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Antimicrobial effect of probiotic microorganisms on clinical and standard *Staphylococcus aureus* isolates

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ABSTRACT: In this study we aimed to identify probiotic microorganisms in various pharmacy preparations, market and homemade probiotic products and investigate the antimicrobial effect of these microorganisms on clinical and standard *Staphylococcus aureus* isolates. The probiotic microorganisms were isolated from probiotic products mentioned above, then identified by Matrix Assisted Laser Ionization Mass Spectrophotometer (MALDI-TOF, Biomeriux) and also by conventional methods. The tolerance of these probiotic microorganisms to different salt, pH and temperature conditions was also detected. The antimicrobial activity of the specified probiotic microorganisms on *S. aureus* was designated by using spot on lawn and agar well diffusion methods. In our study among 16 probiotic products, 27 various probiotic microorganisms were specified by MALDI-TOF. Additionally, 15 of these probiotic microorganism species had an isolated reliability value above 90%. In our study 10 probiotic microorganism species, 7 of which were different from each other were found to maintain their viability in three various pH, salt and temperature conditions. These probiotic microorganisms were *Lactobacillus rhamnosus* (P.2 probiotic pharmaceutical product), *Enterococcus gallinarum* (homemade whey), *Lactobacillus kefiri* 1 (homemade kefir), *Bacillus megaterium* (homemade pickled juice), *Lactobacillus kefiri* 2 (homemade kefir), *Lactobacillus rhamnosus* (P.1 company preparation) *Lactobacillus plantarum* (P.3 company preparation), *Bifidobacterium spp* (M.4 company pure kefir), *Enterococcus faecium* (P.3 company preparation). We determined that *Lactobacillus plantarum* isolated from homemade whey had the highest antimicrobial activity on clinical MRSA 3 (Methicillin resistant *Staphylococcus aureus*) strain (inhibition zone =45.62 mm ± 0.84. Also, we have found that, probiotic microorganisms isolated from pharmacy preparations, market and homemade probiotic products showed antibacterial effect on clinical and standard *Staphylococcus aureus* by spot on lawn method. We designated that besides probiotic microorganisms isolated from various pharmacy preparations, probiotic microorganisms isolated from market and homemade products were efficient against clinical and standard *S. aureus* isolates.

KEYWORDS: Probiotic products; antimicrobial activity; probiotic microorganisms; *Staphylococcus aureus*; spot on lawn.

1. INTRODUCTION

The word probiotic comes from the Greek words "pros" and "bios" which means "for life". Probiotics are defined as a single or mixed culture of microorganisms that develop microflora properties with beneficial effects on the gastrointestinal tract, urogenital canals, and upper respiratory tract [1,2,3] Metchnikoff was the first scientist to observe the beneficial effects of these bacteria. Metchnikoff attributed the longevity of Balkan peasants to the consumption of excess fermented dairy products and the probiotic bacteria involved in them [4-6].

Lactic acid bacteria (LAB) are the most well-known bacteria used as probiotics [7]. Lactic acid, organic acids, hydrogen peroxide, bacteriocin and other antimicrobial properties of synthesized substances produced by LAB prevent the development of undesired microorganisms and pathogens in food [4,6,7]. In addition to modulating intestinal functionality, probiotic microorganisms are also known to have beneficial effects such as boosting immunity of the host [3]. Additionally, it is known that these microorganisms increase the biological mechanisms in the body by lowering cholesterol and promoting metabolic homeostasis. Probiotic

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microorganisms can produce various chain fatty acids, vitamins, enzymes, organic acids and antimicrobial peptides [8].

As it is known staphyloxanthin which is found in *Staphylococcus aureus;* is membrane bound and primarily protects the membrane lipid against reactive oxygen radical challenge. Also staphyloxanthin is a protective protein and protects DNA from free radicals. Ong et al. [6] stated that *L. plantarum* USM8613 reduced the survival and resistance of *S. aureus* via autolysis pathways under stress conditions by inhibiting the biosynthesis of staphyloxanthin in their study.

On the other hand, *S. aureus* is a common bacterial pathogen with the potential to cause serious infections in humans and a variety of wild and agricultural animal species. *S. aureus* isolates are among the first community and hospital-acquired pathogens all over the world in terms of metabolic potential, virulence and antibiotic resistance (ABR). *S. aureus* strains have been implicated in causing serious hospital infections with tissue and organ pathogens, particularly methicillin-resistant *S. aureus* (MRSA) strains causing acute and pyogenic infections, skin and soft tissue infections, urinary tract infections, postoperative wound infections and infections in a broad spectrum including bacteremia [9,10].

Accordingly, there is a need for alternatives to antibiotics in the treatment and prevention of *S. aureus* infections. Specific probiotics that can inhibit the colonization and growth of *S. aureus*, such as lactic acid bacteria, have been proposed as a possible alternative to antibiotics and are of great interest [11].

Hence, in our study we aimed to identify probiotic microorganisms in various pharmaceutical preparations, market and homemade probiotic products and investigate the antimicrobial effect of these microorganisms against clinical and standard *S. aureus* strains.

2. RESULTS

All the 77 probiotic isolates in our study were gram positive, Voges Proskauer test negative and homofermentative. The catalase test was positive in 5 and negative in 72 probiotic isolates among 77 isolates. The arginine hydrolysis test of 35 probiotic isolates was positive and negative in 42 isolates. A total of 27 species were identified by MALDI-TOF, among these microorganisms 15 species had an isolated reliability value above 90% (Table 1).

2.1. Detection of the inhibition percentages of probiotic microorganisms at various pH, salt and temperatures

Bacteria isolated from the products and preparations were found to be low in density and high percentage inhibition compared to the control in the medium of pH 1.5, pH 2 and pH 3.2 and also the bacteria could not survive in environments other than pH 3.2. It was observed that cell densities did not increase significantly in the medium which pH was 3.2 when compared to initial hours however as a result of spot sowing, *L. kefiri* 1(1a-1A), *L. rhamnosus* (2f-2A), *L .plantarum* (1c-2.2.A), *L. plantarum* (2a-2A), *Bifidobacterium spp.* (3b-1A), *E. gallinarum* (1c-1.1.2), *B. megaterium* (1e-3), *L. kefiri* 2 (1a-2.1), *L. rhamnosus* (2b-6) and *E. faecium* (2a-2M) strains were found to be alive. This was the main criterion for choosing the suitable the probiotic bacteria for the antimicrobial tests in our study (Figure 1, 2 and 3).

In general, the isolated bacteria were found to have lower OD_2 values in the medium containing 1%, 0.30% and 0.15% bile salt compared to the control values OD_1. When the growth of the bacteria on the medium containing bile salt in three various ratios was examined, their concentration (OD_2) was found to be highest in the medium value containing 0.15% bile salt. On the other hand, it was found that the strains other than *L. rhamnosus* (2f-2A), *L. helveticus* (3a-A), *L. paraplantarum* (1d-3A), *L. plantarum* (1c-2.2.A), *L. pseudomesenteroides* (1d-3A), *L. lactis ssp. lactis* (3d-4A), *E. faecium* (2b-1A), *L. plantarum* (2a-2A) did not survive despite the increase in OD values compared to 0 hours. In addition, *L. kefiri* (1a-1A), *L. rhamnosus* (2f-2A), *L. helveticus* (3a-A), *L. paraplantarum* (1d-3A), *L. plantarum* (1c-2.2.A), *L. pseudomesenteroides* (1d-3A), *L. lactisssp.lactis* (3d-4A), *E. faecium* (2b-1A), *L. plantarum* (2a-2A) strains were found to survive in all three salt conditions (Figure 4, 5 and 6).

Temperature values of bacteria isolated from products and preparations generally have been found to have low OD values at temperatures of 4 °C, 22 °C and 45 °C when compared to control. However, *L. rhamnosus* (2f-2A), *Lactococcus lactisssp.lactis* (3d-4A), *B .megaterium* (1e-3), *L. kefiri* (1a-2.1), *L. pseudomesenteroides* (3b), *S. salivarus ssp. thermophilus* (2e-M) strains were found to have higher OD values than the control group at 45 °C. According to the results of spot-on lawn, it was observed that all probiotic bacteria survived at 22 °C and 45 °C (Figure 7, 8 and 9).

Table 1. Species identified by MALDI-TOF MS

	Isolate code	*Isolated source*	*Species identified by MALDI-TOF MS*	*Reliability value*
1	1a- A1	Homemade kefir 1	*Lactobacillus kefiri 1*	99.9
2	2f-2A	P.1 company preparation	*Lactobacillus rhamnosus*	99.9
3	3a-A	M.2 commercial company product	*Lactobacillus helveticus*	99.1
4	1d-3A	Homemade whey	*Lactobacillus paraplantarum*	99.9
5	1c-2.2.A	Homemade whey	*Lactobacillus plantarum*	99.9
6	1d-3A	Homemade whey	*Leuconostoc pseudomesenteroides*	99.9
7	3d-4A	P.5 commercial company product	*Lactococcus lactis ssp.lactis*	99.9
8	2b-1A	P.2 company preparation	*Enterococcus faecium*	99.9
9	2a-2A	P.3 company preparation	*Lactobacillus plantarum*	99.9
10	1b-2A	Homemade Yogurt	*Lactobacillus delbruecki*	99.9
11	3b-1A	M.4 company pure kefir	*Bifidobacterium spp*	92.1
12	1d-2A	Homemade whey	*Lactobacillus curvatus*	99.9
13	3a-1	M.2 commercial company product	*Leuconostoc pseudomesenteroides*	99.9
14	1e-2	Homemade pickled juice	*Bacillus pumilus*	99.9
15	1c-1.1.2	Homemade whey	*Enterococcus gallinarum*	98.4
16	1e 3	Homemade pickled juice	*Bacillus megaterium*	99.9
17	3d-1	M.1 commercial company product	*Leuconostoc pseudomesenteroides*	99.9
18	1a-2.1	Homemade kefir 2	*Lactobacillus kefiri 2*	99.9
19	2b-6	P.2 company preparation	*Lactobacillus rhamnosus*	99.9
20	2b-3	P.2 company preparation	*Enterococcus faecium*	99.9
21	1d-3	Homemade whey	*Lactobacillus paraplantarum*	99.9
22	3b	M .4 company pure kefir	*Leuconostoc pseudomesenteroides*	97.9
23	3c-2	M.6 company fruity kefir	*Lactococcus lactis ssp. lactis*	99.9
24	3e-1	M.7 company fruity kefir	*Lactococcus lactis ssp. lactis*	99.9
25	2e-M	P.4 company preparation	*Streptococcus salivarus ssp. thermophilus*	99.9
26	2b-M	P.2 company preparation	*Enterococcus faecium*	99.9
27	2a-2M	P.3 company preparation	*Enterococcus faecium*	99.9

Among 27 species identified in our study, 10 species, 7 of which were different from each other were found to maintain their viability in three various pH, salt and temperature conditions. These probiotic microorganisms were *L. rhamnosus* (P.2 probiotic pharmaceutical product), *E. gallinarum* (homemade whey), *L. kefiri* 1 (homemade kefir), *B. megaterium* (homemade pickled juice), *L. kefiri* 2 (homemade kefir), *L. rhamnosus* (P.1 company preparation) *L. plantarum* (P.3 company preparation), *Bifidobacterium* spp. (M.4 company pure kefir), *E. faecium* (P.3 company preparation). These probiotic bacteria were included in the study in order to find out their antibacterial activity against clinical and standard *S. aureus* (Table 2, 3 and 4).

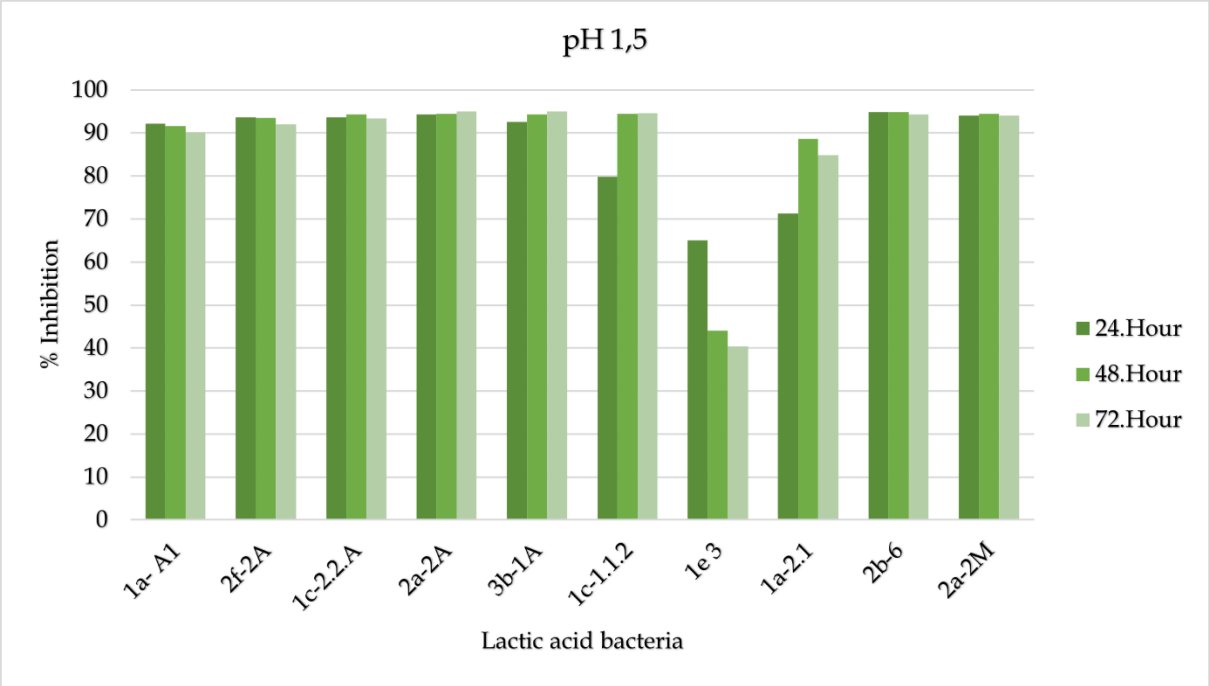

Figure 1. Inhibition percentages of probiotic microorganisms at pH 1.5
1a-1A: *Lactobacillus kefiri* 1, 2f-2A: *Lactobacillus rhamnosus*, 1c-2.2A: *Lactobacillus plantarum*, 2a-2A: *Lactobacillus plantarum*, 3b-1A: *Bifidobacterium* spp, 1c-1.1.2: *Enterococcus gallinarum*, 1e-3: *Bacillus megaterium*, 1a-2.1: *Lactobacillus kefiri* 2, 2b-6: *Lactobacillus rhamnosus*, 2a-2: *Enterococcus faecium*.

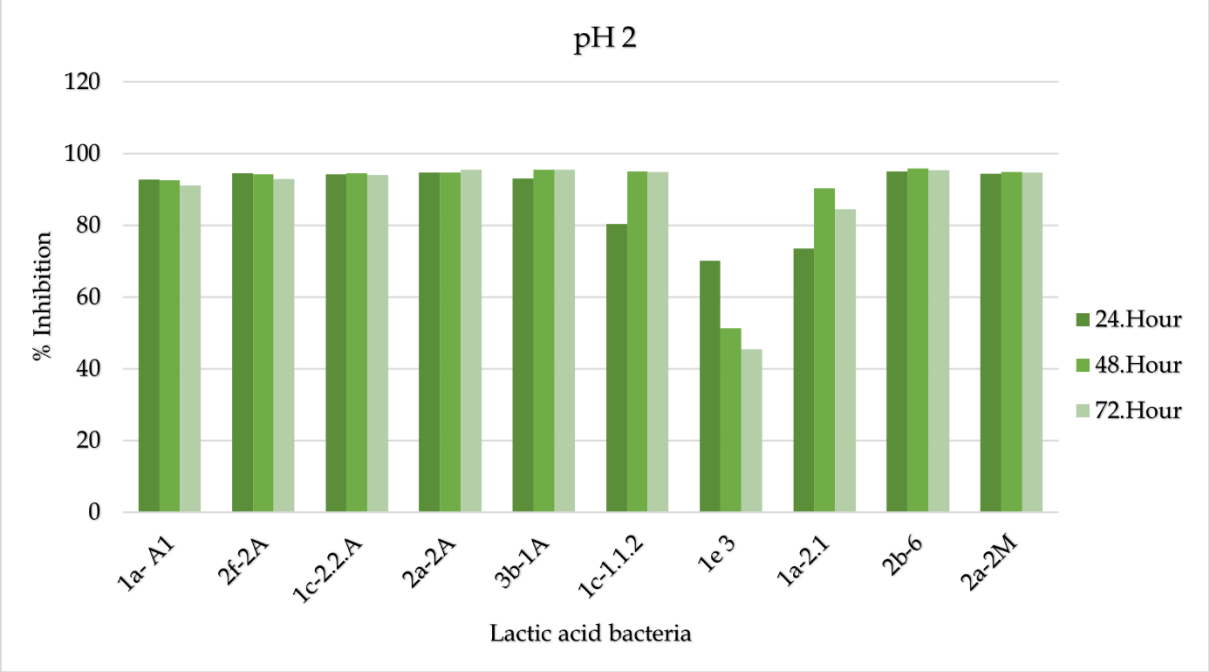

Figure 2. Inhibition percentages of probiotic microorganisms at pH 2
1a-1A: *Lactobacillus kefiri* 1, 2f-2A: *Lactobacillus rhamnosus*, 1c-2.2A: *Lactobacillus plantarum*, 2a-2A: *Lactobacillus plantarum*, 3b-1A: *Bifidobacterium* spp, 1c-1.1.2: *Enterococcus gallinarum*, 1e-3: *Bacillus megaterium*, 1a-2.1: *Lactobacillus kefiri* 2, 2b-6: *Lactobacillus rhamnosus*, 2a-2: *Enterococcus faecium*.

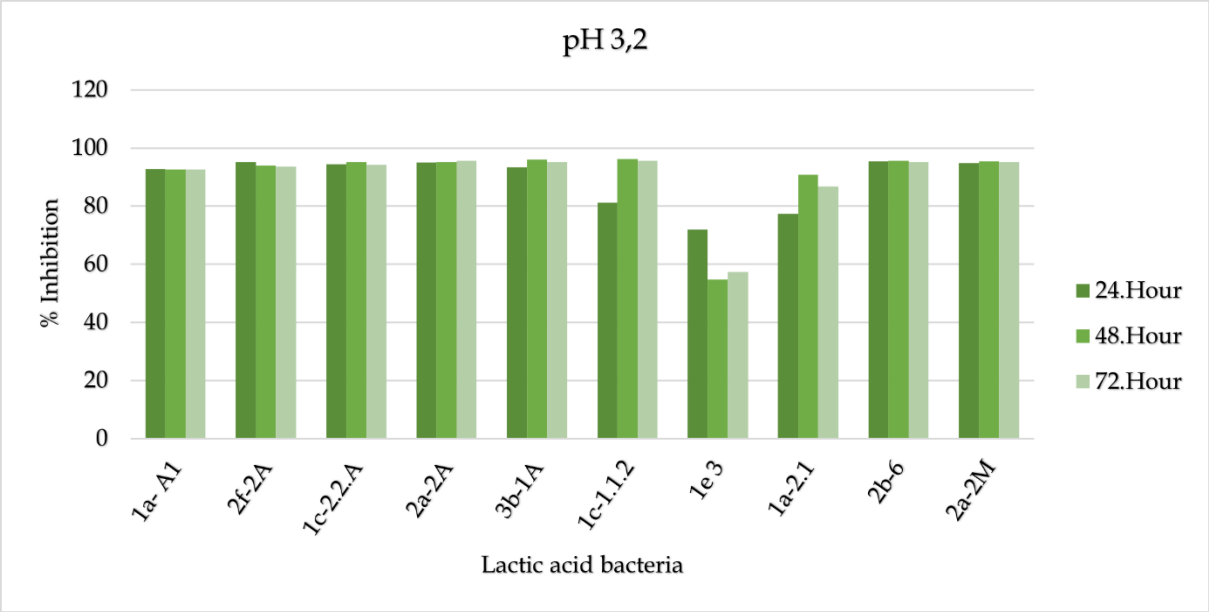

Figure 3. Inhibition percentages of probiotic microorganisms at pH 3.2
1a-1A: *Lactobacillus kefiri 1,* 2f-2A: *Lactobacillus rhamnosus,* 1c-2.2A: *Lactobacillus plantarum,* 2a-2A: *Lactobacillus plantarum,* 3b-1A*: Bifidobacterium spp,* 1c-1.1.2: *Enterococcus gallinarum,* 1e-3: *Bacillus megaterium,* 1a-2.1: *Lactobacillus kefiri 2,* 2b-6: *Lactobacillus rhamnosus,* 2a-2: *Enterococcus faecium*.

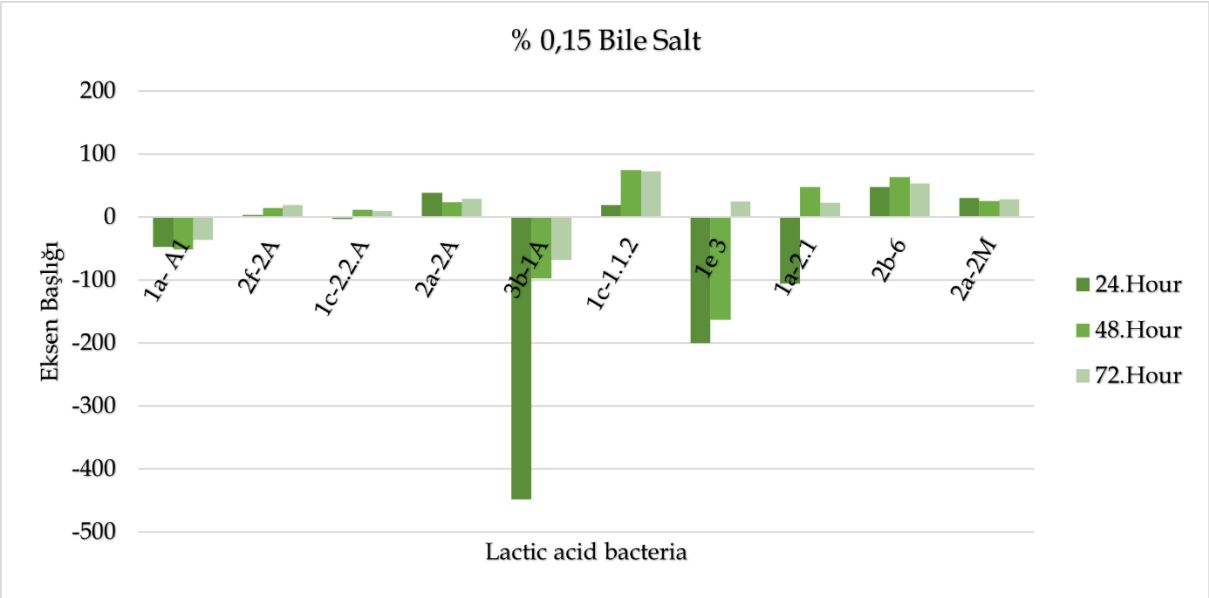

Figure 4. Inhibition percentages of probiotic microorganisms at 0.15% bile salt
1a-1A: *Lactobacillus kefiri 1,* 2f-2A: *Lactobacillus rhamnosus,* 1c-2.2A: *Lactobacillus plantarum,* 2a-2A: *Lactobacillus plantarum,* 3b-1A*: Bifidobacterium spp,* 1c-1.1.2: *Enterococcus gallinarum,* 1e-3: *Bacillus megaterium,* 1a-2.1: *Lactobacillus kefiri 2,* 2b-6: *Lactobacillus rhamnosus,* 2a-2: *Enterococcus faecium*.

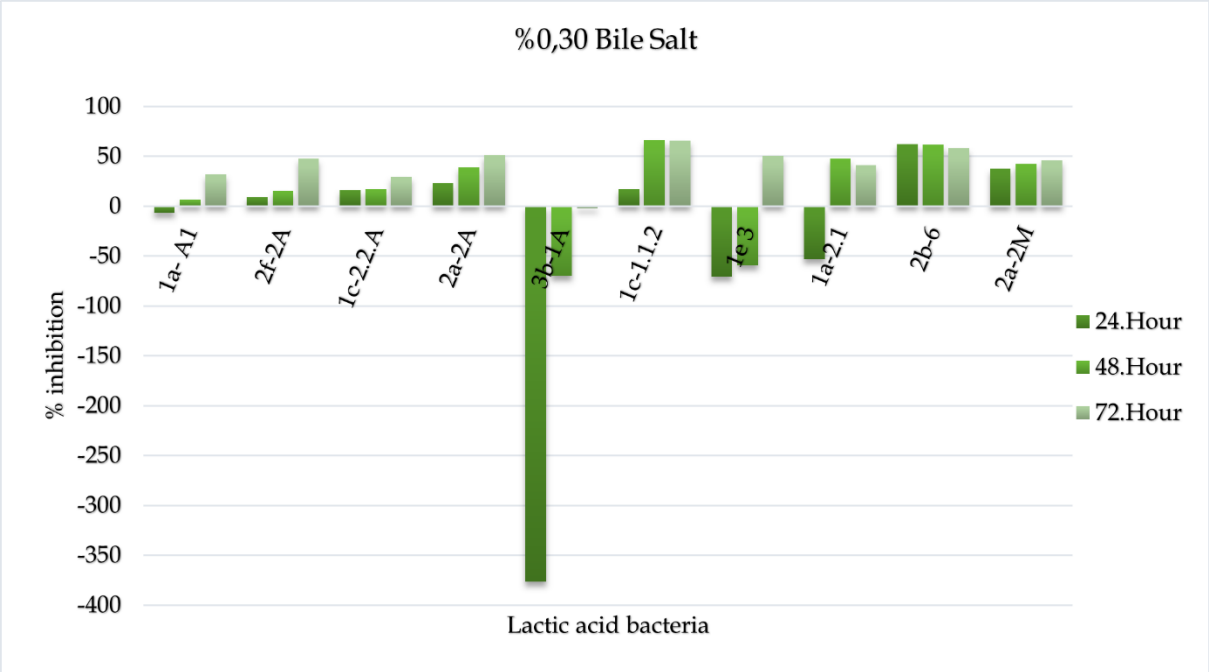

Figure 5. Inhibition percentages of probiotic microorganisms at 0.30% bile salt
1a-1A: *Lactobacillus kefiri 1,* 2f-2A: *Lactobacillus rhamnosus,* 1c-2.2A: *Lactobacillus plantarum,* 2a-2A: *Lactobacillus plantarum,* 3b-1A*: Bifidobacterium spp,* 1c-1.1.2: *Enterococcus gallinarum,* 1e-3: *Bacillus megaterium,* 1a-2.1: *Lactobacillus kefiri 2,* 2b-6: *Lactobacillus rhamnosus,* 2a-2: *Enterococcus faecium*.

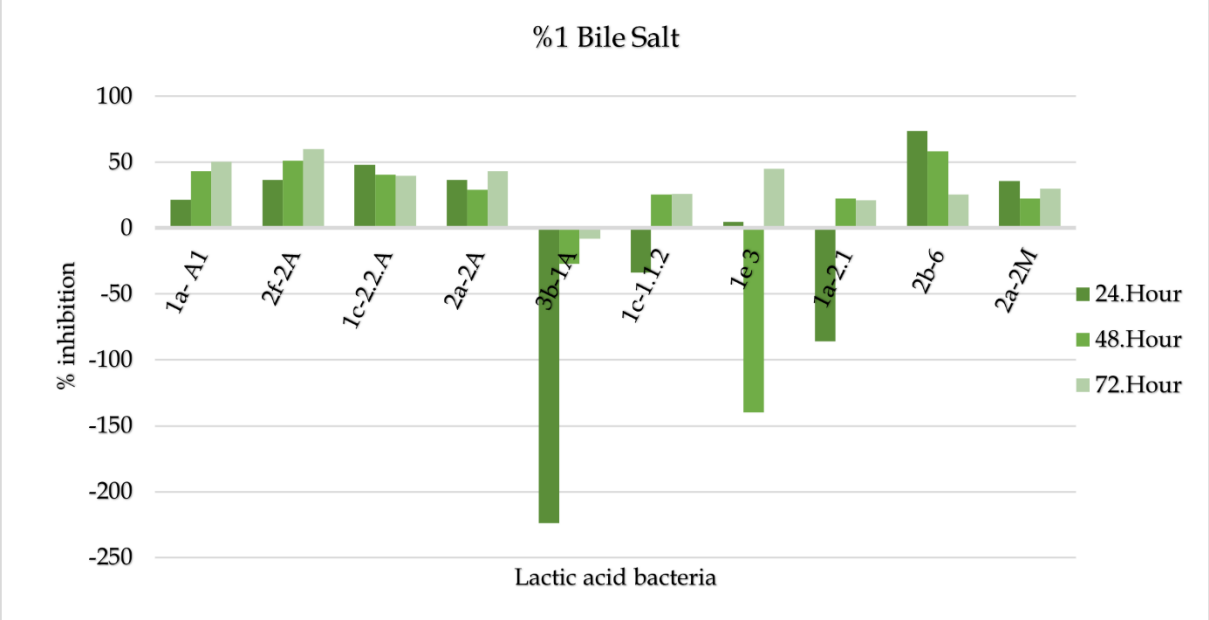

Figure 6. Inhibition percentages of probiotic microorganisms at 1% bile salt
1a-1A: *Lactobacillus kefiri 1,* 2f-2A: *Lactobacillus rhamnosus,* 1c-2.2A: *Lactobacillus plantarum,* 2a-2A: *Lactobacillus plantarum,* 3b-1A*: Bifidobacterium spp,* 1c-1.1.2: *Enterococcus gallinarum,* 1e-3: *Bacillus megaterium,* 1a-2.1: *Lactobacillus kefiri 2,* 2b-6: *Lactobacillus rhamnosus,* 2a-2: *Enterococcus faecium*

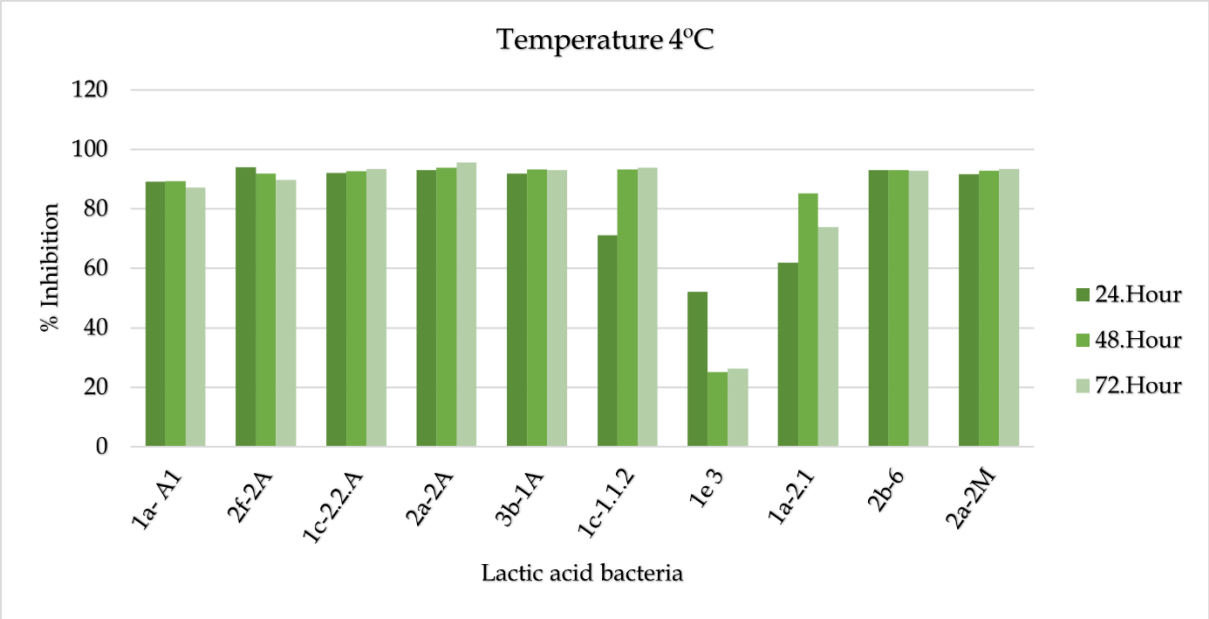

Figure 7. Inhibition percentages of probiotic microorganisms at 4°C.
1a-1A: *Lactobacillus kefiri 1,* 2f-2A: *Lactobacillus rhamnosus,* 1c-2.2A: *Lactobacillus plantarum,* 2a-2A: *Lactobacillus plantarum,* 3b-1A*: Bifidobacterium spp,* 1c-1.1.2: *Enterococcus gallinarum,* 1e-3: *Bacillus megaterium,* 1a-2.1: *Lactobacillus kefiri 2,* 2b-6: *Lactobacillus rhamnosus,* 2a-2: *Enterococcus faecium*.

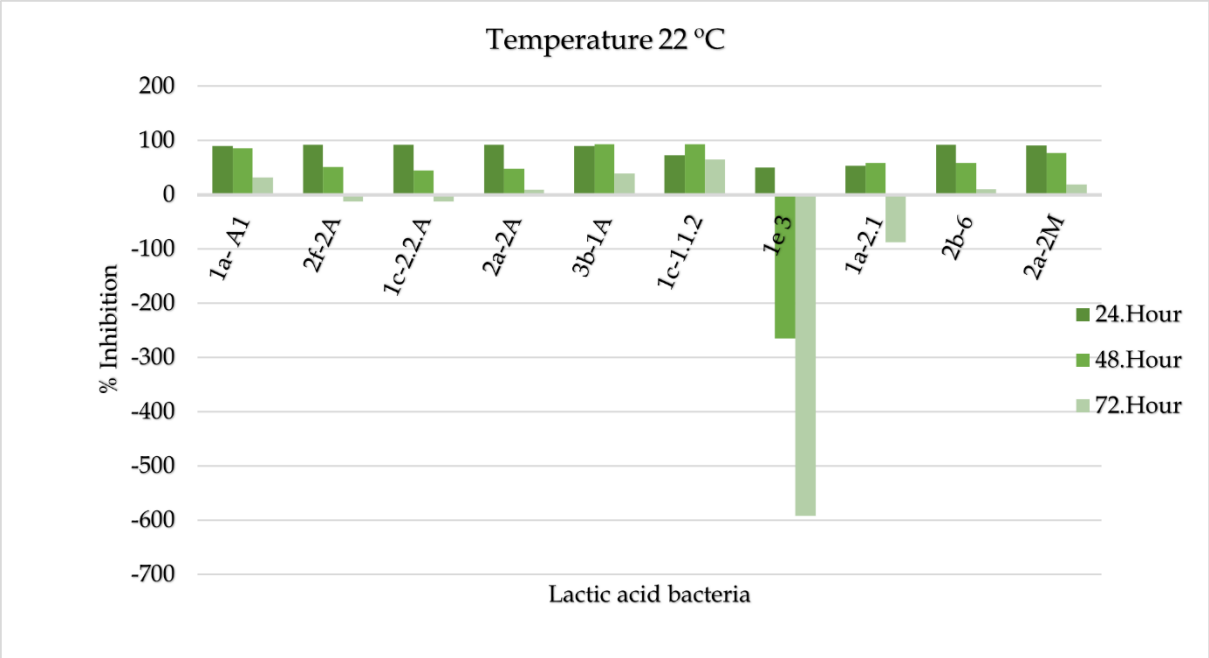

Figure 8. Inhibition percentages of probiotic microorganisms at 22°C
1a-1A: *Lactobacillus kefiri 1,* 2f-2A: *Lactobacillus rhamnosus,* 1c-2.2A: *Lactobacillus plantarum,* 2a-2A: *Lactobacillus plantarum,* 3b-1A*: Bifidobacterium spp,* 1c-1.1.2: *Enterococcus gallinarum,* 1e-3: *Bacillus megaterium,* 1a-2.1: *Lactobacillus kefiri 2,* 2b-6: *Lactobacillus rhamnosus,* 2a-2: *Enterococcus faecium*.

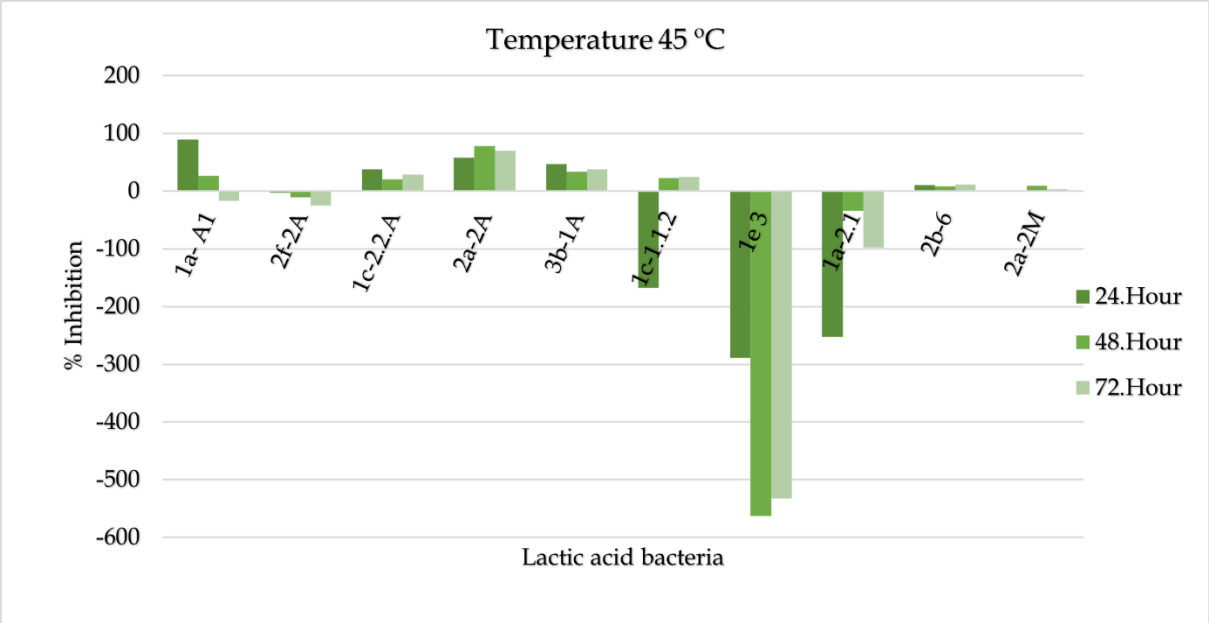

Figure 9. Inhibition percentages of probiotic microorganisms at 45ºC
1a-1A: *Lactobacillus kefiri 1,* 2f-2A: *Lactobacillus rhamnosus,* 1c-2.2A: *Lactobacillus plantarum,* 2a-2A: *Lactobacillus plantarum,* 3b-1A*: Bifidobacterium spp,* 1c-1.1.2: *Enterococcus gallinarum,* 1e-3: *Bacillus megaterium,* 1a-2.1: *Lactobacillus kefiri 2,* 2b-6: *Lactobacillus rhamnosus,* 2a-2: *Enterococcus faecium*.

Table 2. pH values of supernatants

	Probiotic Bacteria	*pH values*
1	**2b-6** *Lactobacillus rhamnosus*	4.13
2	**1c.1.1.2** *Enterococcus gallinarum*	4.09
3	*Lactobacillus sakei* **ATCC 15521**	4.87
4	**1a-1A** *Lactobacillus kefiri*	5.77
5	**2f-2A** *Lactobacillus rhamnosus*	4.04
6	**1c-2.2.A** *Lactobacillus plantarum*	3.89
7	**2a-2A** *Lactobacillus plantarum*	3.96
8	**3b-1A** *Bifidobacterium*	5.10
9	**2a-2M** *Enterecoccus faecium*	3.93
10	**1a-2.1** *Lactobacillus kefiri*	6.06
11	**1e-3** *Bacillus megaterium*	6.32
12	*Lactobacillus acidophilus* **ATCC4356**	4.42

2.2. Detection antimicrobial effect of probiotic microorganisms by spot on lawn method

In our study probiotic bacteria; *Lactobacillus plantarum* isolated from homemade whey showed the highest antimicrobial activity against clinical MRSA strain 3 (45.62 mm /zone) (Table 3)

Antimicrobial activity of (a) *Enterococcus faecium* (2a-2M) on clinical MRSA 3 is seen at Figure 10. Additionally, Figure 11 reflects the antimicrobial activity of (b) *Lactobacillus acidophilus* ATCC 4356 and (c) *Bifidobacterium spp.* (3b-1A) on clinical MSSA strain 9.

Table 3. Antibacterial effect* of probiotic bacteria on against standard and clinical *S. aureus* strains

Bacteria	***L. rhamnosus* 2b-6**	***E. gallinarum* 1c.1.1.2**	***L. sakei* ATCC 15521**	***L. kefiri 1* 1a-1A**	***L. rhamnosus* 2f-2A**	***L. plantarum* 1c-2.2.A**	***L. plantarum* 2a-2A**	***Bifidobacterium ssp.* 3b-1A**	***E. faecium* 2a-2M**	***L. kefiri 2* 1a-2.1**	***B. megaterium* 1e-3**	***L. acidophilus* ATCC 4356**
MRSA 1	23.26	22.45	19.06	13.47	25.44	24.37	22.75	12.67	27.48	4.78	0	19.66
MRSA 2	24.53	23.99	24.45	15.97	29.59	29.66	29.79	13.74	27.99	8.51	0	19.89
MRSA 3	34.56	26.7	37.49	18.98	31.64	45.62	28.09	18.18	30.71	7.78	0	22.45
MRSA 4	18.28	21.11	20.69	8.52	22.92	24.25	19.44	12.04	26.88	3.50	0	26.35
MRSA 5	23.66	17.6	18.55	9.18	21.11	26	26.79	14.71	26.07	1.75	0	16.29
MSSA 6	29.55	20.8	21.68	12.64	21.05	32.54	26.43	17.13	26.64	6.28	0	14.70
MSSA 7	14.39	18.80	15.21	4.32	23.53	22.57	29.35	4.83	22.01	5.10	0	15.39
MSSA 8	34	25.81	29.14	5.87	26.21	26.39	34.63	18.36	32.3	0	0	26.15
MSSA 9	19.83	18.14	19.5	12.28	24.86	29.58	23.01	15.12	25.83	3.21	0	21.49
MSSA 10	20.06	27.46	20.64	3.68	24.08	25.45	24.81	18.70	19.90	8.26	0	11.04
***S. aureus* ATCC 29213**	20.97	25.8	29.09	3.67	23.99	25.73	21.58	16.21	21.24	0	0	18.55
***S. aureus* ATCC 25925**	27.54	28.25	28.09	10.47	32.18	32.77	32.36	25.21	32.47	10.57	0	28.5
S. aureus ATCC 43300	7.2	23.02	16.85	33.37	33.91	27.55	33.89	32.02	23.49	23.62	0	30.25

*The values given in the table express the inhibition zone diameters in mm

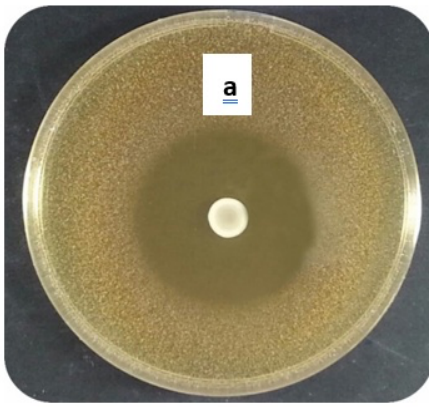

Figure 10. Antimicrobial activity of (a) *Enterococcus faecium* (2a-2M) on clinical MRSA 3

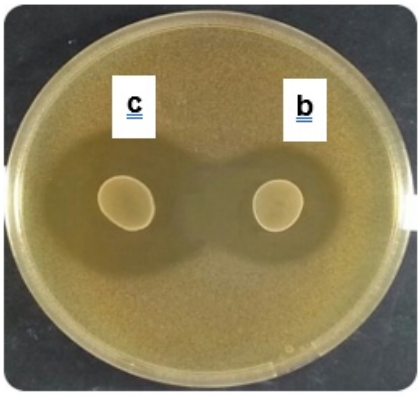

Figure 11. Antimicrobial activity of (b) *Lactobacillus acidophilus* ATCC 4356 and (c) *Bifidobacterium spp.* (3b-1A) on clinical MSSA strain 9.

2.3. Detection of antimicrobial effect of probiotic microorganisms by agar well diffusion method

Antimicrobial activity of probiotic bacteria on *S. aureus* by agar well diffusion method was investigated but no effect was observed. Using the same method, ciprofloxacin showed a 24.17 mm (100 µgmL^{-1}) inhibition zone against *S. aureus* strains.

3. DISCUSSION

Recently the usage of probiotics, known as live microorganisms, which have health benefits to the host when applied in sufficient quantities, has increased worldwide. In parallel with the studies declaring the probiotic properties of microbial species, the number of food containing probiotics, food supplements and drugs are widely used all over the world [12].

Probiotic products, also known as "pharmaceutical preparations" are products that are supplemented with microorganisms, various enzymes, vitamins and aroma components that have beneficial effects on the health of the host, are widely used all over the world (Europe, Japan and other Far East countries) [3]. Tablets or capsules containing probiotics are not used as substitutes in the treatment of diseases, but they are sold as health-promoting products [3,13].

Lactic acid bacteria are common examples of probiotics [7]. In our study, 4 homemade probiotic products, 5 pharmaceutical preparations and 7 market products were identified by using MALDI-TOF MS and conventional methods. These microorganisms are among the probiotic microorganisms identified by Çoşkun (2014) [14].

In various studies it was shown that *Streptococcus salivarius subsp. thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* were isolated from commercial yoghurt [15], *Enterococcus faecalis* from local cheeses [16], *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus kefir* and *Streptococcus thermophilus* from kefir samples[17], *Lactobacillus acidophilus* and *Bifidobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* have been isolated from yogurt and probiotic products [18].

In our study, probiotic microorganisms identified in dairy products and pharmaceutical preparations coincide with the microorganisms identified in the studies mentioned above.

In their study Schillinger and Lucke (1989) investigated the antibacterial effect of 19 *Lactobacillus sakei*, 3 *L. plantarum* and 1*curvatus* strains isolated from meat and meat products against *Lactobacillus* and *Listeria monocytogenes* microorganisms [19]. The investigators reported that while all of the *L. sakei* strains formed inhibition zone against indicator microorganisms by spot on lawn method, only 6 out of 19 *L. sakei* strains formed inhibition zones by agar well diffusion method [19].

In a studythe antimicrobial effects of various lactobacilli species isolated from fermented sausage on was investigated against *L. monocytogenes* and *S. aureus* by spot on lawn and agar well diffusion methods. It was shown that the antibacterial effect of *Lactobacillus* species against *L. monocytogenes* and *S. aureus* varied according to both indicator microorganisms and spot on lawn and agar well diffusion methods and it was found that antibacterial effect of lactobacilli species was higher in spot on lawn method. The investigators reported that although some strains of *L. sakei* showed inhibitory effect against *L. monocytogenes* by spot on lawn method, the same strains did not show inhibitory effect by well diffusion method [20].

In our study, the antimicrobial effect of supernatants of the isolated probiotic microorganisms against clinical MSSA and MRSA strains and standard *S. aureus* was investigated by using agar well diffusion method. However, the cultures of these microorganisms were found to form an inhibition zone against clinical and standard *S. aureus* strains by spot on lawn method. As it is known, supernatants were prepared by centrifugation and filtration processes and do not contain alive microorganisms but contain the metabolic products of the probiotic microorganisms.

The probiotic bacteria which take place in our study had not shown any effect when their supernatant was used (in agar well diffusion method), but when they were used alive (as in spot on lawn method) against clinical (MRSA and MSSA) *S. aureus* and reference strains of the same bacteria were effective.

4. CONCLUSION

In this study, we have found that probiotic microorganisms isolated from pharmacy preparations, market, and homemade probiotic products showed antibacterial activity on clinical and standard *Staphylococcus aureus* by spot on lawn method. We designated that the microorganisms mentioned above were efficient against clinical andstandard *Staphylococcus aureus* isolates.

The results from this study suggest the possible use of probiotic microorganisms as a natural alternative for treatment of *S. aureus* treatment of infections and may eliminate the concerns of antibiotic resistance.

5. MATERIALS AND METHODS

As stated in Table 4, in our study, the 5 clinical MSSA (methicillin-susceptible) and 5 MRSA (clinical methicillin-resistant) *S. aureus* isolates obtained from Haydarpaşa Numune Training and Research Hospital/Microbiology Laboratory together with 3 standard *S. aureus* strain (*S. aureus* ATCC 43300, *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213) were used. The ethics of clinical isolates were obtained from Marmara University Ethics Committee. Additionally, the standard probiotic microorganisms used in our study were *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus sakei* ATCC 15521.

A total of 16 probiotic products were used; 4 are homemade products (yogurt, whey, pickle juice and kefir), 7 are market products (plain and fruit kefir, fruit yogurt whey) and 5 are different pharmaceutical preparations. Dilutions of the probiotic products were made and 100 µL of them was spread on the surface of the MRS and all petri dishes were incubated for 72 hours at 37 ^{o}C under anaerobic and aerobic conditions and at 39 ^{o}C under microaerophilic conditions [15,21].

Morphological and biochemical properties of the isolates were first determined by Gram stain, catalase, gas production from glucose, arginine hydrolysis and Voges-Proskauer tests. Fresh cultures were then

Table 4. Clinical isolates and standard strains

Clinical strains	Source of clinical strains
MRSA 1	Blood
MRSA 2	Tracheal aspirate
MRSA 3	Tracheal aspirate
MRSA 4	Wound
MRSA 5	Wound
MSSA 6	Tracheal aspirate
MSSA 7	Throat
MSSA 8	Wound
MSSA 9	Wound
MSSA 10	Tracheal aspirate
Standard strains 1	*S. aureus* ATCC 43300
Standard strains 2	*S. aureus* ATCC 25925
Standard strains 3	*S. aureus* ATCC 29213

identified by MALDI-TOF MS (Matrix assisted laser desorption ionization time-of-flight mass spectrometry) (Biomeriux, France). Identified isolates were stored at -20 ºC in MRS Broth containing 20% glycerol for use in antimicrobial activity testing [15,21].

5.1. Identification of isolated microorganisms by MALDI-TOF-MS

A single colony was taken from the fresh culture on MRS agar plate and spread on a slide. Then 0.9 µL of VITEK MS CHCA (matrix solution) was added and allowed to dry. *E. coli* was used as the control strain. After the slides were then read on the MALDI-TOF MS device and expressed in Table 1 [22].

5.2. Determination of probiotic properties of isolates

Probiotic bacteria were activated twice by passage in MRS broth. Optical densities of active cultures were adjusted to 0.60 ± 0.02 at 600 nm on a spectrophotometer (Helios-Thermo, UK). Bacteria adjusted to 2% of the medium volume (60 µl) were inoculated into 3 ml MRS broth tubes containing pH 1.5, 2, 3.2. Next, 200 µl of the suspension from the test tubes was dispensed into each of the flat-bottomed 96-well microplate wells [22].

Microplates were then incubated for 24, 48, 72 hours at anaerobic, aerobic 37 ºC or 39 ºC under microaerophilic conditions. After incubation, 5 µL was taken from each well, stained on MRS agar, and petri dishes were incubated. In addition, the microplates were recorded as OD_2 by measuring optical densities at 630 nm. As a control, each bacterial strain was incubated in MRS broth medium with pH 6.4 ± under anaerobic, aerobic at 37 ºC or microaerophilic conditions at 39 ºC for 24, 48, 72 hours own growth conditions and after 24, 48, 72 hours its optical density was measured at 630 nm by microplate reader and recorded as OD_1.

Experiments were carried out in triplicate and after that the average value was obtained. The growth of bacteria in various pH media compared to the control was determined using the % inhibition formula below. The growth of bacteria at various pH conditions compared to control was determined using the formula below. In these conditions, the presence or absence of bacteria was determined according to the formation of stains [22].

% **Inhibition =** OD_1-OD_2 / OD_1 x100
OD_1: Control
OD_2: Growth rate of bacteria in various pH, bile salt and temperature environments.

5.4. Detection of the inhibition percentages of probiotic microorganisms at various bile salt concentrations

In order to determine the growth of the bacteria at various bile salt concentrations. The optical density values were taken from the adjusted bacteria (0.60 ± 0.02) After that OD values were taken from the adjusted bacteria by 2% and inoculated into 3 tubes containing MRS broth medium 0.15%, 0.30%, 1% bile salt. Then 200 µl from the test tubes of suspension was dispensed into each of the flat bottom 96 well microplate wells. The microplates were then incubated under anaerobic, aerobic at 37 °C or microaerophilic conditions at 39 °C for 24, 48, 72 hours. Following incubation, 5 µL was taken from each well and spot seeded on MRS agar and the petri dishes were incubated. In addition, microplates were recorded as OD_2 by measuring their optical density at 630 nm. As a control, each bacterium was incubated in MRS broth medium without bile salt. The growth of the bacteria at various pure salt conditions according to the control was determined by the formula below. The presence or absence of bacteria in these conditions was determined according to the spot formations [22].

5.5. Detection of the inhibition percentages of probiotic microorganisms at various temperature values

In order to determine the bacterial growth at various temperatures, the method applied at 5.4 was used. The growth of the bacteria at various temperature conditions compared to the control was determined using the formula below. Also, the viability of bacteria in these conditions was determined according to spot formulations on MRSA agar which were taken from the examples in wells described above [22].

5.6. Detection antimicrobial activity of probiotic microorganisms by spot on lawn method

The antimicrobial effect of lactic acid bacteria on clinical *S. aureus* microorganisms that were found to be alive at all three various pH, salt and temperature conditions was firstly determined by spot on lawn method. After the isolates were activated twice on MRS broth medium, 5 µl of each active culture was added to 15 mL MRS agar medium and spot was cultured. Then the petri dishes were incubated under anaerobic, aerobic at 37 °C or microaerophilic conditions at 39 °C for 24 hours. After incubation, suspensions of *S. aureus* strains used as test microorganism equivalent to the turbidity standard of McFarland 0.5. Then 50 µL of the prepared suspensions were transferred to TSA (0.7% agar) tubes containing 5 mL of soft agar and vortexed. The mixture was poured onto the petri with colonies of lactic acid bacteria isolates and allowed to solidify for 30 minutes at room temperature. The petri dishes were incubated at 37 °C for 24 hours. Then the diameters of the inhibition zones formed as a result of incubation were measured by digital caliper and recorded in mm. All experiments were performed in triplicate and the average results were taken [23, 24,25].

5.7. Preparation of probiotic bacteria supernatant

Probiotic bacteria were seeded into tubes containing MRS broth medium and subcultured twice at 37 °C for 18 hours. At the end of the incubation, MRS broth medium containing bacteria was centrifuged at 4000 *g* for 30 minutes and the cells were precipitated, and the resulting supernatant was filtered (0.22 µm) and then used in agar diffusion method [24].

5.8. Detection antimicrobial activity of probiotic microorganisms by agar well diffusion method

Another method used to determine the antimicrobial activity of lacticacid bacteria on *S. aureus* in our study was agar well diffusion method. The pH of the prepared probiotic bacteria supernatants was measured and recorded using a pHmeter (Hanna,Germany) and sterilized again by filtration (0.22 µm). Then 0.1mL of *S. aureus* suspensions prepared equivalent to McFarland 0.5 turbidity standard was added to Mueller Hinton Agar (MHA) (which was cooled to 40-45 °C and shaken well, later poured into sterile petri dishes of 90 mm diameter in 4 ± 0.5 mm thickness. After that, 6 mm diameter wells were punched by using sterile punch at 2.5 cm intervals. A volume of 50 µL of the bacterial supernatant was placed into each well one by one from the supernatant and controls. The petri dishes were incubated under aerobic conditions at 37°C for 24 h. and the diameter of the inhibition zones formed was measured by using a digital caliper and recorded in mm. All experiments were performed in triplicated. Ciprofloxacin (200 µg mL^{-1}) was used as the positive control and sterile distilled water was used as the negative control [25-28].

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