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Investigation of vitality, antibacterial properties, and antagonistic effects of probiotic bacteria in probiotic dairy products

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Abstract

Probiotics play a significant role in the diet, and their contribution to the immune system has been recognised. Their effects on the gastrointestinal system have been evaluated for decades, and the mechanisms of the effects may differ. The aim of the present work was (i) to observe the changes in pH and bacterial counts in common probiotic dairy products, (ii) to isolate probiotic bacteria, (iii) to evaluate antibacterial resistance, and (iv) to evaluate their metabolites' antibacterial effects against common foodborne pathogens. To this end, 20 dairy products labelled "probiotics included" were collected. Isolation and enumeration of *Lactobacillus* spp., *L. acidophilus*, and *Bifidobacterium* spp. were carried out using de Man-Rogosa-Sharp agar (MRS), clindamycin/ciprofloxacin-included MRS agar (MRS-CC), and mupirocin (MUP) supplemented *Bifidobacterium* selective count agar (BSC-MUP), respectively. Isolates were identified using MALDI-TOF MS analyses, enumerated, and evaluated for their pH values at 1 to 28 d after production, at 1-w intervals. Selected isolates were analysed for antibacterial resistance using the disc diffusion method. Supernatants were then collected from selected probiotics grown in broth, and studied for their antagonistic effects against pathogens using disc diffusion and agar-well diffusion tests. IBM SPSS software was used for statistical analyses. Tests of normality and non-parametric analyses were performed. On the last day of analyses, 75% of the products met the probiotic bacteria vitality requirement of 10^6 CFU/g. Statistical analyses showed no correlation between increased acidity and bacterial decrease ($p > 0.05$), while the decrease in pH and bacterial count had significant relationship ($p < 0.05$). All selected isolates of probiotic bacteria ($n = 10$) showed multi-drug resistance (MDR) to 10 different common antibiotics. Antagonistic effects were present but weak (inhibition zones were 0 - 4 mm in diameter). When consumed in sufficient amounts, probiotics may inhibit possible pathogen growth in the gut microbiota *via* metabolites.

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Introduction

Probiotic bacteria are living microorganisms that can provide health benefits to the host when consumed in adequate amounts (Smith and Jones, 2012; Nadelman *et al*., 2018). Examples include *Bifidobacterium* and lactic acid bacteria (LAB), such as *Lactobacillus*, *Enterococcus*, and *Lactococcus*. Probiotic dairy products such as yogurt, cheese, kefir, and fermented milk contain probiotic bacteria, and are widely consumed for their nutritional and therapeutic properties. The vitality of these bacteria in the products affects their sensory quality and safety characteristics, such as flavour, texture, acidity, and shelf life (Vinderola *et al*., 2011). The amount of probiotic bacteria must be at least 10^6 CFU/g in dairy

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products to enhance their nutritional and functional properties during their shelf life (Laličić-Petronijević *et al*., 2017; Widyastuti *et al*., 2021).

The vitality of probiotic bacteria refers to their ability to survive and maintain their metabolic activity during the production, storage, and consumption of probiotic dairy products. The vitality of probiotic bacteria can be affected by various environmental conditions, such as temperature, pH, oxygen, moisture, and food matrix (Vinderola *et al*., 2011). The vitality of probiotic bacteria is essential for their functionality and stability in the product and the host's gastrointestinal tract. Therefore, it is necessary to monitor and optimise the vitality in such products at different stages before their expiration date to ensure the functionality and quality of the viable bacteria (Ouwehand *et al*., 2016).

On the other hand, probiotic bacteria may also carry antibacterial resistance genes that could pose a risk of horizontal gene transfer to potential food or gut pathogens. Antibacterial resistance can be intrinsic, acquired by mutation, or acquired by horizontal gene transfer. Therefore, the digestive tract could act as a gene pool for transmitting antibiotic-resistance genes among the gut microflora, leading to the evolution of multidrug-resistant bacteria (Smith and Jones, 2012). Some strains of LAB and *Bifidobacterium* may show different levels of resistance depending on their origin (*e.g*., human, animal, or plant) or their exposure to antibiotics in their environment (*e.g*., dairy products, fermented foods, or probiotic supplements) (Gueimonde and Arboleya, 2021). Antibacterial resistance genes such as tetracycline-, erythromycin-, chloramphenicol-, and vancomycin-resistance genes have been detected in *Lactobacillus* and *Bifidobacterium* strains. Therefore, it is essential to evaluate the antibacterial susceptibility pattern and the genetic basis of resistance in probiotic strains before their application in functional foods or feeds (Gueimonde *et al*., 2013; Anisimova and Yarullina, 2019; Cizeikiene and Jagelaviciute, 2021).

The antagonistic effect of probiotic bacteria against pathogenic bacteria species is a valuable feature that can contribute to the prevention and treatment of various infectious diseases (Murry *et al*., 2004). The antagonistic effects of probiotic bacteria can be mediated by various mechanisms such as the production of organic acids, hydrogen peroxide, bacteriocins or other antimicrobial substances; competition for nutrients or adhesion sites; modulation of the immune system; or interference with quorum sensing (Servin, 2004; Hütt *et al*., 2006; Nogueira *et al*., 2023). The antagonistic activities of these bacteria against different pathogenic bacteria have been demonstrated *in vitro* and *in vivo* by several studies for some strains of *Lactobacillus* against *Escherichia coli*, *Salmonella enterica*, *Shigella flexneri*, and *Clostridium difficile*. Similarly, *Bifidobacterium lactis* and *B. longum* have shown antagonistic activity against some common foodborne pathogens such as *Staphylococcus aureus*, *Enterococcus faecalis*, and *Campylobacter jejuni* (Hütt *et al*., 2006).

It is important to investigate the vitality, antibacterial resistance, and antagonistic effects of probiotic bacteria in probiotic dairy products to ensure the quality and safety of these products, and to evaluate their potential health benefits. In this context, one of the objectives of the present work was to isolate and identify LAB and *Bifidobacterium* spp. during the shelf life of commercial probiotic dairy products using MALDI-TOF MS, a trusted method for identifying probiotic bacteria (Lewis *et al*., 2016). We also assessed the viability of LAB and *Bifidobacterium* spp. during the shelf life of the products, and determined their antibacterial resistance to commonly used antibiotics. The *in-vitro* antagonistic effect of these bacteria against common foodborne pathogens was also evaluated.

Materials and methods

Materials

The study was done using all probiotic dairy products that are commercially available in supermarkets (n = 20), which included probiotic yogurt (n = 11) and probiotic drinks (n = 9; ayran [n = 5] and kefir [n = 4]) with labels indicating "probiotic included" and "includes at least 10^6 CFU/g probiotic bacteria" on the package. Five packs from each category were purchased from supermarkets. The samples were kept cold, brought to the laboratory, and placed in a refrigerator at 4°C for 28 d. All lots were numbered from 1 to 20, and nine samples included fruit (samples 2, 3, 4, 5, 8, 10, 11, 18, and 19). A new package of the same lot was opened and analysed on each day of the analysis (28, 21, 14, 7, and 0 days to the expiration date).

Isolation and enumeration

For the isolation and enumeration of LAB, *Lactobacillus acidophilus* (Turgut and Cakmakci,

2018), and *Bifidobacterium* spp. (ISO, 2010), 10 g of samples were taken from each product, and homogenised with 90% ¼ Ringer's solution (Biokar, Pantin, France) to obtain 10^{-1} dilutions. Then, decimal serial dilution (10^{-1} - 10^{-9}) was prepared and used for microbiological analysis. From this dilution, *Bifidobacterium* spp., LAB/*Lactobacillus* spp., and *Lactobacillus acidophilus* were isolated and enumerated by preparing and inoculating mupirocin (MUP) selective supplement added *Bifidobacterium* selective count agar base (BSC-MUP) (Himedia, Mumbai, India), de Man, Rogosa, and Sharpe (MRS) agar (Biokar, Pantin, France), and clindamycin/ciprofloxacin-included MRS agar (MRS-CC) (Himedia, Mumbai, India).

Inoculated media were incubated in anaerobic conditions using an Anaerogen kit (Anaerogen, ThermoFisher, MA, USA) in anaerobic jars at 37°C for 48 - 72 h. After the incubation, colonies were counted within 25 - 250 colonies range, and noted in CFU/g. Then, isolates were subcultured and Gram-stained, evaluated biochemically and morphologically, inoculated into MRS broth (Merck, Darmstadt, Germany) containing 20% glycerol cryotubes, and kept at -20°C until further identification (Bamgbose *et al*., 2022).

Identification

After the isolation steps, MALDI-TOF MS analyses were done for identification (Bruker Daltonik, Bremen, Germany), and 31 isolates from the samples were identified. Briefly, isolates were taken from tubes containing glycerol, inoculated into MRS broth using a loop, and left to incubate in an Anaerogen jar for 72 h at 37°C. At the end of the incubation, isolates were inoculated rapidly on MRS agar, and incubated in an anaerobic environment at 37°C for 48 h. After incubation, ethanol-formic acid extraction was applied to the bacterial colonies, and then spread onto MALDI-TOF MS plates before adding 1 µL of matrix mix, and analysing the colonies (Huang *et al*., 2016). Criteria reported by Seng *et al*. (2009) were used to evaluate the results, and the samples with two or more spectrum points were further assessed.

pH evaluation

Before microbiological analyses, pH levels were measured using a pH meter (Mettler Toledo, Columbus, OH, USA) (Turgut and Cakmakci, 2018). A volume of 20 mL from each dairy product sample was taken in 50-mL sterile beakers to evaluate the relationship between the number of probiotic bacteria and the products' pH levels during the shelf life.

Reviving the isolates

LAB and *Bifidobacterium* sp. isolates and pathogens that had been frozen at -20°C were brought to room temperature. All isolates were inoculated separately into Brain Heart Infusion broth (Oxoid, United Kingdom). LAB and *Bifidobacterium* sp. isolates were also inoculated into MRS 0.2 broth (MRS broth with 2% glucose addition) (Merck, Germany) to obtain the cell-free supernatant. Inoculated media were incubated in anaerobic conditions at 37°C for 24 h (Nemska *et al*., 2022).

Preparing cell-free supernatants

Broth tubes with bacterial growth were centrifuged at 3,260 *g* (4,500 rpm) at 4°C for 30 min (SL 8R Centrifuge, ThermoFisher Scientific, Waltham, MA, USA). The supernatant was pipetted out and sterilised by filtering through 0.22-µm filter papers, and then centrifuged again at 3,260 *g* (4,500 rpm) and 4°C for 10 min (Chen *et al*., 2019).

Detection of antibacterial resistance of LAB and Bifidobacterium sp.

One isolate of each species identified by MALDI TOF MS with the highest score was used to determine the antibacterial resistance and antagonistic effect. An agar disc diffusion assay was used to observe the antibacterial resistance since the bacteria might perform gene transfer in the gut microbiota. For this method, discs of the following antibiotics (Oxoid, United Kingdom) were used: chloramphenicol (30 µg), ampicillin (10 µg), oleandomycin (15 µg), penicillin (10 µg), kanamycin (30 µg), erythromycin (15 µg), neomycin (30 µg), tetracycline (30 µg), polymyxin (300 µg), and vancomycin (30 µg).

Mueller-Hinton agar (Oxoid, United Kingdom) was prepared and poured into Petri dishes to a thickness of approximately 4 mm, and left in an incubator to dry at 25°C overnight. Samples were taken from the Mueller-Hinton broths (Oxoid, United Kingdom) with bacterial growth, and inoculated into sterile saline tubes to fix the solutions to the 0.5 McFarland standard. After setting the suspension, samples were taken using sterile swabs and spread onto Petri dishes. At 15 min after inoculation, antibiotic discs were laid onto the surface, and

incubated at 37°C in anaerobic conditions for 72 h, and inhibition zones were measured (Celik *et al*., 2016; Prabhurajeshwar and Chandrakanth, 2017; 2019).

Detection of antagonistic effects of LAB and Bifidobacterium spp.

Detection of antagonistic activity using agar disc diffusion method

Antagonistic effects of the selected isolates were evaluated using the agar disc diffusion method with modifications developed by Soomro *et al*. (2007). Briefly, Nutrient agar (Oxoid, United Kingdom) was prepared and poured into Petri dishes to a thickness of approximately 4 mm. The test-sample pathogens were set to the 0.5 McFarland standard, and the bacterial suspension was spread onto the media, and dried in an incubator for 30 min. At 15 min after inoculation, discs soaked with 20 µL of cell-free supernatant were carefully laid onto the media. After incubation with the proper time and conditions, inhibition zones were measured using a ruler and recorded in millimetres (mm) (Fijan, 2016).

Detection of antagonistic activity using agar well diffusion method

Nutrient agar (Oxoid) was prepared and inoculated with pathogens at 1% and set at the 0.5 McFarland standard (Den-1B McFarland Densitometer, Biosan, Latvia) for an agar well diffusion assay. It was then poured into sterile Petri dishes to a thickness of approximately 6 mm. After solidification, 6-mm wells were made in the agar, and sealed with 0.05 mL (1 drop) of liquid agar at the bottom. Then, wells were inoculated with 100 µL of cell-free supernatant, and incubated with proper temperatures and conditions. After incubation, inhibition zones were measured using a ruler and recorded in millimetres (mm) (Fijan, 2016). The pathogens used were *E. coli* ATCC 8759, *E. coli* O157 ATCC 43895, *S. aureus* ATCC 29213, *S. enterica* spp. *enterica* ATCC 14028, and *Listeria monocytogenes* ATCC 19115.

Statistical analyses

The data collected were evaluated using non-parametric tests in SPSS software. A Shapiro-Wilk normality test was performed on the data to see whether the distribution was normal. Then, Friedman tests were performed on data to detect any differences, and the Wilcoxon test was performed to see whether the differences were significant. A correlation test was performed on the pH values and bacterial count to see whether there was any relation between them.

Results

The pH values for each product were evaluated, and median values were calculated. The results showed that over time, the pH values decreased. The statistical analyses indicated that the decrease from day 0 to 28 was significant ($p = 0.025$). Table 1 shows the median numbers and mean pH values of LAB/*Lactobacillus* spp., *L. acidophilus*, and *Bifidobacterium* spp. from each sample from weekly measurements. Table 2 shows the MALDI-TOF MS results. Table 3 shows that the numbers of LAB/*Lactobacillus* spp. decreased tenfold during 28 d. This indicated that the amount of LAB decreased significantly ($p < 0.001$).

Only six of the products (30%) had *L. acidophilus*. During this period, the count of *L. acidophilus* per millilitre of the products was not found to decrease significantly ($p > 0.005$). All products (100%) had *Bifidobacterium* as probiotics. Based on the MALDI-TOF MS assay results, *Bifidobacterium animalis* subsp. *lactis* was the only species from its genus in the products as a probiotic. The bacteria count was sufficient on the last measuring day, even though the numbers decreased significantly ($p = 0.001$). The pH of probiotic products varied between 3.71 and 4.41, but we could not find any statistical connection between pH and bacteria count.

The antibiotic resistance of obtained LAB isolates was evaluated *via* the zone diameters around the antibiotic discs plated onto the inoculated plates. All selected probiotic isolates (100%) were resistant to kanamycin, polymyxin, and vancomycin. Nine isolates (90%) were resistant to penicillin, while one was intermediately resistant. Eight isolates (80%) were resistant to chloramphenicol, neomycin, and tetracycline, while one had intermediate resistance (10%), and one (10%) was susceptible to chloramphenicol. Two (20%) were sensitive to neomycin, and two (20%) had intermediate resistance to tetracycline.

Table 1. Some properties of samples, mean pH, and median counts of LAB, *Bifidobacterium* sp., and *L. acidophilus* (CFU/g).

Sample no.	Type	Fruit*	pH ± SD	LAB ± SD	*Bifidobacterium* spp. ± SD	*L. acidophilus* ± SD
1	Y	No	4.28 ± 0.03	$2.3\times10^{7} \pm 1.7 \times 10^{7}$	$8.4 \times 10^{7} \pm 4.9 \times 10^{7}$	0
2	Y	Yes	4.27 ± 0.03	$4.1 \times 10^{7} \pm 2.5 \times 10^{7}$	$4.8 \times 10^{8} \pm 4.4 \times 10^{8}$	0
3	Y	Yes	4.25 ± 0.03	$4.3 \times 10^{7} \pm 2.6 \times 10^{7}$	$4.7 \times 10^{7} \pm 3.0 \times 10^{7}$	0
4	Y	Yes	4.22 ± 0.06	$2.4 \times 10^{7} \pm 1.4 \times 10^{7}$	$2.2 \times 10^{8} \pm 2.3 \times 10^{8}$	0
5	Y	Yes	4.30 ± 0.01	$5.2 \times 10^{7} \pm 1.6 \times 10^{7}$	$5.1 \times 10^{8} \pm 5.4 \times 10^{8}$	0
6	Y	No	4.00 ± 0.03	$3.0 \times 10^{7} \pm 1.2 \times 10^{7}$	$2.8 \times 10^{7} \pm 3.1 \times 10^{7}$	$3.7 \times 10^{7} \pm 3.5 \times 10^{7}$
7	Y	No	4.37 ± 0.05	$1.1 \times 10^{8} \pm 9.6 \times 10^{7}$	$1.2 \times 10^{8} \pm 2.3 \times 10^{7}$	0
8	Y	Yes	4.36 ± 0.06	$1.3 \times 10^{8} \pm 3.0 \times 10^{7}$	$6.9 \times 10^{7} \pm 6.4 \times 10^{7}$	$2.7 \times 10^{8} \pm 2 \times 10^{8}$
9	D	No	3.85 ± 0.03	$2.6 \times 10^{7} \pm 6.6 \times 10^{6}$	$8.3 \times 10^{6} \pm 9.3 \times 10^{6}$	0
10	D	Yes	4.04 ± 0.02	$8.8 \times 10^{7} \pm 5.1 \times 10^{7}$	$8.9 \times 10^{7} \pm 1.2 \times 10^{8}$	0
11	D	Yes	4.21 ± 0.03	$1.3 \times 10^{8} \pm 5.4 \times 10^{7}$	$4.8 \times 10^{8} \pm 1.9 \times 10^{8}$	0
12	Y	No	4.17 ± 0.01	$5.1 \times 10^{8} \pm 4.6 \times 10^{8}$	$2.7 \times 10^{9} \pm 2.7 \times 10^{9}$	0
13	Y	No	4.10 ± 0.01	$2.8 \times 10^{8} \pm 2.7 \times 10^{8}$	$1.9 \times 10^{6} \pm 4.3 \times 10^{5}$	0
14	D	No	4.08 ± 0.04	$7.9 \times 10^{7} \pm 7.3 \times 10^{7}$	$7.4 \times 10^{7} \pm 7.0 \times 10^{7}$	$1.1 \times 10^{7} \pm 1.2 \times 10^{7}$
15	D	No	4.09 ± 0.01	$3.2 \times 10^{7} \pm 1.8 \times 10^{7}$	$9.6 \times 10^{6} \pm 3.2 \times 10^{6}$	0
16	D	No	4.32 ± 0.00	$4.4 \times 10^{7} \pm 4.5 \times 10^{7}$	$1.8 \times 10^{7} \pm 2.9 \times 10^{7}$	$3.4 \times 10^{7} \pm 3.3 \times 10^{7}$
17	D	No	3.91 ± 0.14	$4.5 \times 10^{7} \pm 2.7 \times 10^{7}$	$1.4 \times 10^{7} \pm 2.9 \times 10^{7}$	$2.1 \times 10^{6} \pm 4.1 \times 10^{5}$
18	Y	Yes	4.15 ± 0.02	$3.2 \times 10^{7} \pm 6.6 \times 10^{7}$	$3.0 \times 10^{8} \pm 1.1 \times 10^{8}$	0
19	D	Yes	4.13 ± 0.02	$4.6 \times 10^{7} \pm 2.7 \times 10^{7}$	$5.0 \times 10^{3} \pm 4.6 \times 10^{3}$	0
20	D	No	4.28 ± 0.03	$7.8 \times 10^{7} \pm 4.6 \times 10^{7}$	$2.4 \times 10^{6} \pm 1.3 \times 10^{6}$	$4.1 \times 10^{7} \pm 2.9 \times 10^{7}$

Y: yogurt; and D: drink. (*) Whether the product has fruit or not.

Table 2. MALDI-TOF MS identification results of LAB and *Bifidobacterium* spp. from samples.

Sample no.	Identified probiotic	Sample no.	Identified probiotic
1	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus rhamnosus* *Lactococcus lactis*	11	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus rhamnosus* *Lactobacillus zeae* *Lactobacillus casei*
2	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus rhamnosus*	12	*Bifidobacterium animalis* ssp. *lactis*
3	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus rhamnosus* *Lactococcus lactis*	13	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus delbrueckii* ssp. *bulgaricus* *Lactobacillus casei* *Lactobacillus coryniformis*
4	*Bifidobacterium animalis* ssp. *lactis* *Lactococcus lactis*	14	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus rhamnosus* *Lactobacillus delbrueckii* ssp. *bulgaricus* *Lactobacillus acidophilus* *Lactobacillus zeae* *Lactobacillus coryniformis*
5	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus coryniformis* *Enterococcus durans*	15	*Bifidobacterium animalis* ssp. *lactis*
6	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus rhamnosus* *Lactobacillus acidophilus*	16	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus acidophilus* *Lactobacillus coryniformis*
7	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus rhamnosus*	17	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus acidophilus* *Lactobacillus coryniformis* *Lactobacillus kefiri* *Enterococcus durans*
8	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus rhamnosus* *Lactobacillus acidophilus* *Lactococcus lactis*	18	*Bifidobacterium animalis* ssp. *lactis* *Lactococcus lactis*
9	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus rhamnosus*	19	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus coryniformis* *Lactobacillus kefiri*
10	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus zeae*	20	*Bifidobacterium animalis* ssp. *lactis* *Lactobacillus acidophilus* *Lactobacillus kefiri*

Table 3. pH values and LAB, *Bifidobacterium* sp., and *L. acidophilus* counts (CFU/g) of samples on days 28 and 0 of analyses.

Sample no.	pH		LAB		*Bifidobacterium* spp.		*L. acidophilus*	
	28*	0*	28*	0*	28*	0*	28*	0*
1	4.31	4.25	4.4×10^7	2×10^6	1.2×10^8	7.8×10^6	0	0
2	4.31	4.26	6.6×10^7	1.4×10^6	8.9×10^8	5×10^6	0	0
3	4.30	4.25	6.5×10^7	1×10^4	8.8×10^7	1.8×10^7	0	0
4	4.25	4.12	3.9×10^7	3.6×10^6	5.2×10^8	8.1×10^6	0	0
5	4.29	4.29	7.4×10^7	4×10^7	1.4×10^9	5×10^7	0	0
6	4.05	3.99	4×10^7	9×10^6	8×10^7	2.9×10^7	8.1×10^7	7×10^6
7	4.40	4.30	2.2×10^8	1.3×10^7	1×10^8	1.6×10^8	0	0
8	4.39	4.26	1.6×10^8	1.4×10^8	1.1×10^8	3×10^6	5.5×10^8	7.8×10^7
9	3.83	3.91	3.1×10^7	1.6×10^7	2.3×10^7	5×10^5	0	0
10	4.07	4.04	1.3×10^8	1.3×10^6	3×10^8	6×10^6	0	0
11	4.25	4.21	2×10^8	4.9×10^7	7×10^8	3.2×10^8	0	0
12	4.18	4.17	9×10^8	3×10^6	6×10^9	2.4×10^7	0	0
13	4.13	4.10	3.5×10^8	7×10^7	2.2×10^6	2×10^6	0	0
14	4.13	4.10	7.2×10^7	8×10^7	1.5×10^8	1.5×10^7	2.4×10^7	3×10^5
15	4.09	4.11	2.2×10^7	4×10^7	1.2×10^7	4.3×10^6	0	0
16	4.32	4.31	1×10^8	1.5×10^6	6.7×10^7	1.2×10^3	7×10^7	7×10^5
17	3.71	4.02	8×10^7	1×10^7	6.6×10^7	2×10^2	2×10^6	2.8×10^6
18	4.16	4.13	1.5×10^8	3.2×10^5	1.7×10^8	2×10^8	0	0
19	4.15	4.16	8×10^7	1.5×10^7	1×10^4	4.2×10^2	0	0
20	4.32	4.26	1.2×10^8	2.4×10^7	4.6×10^6	1.8×10^6	5×10^7	4×10^7

(*) 28 indicates 28 days to expiration date, and 0 indicates expiration date.

Seven (70%) of the isolates were resistant to ampicillin, while two (20%) had medium resistance, and one (10%) was susceptible. Five (50%) of the isolates were resistant to both oleandomycin and erythromycin, five (50%) had intermediate resistance to oleandomycin, three (30%) had intermediate resistance to erythromycin, and two (20%) were susceptible to it. All selected isolates ($n = 10$) showed multi-drug resistance (MDR) (Table 4). The results of the antagonistic activities against a wide range of pathogenic microorganisms are shown in Table 5.

Discussion

In many cases, especially in the current COVID-19 epidemic, probiotics are an alternative to drugs for strengthening immunity and maintaining a healthier life by reducing the risk, duration, or severity of many diseases (Monteiro *et al*., 2022). It has one of the fastest-growing product markets due to increasing consumer awareness of the positive effects of probiotic foods on health (Kucukgoz and Trzaskowska, 2022). However, the bacterial count must be at least 10^6 CFU/g for the probiotics' benefits to be effective. Based on our study, analysing the effect of time on probiotic bacteria is possible through samples taken 28 d before the expiration date. A decrease in bacterial count was observed, which indicated that it would be best to consume probiotic dairy products sooner than the expiration date.

The pH value of three ayran samples increased during the analyses, unlike other samples. In total, the

Table 4. Results of antibacterial susceptibility testing of selected LAB and *Bifidobacterium* isolates.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
C	(R)	(R)	(I)	(R)	(R)	(R)	(S)	(R)	(R)	(R)
AMP	(I)	(R)	(I)	(R)	(R)	(R)	(S)	(R)	(R)	(R)
OL	(I)	(I)	(R)	(R)	(I)	(R)	(I)	(R)	(I)	(R)
P	(R)	(R)	(R)	(R)	(R)	(R)	(I)	(R)	(R)	(R)
K	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(R)
E	(I)	(R)	(I)	(R)	(I)	(R)	(S)	(S)	(R)	(R)
N	(R)	(R)	(R)	(R)	(R)	(R)	(S)	(S)	(R)	(R)
TE	(R)	(I)	(R)	(R)	(R)	(R)	(I)	(R)	(R)	(R)
PB	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(I)	(R)	(R)
VA	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(R)

C: chloramphenicol (30 µg); AMP: ampicillin (10 µg); OL: oleandomycin (15 µg); P: penicillin (10 µg); K: kanamycin (30 µg); E: erythromycin (15 µg); N: neomycin (30 µg); TE: tetracycline (30 µg); PB: polymyxin (300 µg); and VA: vancomycin (30 µg). Numbers indicate a specific bacterium. 1: *Lactobacillus rhamnosus*; 2: *Lactobacillus delbrueckii* ssp. *bulgaricus*; 3: *Lactobacillus coryniformis*; 4: *Lactococcus lactis*; 5: *Bifidobacterium animalis* spp. *lactis*; 6: *Enterococcus durans*; 7: *Lactobacillus zeae*; 8: *Lactobacillus kefiri*; 9*: Lactobacillus casei*; and 10*: Lactobacillus acidophilus.* R: resistant; I: intermediate; and S: susceptible.

Table 5. Zone diameters (mm) from antagonistic tests of selected LAB and *Bifidobacterium* isolates against foodborne pathogens.

	E. coli **ATCC 8759**		***S. aureus*** **ATCC 29213**		***Salmonella*** **ATCC 14028**		***L. monocytogenes*** **ATCC 19115**		***E. coli*** **ATCC 43895**	
	Well	**Disc**	**Well**	**Disc**	**Well**	**Disc**	**Well**	**Disc**	**Well**	**Disc**
1	1	0	0	0	1.5	0	0	0	0	0
2	2	2	1	1	2	2	0	0	1	1
3	3	0	0	0	1	1	0	0	2	2
4	4	2	2	0	1	1.5	0	0	1	1
5	4	2	3	1	2	1	0	0	3	1.5
6	1	0	2	0	0.5	0.5	0	0	1	1
7	1	0	0	0	2	0	0	0	0	0
8	3	0	0	0	1	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0

Numbers indicate a specific bacterium. 1: *Lactobacillus rhamnosus*; 2: *Lactobacillus delbrueckii* ssp. *bulgaricus*; 3: *Lactobacillus coryniformis*; 4: *Lactococcus lactis*; 5: *Bifidobacterium animalis* spp. *lactis*; 6: *Enterococcus durans*; 7: *Lactobacillus zeae*; 8: *Lactobacillus kefiri*; 9*: Lactobacillus casei*; and 10*: Lactobacillus acidophilus.*

mean pH values decreased from 4.18 to 4.16, which agreed with Akalin *et al*. (2004). Although this change was significant (p = 0.025), there was no correlation between the pH change and the viability of the culture. Similar to the results of Shah *et al*. (1995), our samples had significant decrease in the pH values.

Yogurt samples showed more diversity in bacteria (seven different species) than drinkable products (four other species). *B. animalis* subsp. *lactis* and *Lactobacillus rhamnosus* were seen in yogurt and ayran/kefir samples. On the other hand, the bacterial counts were close to each other. The number of LAB decreased tenfold in nine products (45%), while it decreased more in five products (25%), but it did not significantly decrease in six products (30%). Except for two samples (samples 3 and 18), bacterial counts were adequate according to the Turkish Food Codex (TFC, 2009).

On the last day of analyses, 15 out of 20 dairy products (75%) met the regulations indicated by the Turkish Food Codex (TFC, 2009), which states that "fermented dairy products must have at least 10^6 CFU/g of probiotic bacteria" During incubation, *Bifidobacterium* spp. count decreased tenfold, as seen in the other bacteria. The highest *Bifidobacterium* spp. count on the final day was 3.2×10^8 CFU/mL in the "fruity-lactose-free probiotic drink" (sample 11). The lowest *Bifidobacterium* spp. count was seen in "plain kefir," which decreased to 2×10^2 CFU/mL (sample 17). Live bacteria found in fermented foods such as kefir fall below the criteria for a product to be considered a probiotic. Therefore, it has been suggested that foods containing live bacteria, such as fermented milk products, should not be defined as probiotics but rather as "containing live and active cultures" (Hill *et al*., 2014).

Table 4 indicates that LAB had antimicrobial activity against pathogenic bacteria, although the effects may be weak. It was observed that the antimicrobial activity was the lowest against *L. monocytogenes* since there were no inhibition zones. *Bifidobacterium animalis* subsp. *lactis* was the most effective antibacterial agent against pathogens, and inhibited *E. coli* the most. There were more extensive inhibition zones in the agar well diffusion assay than in the agar disc diffusion assay.

Compared to the study by Celik *et al*. (2016), our isolates showed 80% resistance to tetracycline, while they found that none of their LAB isolates had resistance to tetracycline. Most isolates were resistant to antibiotics in our study, whereas Celik *et al*. (2016) found susceptible isolates. Prabhurajeshwar and Chandrakanth (2019) observed approximately 65% susceptibility to kanamycin, chloramphenicol, and neomycin, and 70% susceptibility to vancomycin and tetracycline.

Prabhurajeshwar and Chandrakanth (2017) showed that half of the isolates were resistant to chloramphenicol, 69% were sensitive to erythromycin, 63% were susceptible to tetracycline, and 19% were susceptible to vancomycin, unlike our results, which mostly showed resistant isolates. Reuben *et al*. (2019) observed 100% resistance to penicillin and 83% resistance to chloramphenicol, erythromycin, and vancomycin. These results indicated that LAB might have developed antibiotic resistance or that commercial isolates in Turkey have become resistant. It can be suggested that the LAB isolated from commercial products have MDR, which is a severe public health problem.

The bacteria isolated from the probiotic dairy products were similar to those found by Van de Casteele *et al*. (2006). It was reported that a fruit yogurt formulation and fruit mixtures' pH might affect the quality of probiotic bacteria (do Espírito Santo *et al*., 2011; Barat and Özcan, 2016), although we have not assessed any differences (samples 2, 3, 4, 5, 8, 10, 11, 18, and 19). The pH should be around 4.5 or lower to produce a high-quality yogurt. A decrease in pH value under 4.4 causes probiotic bacteria to decrease by about 10^3 - 10^4 CFU/g-, as shown in our study (Guler-Akin and Akin, 2007; Salman *et al*., 2020).

According to Tharmaraj and Shah (2009), LAB showed antagonistic effects against some spoilage and pathogenic bacteria. The smallest inhibition zone for *E. coli* was 11 mm, while the largest was 17 mm. Our results showed a different range in inhibition zones of 1 to 4 mm. The inhibition of *S. aureus* observed by Tharmaraj and Shah (2009) was notable, ranging between 12 and 23 mm, while in this study, a range of 1 to 3 mm was found. Their most effective isolate was *L. rhamnosus*, while ours was *Bifidobacterium animalis* subsp. *lactis*.

It seems possible that pathogens may develop immunity to the LAB metabolites over time. Sayeed *et al*. (2017) showed that commercial LAB has antagonistic effects against pathogenic bacteria. Based on their research, the antimicrobial effects of probiotics were quite adequate for *S. aureus*, and the largest inhibition zones were 15 mm wide.

In contrast, our probiotic isolates were most effective against *E. coli*, with the highest inhibition zone measured at 4 mm. Yesillik (2009) found that commercial probiotic dairy products had antagonistic effects on pathogens with inhibition zones of 17 mm for *E. coli*, 21 mm for *S. aureus*, and 28 mm for *S.* Typhimurium. In that study, it was found that even homemade yogurts had antibacterial effect against foodborne pathogens.

Conclusion

Clinical studies show that consuming probiotics enhances immunity, and helps to regulate intestinal problems, fight illness, and maintain a healthier life. Regarding the effects of probiotics on the pathogens that can diffuse in the gut epithelium, a sufficient number of probiotics inhibit pathogen growth, leading to a healthier gastrointestinal tract. Considering the results, yogurt, ayran, or kefir consumption would provide equal health benefits, but the probiotic species may differ. Furthermore, probiotic products should be consumed as soon as they are produced because microbial vitality decreases over time.

The present work showed a severe case of antibiotic resistance for LAB, which is undesirable. Bacteria can exchange resistance genes in the gut microflora, leading to MDR pathogen strains and health threats. The present work could be helpful for further studies about probiotics, and understanding their mechanism of action in the gut microbiota.

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