerance of isolates

and penicillin G.

Previous studies confirm the generally susceptible of the *Lactobacillus* spp. species studied here to erythromycin, chloramphenicol and tetracycline (Charteris et al. 1998, Katla et al. 2001, Temmerman, 2003, Erginkaya et al. 2018).

A variety of studies as this study stated that *Leuconostoc* spp. are resistant to vancomycin (Tynkkyinen et al., 1998; Salminen et al., 1998; De Paula et al., 2015). While some researchers also found that *Leu. mesenteroides* strains were resistant to tetracycline and streptomycin (Akpınar and Yerlikaya, 2021).

Ahmad et al. (2004) found that *Acetobacter diazotrophicus* isolated from sugarcane was mostly resistance to test antibiotics. Haghshenas et al. (2015), determined that all examined *Acetobacter* strains show high resistance to erythromycin, vancomycin and sulfamethoxazole.

#### Hydrophobicity capability of isolates

In vitro studies showed that the adhesion properties of probiotic bacteria displayed non-competitive exclusion features by affecting their adhesion properties against pathogens (Ouwend et al., 1999; Gopal et al., 2001).

The hydrophobicity with xylene of isolates was identified to be between 58.75% and 11.5%. The hydrophobicity of

*Lactobacillus delbrueckii* subsp. *bulgaricus* was identified to be lower compared to *Lactobacillus paracasei* subsp. *paracasei*. The hydrophobicity values were statistically different between the bacterial strains ( $p < 0.001$ ).

*Lactobacillus paracasei* subsp. *paracasei* and a variety of *Lactobacillus* spp. were determined to adhere to HT29 and Caco-2 cells (Minelli et al., 2004; Schillinger et al., 2005). *L. mesenteroides* spp. strains had lower hydrophobicity capability than some other strains reported in the literature (Aswathy et al., 2008, Raghavendra and Halami 2009, De Paula et al., 2015).

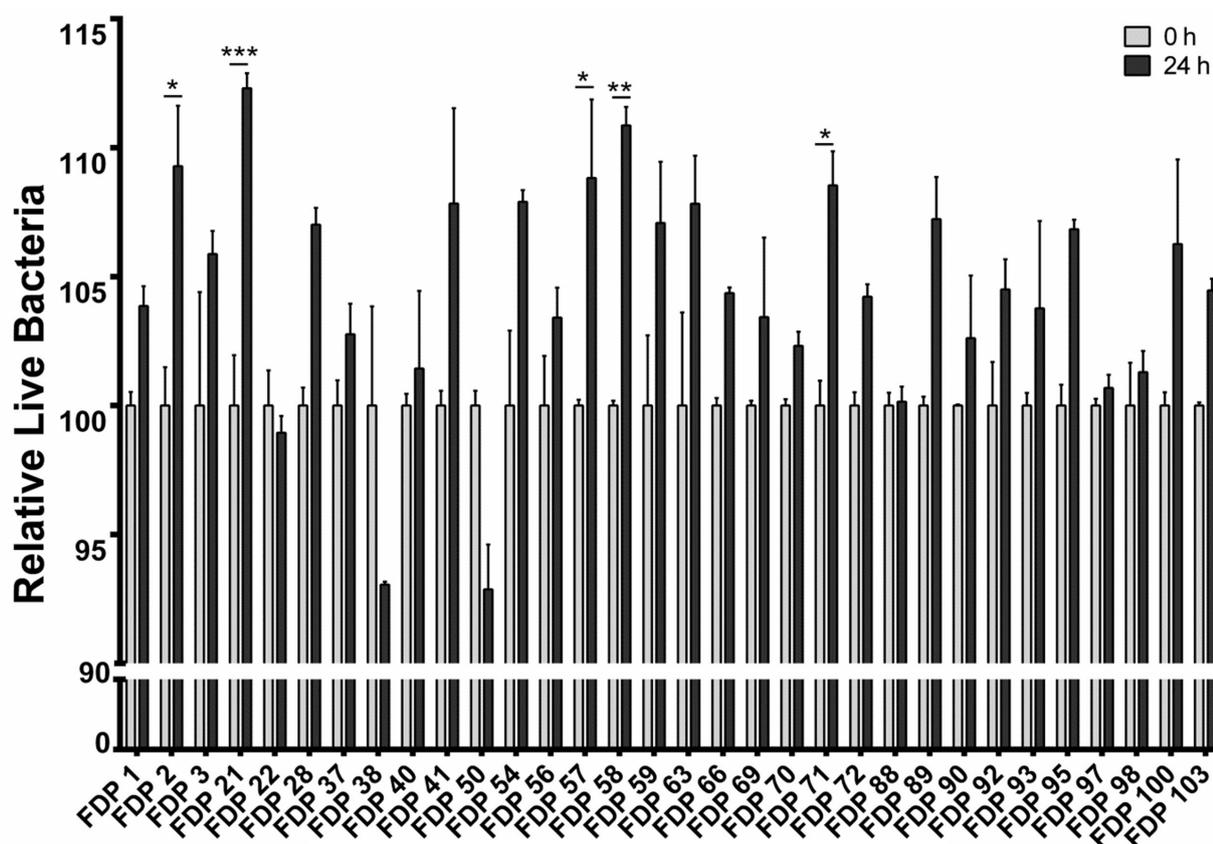
#### Bile salt resistance of isolates

All isolates were resistant against 0.3% bile salt, and were not inhibited after 24-hour incubation in a 0.3% bile salt medium (Figure 2). The bile salt tolerance values of isolates were assessed with two-way ANOVA and the results differed according to time and bacteria ( $p < 0.0001$ ).

Previous studies confirm the generally resistance of the strains isolated from milk products studied here towards to Bile salt (Abosereh et al., 2016; Prasad et al. 1999, Minelli et al. 2004;

Maragkoudakis et al. 2006)

The results in our study are parallel to data reached in



**Figure 2.** Bile Salt Resistance of isolates

studies by Abosereh et al. (2016), Prasad et al. (1999), Minelli et al. (2004), and Maragkoudakis et al. (2006), though there are differences according to strains.

Data analyzed by two-way ANOVA using the Graph Prism 7.0 program. Statistical differences between bacteria groups are depicted on the tops of bars as follows: \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

Haghshenas et al. (2015), reported that *Acetobacter* isolates showed good tolerance to bile (1% bile; ranged from 62% to 95%).

Some researchers reported that bile salt affected the growth rate of *Leuconostoc* spp. strains and limited its viability (Allameh et al., 2012; Todorov et al., 2012; Akpınar and Yerlikaya, 2021). In contrast Some researchers have reported that strains of *Leuconostoc* spp. can survive under different concentrations of bile salts (Chang et al., 2010; Seo et al., 2012; Nakamura et al., 2012).

## CONCLUSION

Fermented milk products that do not use commercial cultures for fermentation but developed their own microbial culture through the years have their own characteristic features. Determination of the microbiota in naturally fermented products not using commercial cultures is important in terms of protecting these products and sustaining them for future generations.

Primary probiotic assessments, including high bile salt and low pH tolerance tests, hydrophobicity test, antibiotic susceptibility, and antagonistic activity test against pathogens confirmed the probiotic properties of TDP 1, TDP 93, TDP 21 isolates, which was identified *Lactobacillus paracasei* subsp. *paracasei* (from cheese), *Lactobacillus delbrueckii* subsp. *bulgaricus* (from yoghurt), and *Acetobacter ghanensis* (from kefir) respectively can be introduced as novel candidate probiotics.

There is an increasing number of studies showing that probiotics can be an important tool in the treatment and prevention of gastrointestinal tract infections and chronic inflammatory disorders. Probiotic candidates with strong antibacterial activity we obtained in our research supports these studies.

## COMPLIANCE WITH ETHICAL STANDARDS

### Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

### Author contribution

Conceptualization; N.T.D., methodology; N.T.D and M.A., formal analysis; N.T.D, M.A., and S.Y., validation; N.T.D., investigation; N.T.D and M.A., supervision; N.T.D., writing-original draft; N.T.D, M.A., and S.Y., review and editing; N.T.D, M.A., and S.Y., All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that

they have not been published before.

#### Ethical approval

Ethics committee approval is not required.

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#### Data availability

Not applicable.

#### Consent for publication

Not applicable

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