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Research Article

Dissecting Probiotic Diversity in a Fermented Cheese: A Multivariate Approach to Strain Characterisation and Safety

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Probiotics improve intestinal microbiota, thereby positively influencing human health. Additionally, most probiotic microorganisms belong to the group of lactic acid bacteria. As these bacteria are involved in producing fermented foods and drinks, they can easily be introduced to the body. Therefore, investigating the probiotic properties of microorganisms in fermented products is crucial for their characterisation. This study investigated 10 Surk strains belonging to the species *Ligilactobacillus acidipiscis*, *Lactiplantibacillus plantarum* and *Companilactobacillus alimentarius*, an important traditional cheese in the Eastern Mediterranean region, in terms of their tolerance to gastric juice and bile salts, auto/co-aggregation, cell surface hydrophobicity, bile salt hydrolase activity, antimicrobial activity, DNase and gelatinase activity, ability to form biofilms, virulence factors and antibiotic susceptibility. The survival rate of all strains was between 76.59% and 82.36% in gastric juice and between 84.44% and 110.28% in bile salts. All the strains were hydrophobic to varying degrees (2.08%–63.37%) and were capable of autoaggregating (32.38%–75.67%). Additionally, almost all of the strains exhibited antimicrobial activity against the pathogens. Finally, the strains were found to lack DNase and gelatinase activity, as well as virulence factors. According to these findings, the Surk strains employed in this investigation may find application as probiotics. However, further studies are needed, particularly with regard to antibiotic resistance, before these isolates can be used in food products.

Keywords: *Companilactobacillus alimentarius*; *Lactiplantibacillus plantarum*; *Ligilactobacillus acidipiscis*; probiotic property; Surk cheese

1. Introduction

Probiotics are described as single or mixed cultures with significant effects on human health that positively modify the indigenous gut bacteria. Despite the many defences of the digestive system, humans can occasionally be exposed to enteric pathogens, including bacteria and viruses. The distribution of microorganisms throughout the gastrointestinal tract is variable and supports a complex community of these organisms [1]. In the gut, microorganisms interact with the host to perform a variety of functions, including the prevention of pathogen colonisation, the breakdown of dietary substances, the production of nutrients, and the establishment and maintenance of healthy mucosal immunity [2].

Probiotic studies and applications have recently gained prominence in a number of disciplines, including medicine, veterinary medicine, pharmacy and the food industry. Dairy products, such as yoghurt, milk and cheese, are among the most important sources of probiotics in the food industry. It has been found that most of the probiotic bacteria in these products are of human origin, often belonging to the genera *Lactobacillus* and *Bifidobacterium* [3]. Yoghurt, kefir and milk-based fermented products have been the most widely used probiotic food production for many years, but there is growing public and scientific interest in other dairy products, such as different types of cheese. Although a wide variety of cheeses are produced in the world and in Türkiye, the use of these cheeses for the probiotic products is still

gaining importance [4, 5]. From a health and wellness perspective, cheese is a particularly adaptable food that offers opportunities to enhance its probiotic potential. Research shows that compared to fermented dairy products, such as yoghurt, cheese can provide a more effective environment for probiotic survival due to its unique physicochemical properties, such as its solid matrix structure, low pH, natural fat content and storage temperatures [6]. There are many types of local cheeses in Türkiye, and one of them is Surk cheese. The Eastern Mediterranean region, especially Türkiye, Lebanon, Syria and other Middle Eastern countries, is where Surk cheese first appeared. Surk is a dairy product made from skimmed milk cheese, which gets its unique sensory characteristics by adding certain spices [7].

In this study, 10 lactic acid bacteria (LAB) strains previously [7] isolated from Surk cheese were comprehensively evaluated for their probiotic potential. The findings highlighted that several strains, particularly belonging to the species *Ligilactobacillus acidipiscis, Lactiplantibacillus plantarum* and *Companilactobacillus alimentarius*, exhibited remarkable tolerance to gastrointestinal conditions, strong antimicrobial activity and an absence of virulence factors—collectively emphasising Surk cheese as a valuable source of novel and safe probiotic candidates.

2. Materials and Methods

2.1. Surk Strains and Growth Conditions. The strains used in this study were obtained from the microbiological culture collection of the Food Technology Department of Atatürk University. In this study, a total of 10 Surk strains (*Ligilactobacillus acidipiscis* P1, *Ligilactobacillus acidipiscis* P2, *Ligilactobacillus acidipiscis* P3, *Ligilactobacillus acidipiscis* P4, *Lactiplantibacillus plantarum* P5, *Lactiplantibacillus plantarum* P6, *Lactiplantibacillus plantarum* P7, *Lactiplantibacillus plantarum* P8, *Companilactobacillus alimentarius* P9 and *Companilactobacillus alimentarius* P10) were used. These strains were genetically identified by the 16S rDNA method in the study of Esen and Çetin [7]. Throughout the study, all strains were incubated on De Man–Rogosa–Sharpe (MRS) agar/broth at 30°C for 48–72 h under anaerobic conditions. Cell concentrations were measured as 10^8 cfu/mL. In Esen and Çetin [7]'s study, both starter LAB and nonstarter LAB were isolated and identified. Of these identified strains, these 10 strains were selected because, according to the literature, these species are potential probiotic candidates.

2.2. Tolerance to Simulated Gastric Juice and Bile Salts. Initially, 10^8 cfu/mL of fresh cultures of these 10 Surk strains was suspended in phosphate-buffered saline (PBS, pH 7.2). To assess tolerance to bile salts, this mixture (1%) was injected into MRS broth (Merck) containing 0.3% bile salts. To assess tolerance to a simulated gastric juice, 1 mL of this suspension was inoculated into 9 mL of a gastric mixture (5 mg/mL NaCl, 3 mg/mL pepsin, pH 3). The viable cells were enumerated after 24 h at 37°C for bile salt tolerance and after 2 and 3 h at 37°C for simulated gastric juice tolerance using the serial dilution method. All incubations were performed under anaerobic conditions. The tolerance to the simulated gastric juice and bile salts was calculated using the following formula: Tolerance (%): (log N1/log N0) × 100, where N1 showed the number of viable cells at 2, 3 or 24 h, and N0 showed the initial number of viable cells [8].

2.3. Auto/Co-Aggregation and Cell Surface Hydrophobicity. Fresh Surk strains were suspended in PBS (10^8 cfu/mL) and cultured for 24 h at 37°C to assess their auto-aggregation capacity. A plate reader (Epoch, BioTek) was used to measure the absorbance of the residual liquid phase at 600 nm following 2, 4 and 24 h of incubation. The calculation was performed with the following formula: Auto-aggregation $(\%) = 1 - (A_t/A_0) \times 100$, where A_t showed absorbance after 2, 4 or 24 h of incubation, and A_0 showed initial absorbance [9].

To determine the co-aggregation, Surk strains in PBS (10^8 cfu/mL) and pathogens (*Staphylococcus aureus* ATCC 29213, *Escherichia coli* O157:H7, *Listeria monocytogenes* ATCC 7644 and *Salmonella* Typhimurium RSHMB 95091) were mixed (2:2 mL) and homogenised by vortexing for 30 s. The absorbance of the upper liquid phase was determined at 600 nm via a plate reader (Epoch, BioTek) after 2 h at 37°C. The co-aggregation ability was calculated using the following formula: Co-aggregation $(\%) = [((A_x + A_y)/2) - A(x + y)]/((A_x + A_y)/2)] \times 100$, where $(A_x + A_y)$ showed the first absorbance for the isolates and pathogens, and $A(x+y)$ showed the absorbance of the mixture at 2 h [10].

For cell surface hydrophobicity, a fresh suspension of Surk strains in PBS (10^8 cfu/mL) and xylene (Merck) was mixed in a ratio of 3:1, vortexed for two min and allowed to stand at 25°C until phase separation occurred. A plate reader (Epoch, BioTek) was used to detect the absorbance of the underlying liquid phase at 600 nm, and the following formula was used to calculate the percentages of cell surface hydrophobicity: Cell surface hydrophobicity $(\%) := 1 - (A_1/A_0) \times 100$, where A_1 showed the absorbance of the underlying liquid phase after phase separation, and A_0 showed initial absorbance [9].

2.4. Bile Salt Hydrolase (BSH) Activity. The BSH activity was determined by the method described by Byakika et al. [11]. Briefly, 10 μL of fresh Surk strains suspended in PBS (10^8 cfu/mL) was dropped onto bile esculin agar (Condalab) and anaerobically incubated at 37°C for 24 h. At the end of incubation, BSH activity was categorised using the diameter of the dark brown zones formed around the isolates: zone diameter ≤ 10 mm: weak activity, zone diameter 11–15 mm: moderate activity, and zone diameter ≥ 16 mm: strong activity.

2.5. Antimicrobial Activity. The antimicrobial activity of the Surk strains against the indicator pathogens (*Staphylococcus aureus* ATCC 29213, *Escherichia coli* O157:H7, *Listeria monocytogenes* ATCC 7644 and *Salmonella Typhimurium* RSHMB 95091) was determined using the agar well diffusion

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method [12]. For preparation of the cell-free supernatants (CFS), fresh Surk strains were cultured anaerobically in MRS broth (Merck) at 30°C for 24 h, followed by centrifugation at 8000 × g for 10 min. The resulting supernatants were filtered through 0.22-μm sterile syringe filters (Sartorius, Germany) to remove any residual cells, and the pH of the filtrates was adjusted to 6.5 ± 0.1 using 1 M NaOH to minimise the influence of organic acids. Wells (6 mm diameter) were made in Brain Heart Infusion Agar (BHIA) plates previously inoculated with the pathogens, and 100 μL of the CFS was added to each well. The plates were incubated at 37°C for 18–24 h, and antimicrobial activity was assessed by measuring the diameters of inhibition zones.

2.6. DNase and Gelatinase Activity. For DNase activity, fresh Surk strains on DNase agar (Merck) were incubated anaerobically at 37°C for 72 h, and the plate was then covered with 1 N HCl (Merck). The strains with a clear zone were evaluated as positive [13].

For gelatinase activity, a modified medium consisting of 30 g/L gelatin (Sigma-Aldrich), 5 g/L peptone (Merck), 3 g/L yeast extract (Merck) and 15 g/L bacteriological agar (Merck) was used. Fresh Surk strains in PBS (10^8 cfu/mL) were dropped 10 μL onto this modified medium, and the plates were incubated anaerobically at 37°C for 48 h. The surface of the modified medium was then coated with saturated ammonium sulphate, and isolates forming a transparent zone were considered positive [14].

2.7. Biofilm Formation Ability. The tissue culture plate method was used to determine the biofilm formation ability of the Surk strains [13]. Fresh Surk strains suspended in PBS were mixed (10:190, v/v) with MRS broth (Merck) and incubated anaerobically in a flat-bottomed 96-well plate at 37°C for 24 h. After removing the planktonic cells from the broth, the plates underwent three PBS washes. The wells were then filled with sodium acetate solution (2%, w/v) and crystal violet (0.1%, w/v). The plates were cleaned with deionised water and dried under a constant air stream after 30 min of incubation at 25°C. The amount of biofilm in the wells of the plate was read at 570 nm via a plate reader (Epoch, BioTek), and the biofilm formation ability of the strains was classified as follows: $OD_{570} < 0.12$: nonbiofilm or weak biofilm, $0.12 < OD_{570} > 0.24$: moderate biofilm, and $OD_{570} > 0.24$: strong biofilm.

2.8. Detection of Virulence Genes. To determine the virulence factors in the genomes of the Surk strains, primers specific for *ace* (collagen adhesion) [15], *asa* (aggregation substance) [16], *cylA* (cytolysin) [17], *efaAfs* (*Enterococcus faecalis* specific endocarditis antigen), *gelE* (gelatinase), *esp* (enterococcal surface protein) [18] and *hyl* (hyaluronidase) [16] genes were used, and the analysis was performed by PCR. Genomic DNA of the Surk strains was isolated using the method of Singh and Ramesh [19]. The PCR mixture (total 50 μL) consisted of 25 μL Taq OptiMix CLEAR Master Mix (AMPLIQON), primers (1 μL) (reverse and forward), template DNA (50 ng/μL) and nuclease-free water. The thermal cycle was as follows: 5 min predenaturation at 95°C, 30 s denaturation at 95°C, 35 s annealing at 52°C (Tm) and 30 s extension at 72°C, a total of 35 cycles and 5 min post-extension at 72°C. Then, the products were visualised under UV light [20].

2.9. Antibiotic Susceptibility. The disc diffusion method was used to detect the antibiotic susceptibility profiles of the Surk strains, as reported by Abbasiliasi et al. [21]. The antibiotics used in this analysis were ampicillin (20 μg/disc), chloramphenicol (30 μg/disc), clindamycin (2 μg/disc), erythromycin (15 μg/disc), gentamicin (10 μg/disc), kanamycin (30 μg/disc), streptomycin (10 μg/disc), tetracycline (30 μg/disc) and vancomycin (30 μg/disc). Briefly, fresh Surk strains in PBS (10^8 cfu/mL) were well spread on MRS agar (Merck), and antibiotic discs were placed on it. After the incubation anaerobically at 30°C for 18–24 h, the clear zone diameters formed around the antibiotic discs were detected via a ruler. The antibiotic susceptibility status of the strains was classified as follows: zone diameter ≥ 21 mm: susceptible, 16 < zone diameter > 20: intermediate, and zone diameter ≤ 15 mm: resistant. The multiple antibiotic resistance (MAR) index of the Surk strains was calculated by the following formula: $MAR = a/b$, where a showed the antibiotics to which the strain is resistant, and b showed the total number of antibiotics [22].

2.10. Statistical Analysis. In this study, all analyses were carried out in 2 replicates, and all results were given as mean ± standard deviation. All results of this study were analysed using the SPSS 22.0 statistical program. One-way ANOVA and Duncan's multiple comparison analysis (significance level $p < 0.05$) were used to detect statistical differences between strains. In addition, SIMCA 14.1 software was used for the principal component analysis.

3. Results and Discussion

3.1. Tolerance to Simulated Gastric Juice and Bile Salts. Probiotics are characterised by their resistance to gastric acid and bile salts, which allows them to reach the colonic microbiota of humans and animals in vivo and form their natural microbiota. Probiotics are generally considered safe because they do not produce toxic metabolites, cause immunological sensitisation in the host or promote the emergence of resistant pathogenic microorganisms [23]. In this study, the tolerance of 10 Surk strains to gastrointestinal conditions was evaluated in a simulated gastric juice and in the presence of bile salts. It was found that all Surk strains were able to maintain viability in the simulated gastric juice. Furthermore, the survival percentages of the strains in the simulated gastric juice were in the range of 77.79%–84.84% at the end of 2 h (Figure 1(a)) and in the range of 76.59%–82.36% at the end of 3 h (Figure 1(b)). There was no statistical difference between the Surk strains in terms of survival after both 2 and 3 h in the simulated gastric juice

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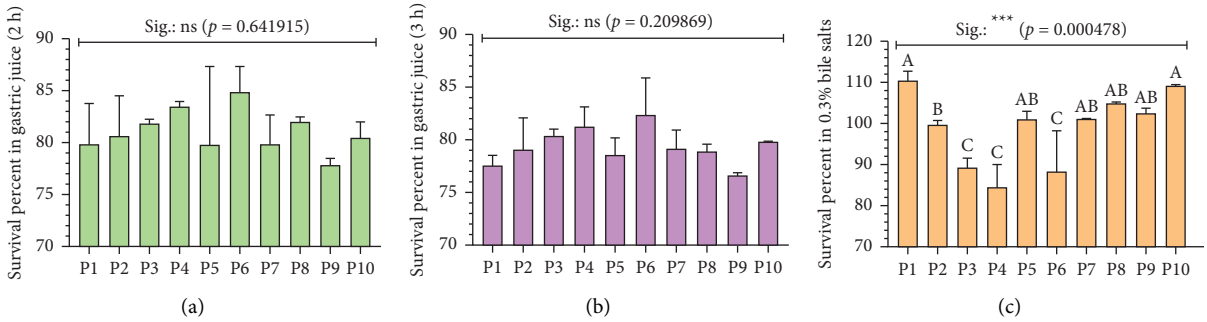


FIGURE 1: Tolerance of strains (P1–P10) isolated from Surk cheese to simulated gastric juice after 2 h (a) and 3 h (b), and bile salts after 24 h (c). All values are given as mean ± standard deviation. Uppercase letters indicate statistically significant differences between strains. Sig: level of statistical significance, ns: not statistically significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

($p < 0.05$). These results indicate that all Surk strains used in this study are resistant to gastric juice and have the potential to survive the upper part of the digestive tract. Similarly, Arellano et al. [24] reported that *C. alimentarius* and *L. plantarum* strains isolated from different food samples maintained viability in the gastric environment. Furthermore, Melgar-Lalanne et al. [25] investigated the gastric tolerance of *L. plantarum* and *L. acidipiscis* strains isolated from tropical Mexican cheeses, and the results showed that both isolates were able to survive in gastric conditions with both pH 2 and pH 3.

It was found that the survival of the Surk strains in the medium containing 0.3% bile salts ranged from 84.44% to 110.28%, and there were statistically significant differences between the strains' tolerance to bile salts ($p < 0.001$) (Figure 1(c)). *L. acidipiscis* P1 and *C. alimentarius* P10 had the highest tolerance to bile salts, while *L. acidipiscis* P3, *L. acidipiscis* P4 and *L. plantarum* P6 had the lowest tolerance ($p < 0.001$). This suggests that some strains are resistant to the damaging effects of bile salts on the cell membrane and that there may be variation between species [26]. The fact that the viability concentration of these strains is higher than the inoculation concentration indicates that they are likely to use bile salt as a substrate and grow, and that their adaptation to the acid environment is likely to be high. In a study by Melgar-Lalanne et al. [27], *L. plantarum* and *L. acidipiscis* strains isolated from Chiapas cheese showed the ability to grow in the presence of up to 1% bile salt, while their growth was inhibited between 1.0% and 3.0% bile salt concentration.

There are many studies reporting that *L. plantarum* isolated from cheeses are tolerant to bile salts and gastric acidity [28, 29]. *L. acidipiscis* was originally isolated from salt-tolerant sources, primarily fermented fish, but it is also present in many foods, including different cheeses (such as halloumi and cotija), fermented meats, pickles, vinegar and table olives [30]. This supports the idea that these species may have broad environmental tolerance and therefore probiotic potential by surviving in body barriers, such as the gastric acidity and bile salts. However, there are very limited studies on the determination of probiotic properties of *C. alimentarius*. This suggests that *C. alimentarius* P9 and P10, which showed high bile tolerance in this study, should be further investigated. In general, all strains can be considered successful in terms of tolerance to gastric juice and bile salts. These tolerances are the first criteria for probiotic selection and are critical in the selection of potential probiotic candidates [20]. This study shows that traditional fermented products, such as Surk cheese, are an important source not only of rich microbial diversity but also of microorganisms with functional properties. However, beyond basic in vitro criteria, such as gastrointestinal tolerance, these strains need to be supported by advanced probiotic evaluation parameters, such as their ability to adhere to the mucosa, effects on the immune response and metabolite profiles. In this context, it is suggested that future studies should focus on these aspects.

3.2. Auto/Co-Aggregation and Cell Surface Hydrophobicity. In this study, the auto/co-aggregation and cell surface hydrophobicity properties of the Surk strains were investigated, which are three important criteria for probiotic evaluation, and the results are shown in Figure 2. Auto-aggregation ability refers to the ability of bacteria to cluster together and is known to be important in maintaining colony balance in the gut mucosa [31]. In this study, it was determined that there was no statistical difference between the auto-aggregation abilities of the Surk strains at 2 h ($p > 0.05$), but there was a statistical difference at 4 ($p < 0.01$) and 24 h ($p < 0.001$) (Figure 2(a)). This is an expected result as studies have reported that the ability of lactobacilli to auto-aggregate increases/changes over time [32]. In this study, *L. acidipiscis* P3 (36.57%) had the highest auto-aggregation ability after 4 h of incubation ($p < 0.01$), while *L. acidipiscis* P3 (75.28%), P4 (75.67%) and *L. plantarum* P6 (75.25%) had the highest auto-aggregation ability after 24 h ($p < 0.001$). Similarly, Shen et al. [33] reported that the auto-aggregation ability of *L. acidipiscis* YJ5 was between 55% and 60% after 10 h and 85% and 90% after 24 h. In addition, Isenring et al. [34] reported that the auto-aggregation capacity of *L. plantarum* strains was 80%–85% after 24 h, whereas Ahumada et al. [35] reported that only one of the *C. alimentarius* strains from different sources had auto-aggregationcapacity.

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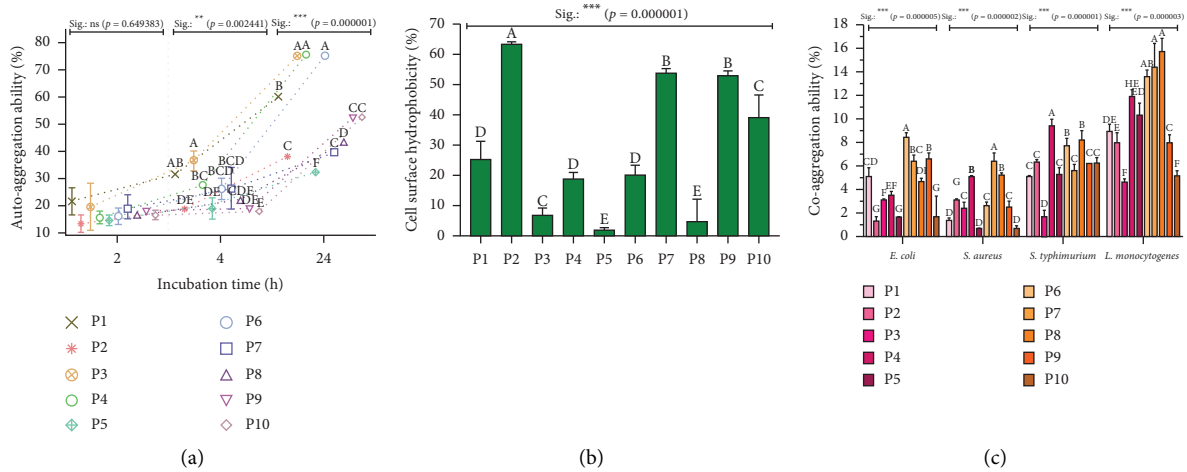


FIGURE 2: The auto-aggregation (a), cell surface hydrophobicity (b) and co-aggregation ability (c) of the strains (P1–P10) isolated from Surk cheese. All values are given as mean ± standard deviation. Uppercase letters indicate statistically significant differences between strains. Sig: level of statistical significance, ns: not statistically significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Cell surface hydrophobicity is an important property that affects the potential of bacteria to bind to the large intestinal mucosa [36]. The study revealed that the cell surface hydrophobicity properties of the Surk strains were statistically different ($p < 0.001$), with *L. acidipiscis* P2 (63.37%) having the highest cell surface hydrophobicity and *L. acidipiscis* P3 (6.87%), *L. plantarum* P5 (2.08%) and P8 (4.89%) having the lowest cell surface hydrophobicity (Figure 2(b)). The fact that LAB are known to be highly adhesive in the gut [37] supports the results obtained in this study. In a study by García-Cayuela et al. [18], the hydrophobicity ability of *L. plantarum* strains was found to be between 65% and 95%. However, in our study, the cell surface hydrophobicity ability of *L. plantarum* strains ranged between 2.08% and 53.80%. Therefore, this indicates that strains belonging to the same species may have different properties.

Co-aggregation refers to the ability of probiotics to physically interact directly with pathogens to inactivate or prevent them from colonising [36]. In this study, the Surk strains have statistically different co-aggregation abilities for all pathogen treatments, including *Escherichia coli* O157:H7 ($p < 0.001$), *Staphylococcus aureus* ATCC 29213 ($p < 0.001$), *Salmonella* Typhimurium RSHMB 95091 ($p < 0.001$) and *Listeria monocytogenes* ATCC 7644 ($p < 0.001$) (Figure 2(c)). The results revealed that *L. plantarum* P6 had the highest co-aggregation ability with 8.43% for *Escherichia coli* O157:H7, *L. plantarum* P7 with 6.47% for *Staphylococcus aureus* ATCC 29213, *L. acidipiscis* P4 with 9.38% for *Salmonella* Typhimurium RSHMB 95091 and *L. plantarum* P7 and P8 with 14.44% and 15.73%, respectively, for *Listeria monocytogenes* ATCC 7644. It is noteworthy that *L. plantarum* strains, especially *L. plantarum* P7, can be highly co-aggregated with multiple pathogens. Shen et al. [33] reported that *L. acidipiscis* strains had higher co-aggregation against the same pathogens, suggesting that co-aggregation properties may differ even within the same species.

These results provide important insights that the Surk strains, in addition to their tolerance to gastric acidity and bile salts, can also have a positive effect on host intestinal health. However, verification of these properties at the in vivo level and investigation of their mechanisms at the genetic/molecular level are important for a complete understanding of the probiotic mechanisms of action.

3.3. BSH Activity. Bile salts are hydrolysed, reducing their molecular weight and allowing them to be excreted from the body in the faeces. This process can lead to a reduction in blood cholesterol levels, significantly reducing the risk of cardiovascular disease associated with high cholesterol [38, 39]. As a result of the analysis performed in our study, none of the Surk strains had BSH activity. Therefore, it is assumed that they have no benefit in terms of cholesterol control. However, some studies have reported that these strains have BSH activity [25, 40, 41]. It is known that there are different methods for the determination of BSH activity; therefore, it is thought that the reasons why BSH activity was not observed in our study may be due to both the method used and the individual characteristics of the strains.

3.4. Antimicrobial Activity. Antimicrobial activity against foodborne pathogens or spoilage microorganisms is one of the expected properties of probiotics. Probiotics are known to improve food safety by inhibiting undesirable microorganisms in food [42]. In this study, the antimicrobial activities of the Surk strains against *Escherichia coli* O157:H7, *Staphylococcus aureus* ATCC 29213, *Salmonella* Typhimurium RSHMB 95091 and *Listeria monocytogenes* ATCC 7644 were investigated, and the results are shown in Table 1. The statistical differences were found between the antimicrobial activities of the Surk strains for each pathogen ($p < 0.001$). According to the results, *L. acidipiscis* P2 and *L. plantarum* P5, P7 and P8 stand out for their high level of antimicrobial

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Table 1: Antimicrobial activity of the Surk isolates against pathogens (zone diameter, mm)[a].

Strains	Pathogens			
	E. coli O157:H7	*S. aureus* ATCC 29213	*S. typhimurium* RSHMB 95091	*L. monocytogenes* ATCC 7644
P1	$19.00^{d} \pm 0.71$	$17.50^{cd} \pm 0.71$	$15.00^{de} \pm 1.41$	$16.00^{cd} \pm 1.41$
P2	$23.00^{c} \pm 0.71$	$0.00^{f} \pm 0.00$	$21.50^{c} \pm 0.71$	$20.00^{b} \pm 1.41$
P3	$17.00^{de} \pm 0.71$	$16.50^{d} \pm 0.71$	$14.50^{de} \pm 0.71$	$0.00^{e} \pm 0.00$
P4	$0.00^{f} \pm 0.00$	$22.00^{b} \pm 1.41$	$16.50^{d} \pm 0.71$	$17.50^{c} \pm 0.71$
P5	$29.50^{a} \pm 2.12$	$21.00^{b} \pm 1.41$	$24.50^{b} \pm 0.71$	$28.50^{a} \pm 1.41$
P6	$24.50^{bc} \pm 0.71$	$0.00^{f} \pm 0.00$	$30.00^{a} \pm 1.41$	$0.00^{f} \pm 0.00$
P7	$26.00^{b} \pm 1.41$	$24.50^{a} \pm 0.71$	$0.00^{g} \pm 0.00$	$26.75^{a} \pm 1.06$
P8	$17.00^{de} \pm 1.41$	$21.50^{b} \pm 0.71$	$23.50^{bc} \pm 0.71$	$22.25^{b} \pm 1.77$
P9	$15.25^{e} \pm 0.35$	$18.50^{c} \pm 0.71$	$11.00^{f} \pm 1.41$	$17.25^{c} \pm 1.06$
P10	$16.50^{de} \pm 0.71$	$11.50^{e} \pm 0.71$	$13.50^{e} \pm 0.71$	$13.75^{d} \pm 0.35$
Sig.	***	***	***	***

Note: Sig.: degree of statistical significance, ns: not statistically significant. Lowercase letters represent statistical differences in the same column.
[a]All results are expressed as zone diameter (mm) and mean ± standard deviation.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

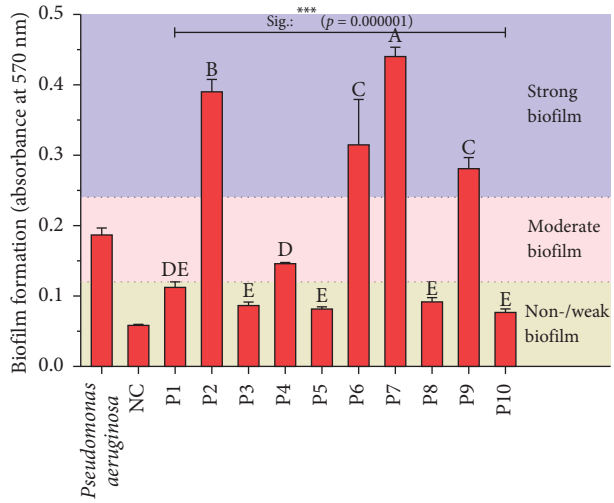


Figure 3: The biofilm formation ability of the strains (P1–P10) isolated from Surk cheese. All values are given as mean ± standard deviation. Uppercase letters indicate statistically significant differences between strains. Sig: level of statistical significance, ns: not statistically significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, NC: negative control.

activity against multiple pathogens. Similarly, Luo et al. [43] and Melgar-Lalanne et al. [25] investigated the antimicrobial activities of *L. plantarum* and *L. acidipiscis* strains against almost identical pathogens and reported that these strains inhibited these pathogens. It is known that organic acids, hydrogen peroxide and bacteriocins produced by microorganisms are mainly responsible for the antimicrobial activity [44]; so, further studies are needed to clearly understand the reason for the antimicrobial activity obtained in this study.

3.5. DNase/Gelatinase Activity and Detection of Virulence Genes. Virulence factors, also known as pathogenicity criteria, reveal the ability of bacteria to cause disease. Virulence factors of bacteria are various exoproteins with cytotoxic and enzymatic activities, such as adhesion to specific substances, secreted alpha, beta and gamma haemolysis, elastase and DNase [45]. Furthermore, chromosomal DNA contains the *gelE* gene, which produces gelatinase. An essential virulence factor, gelatinase is a proteolytic enzyme that damages host tissue and facilitates bacterial motility and dissemination by acting on substrates, such as gelatin, collagen, haemoglobin and endothelium tissue [46]. In this study, the Surk strains were tested for all these undesirable properties, and none of the strains had these properties. The absence of gelatinase and DNase activities and virulence genes in *L. plantarum*, *L. acidipiscis* and *C. alimentarius* strains emphasises the safety of these strains and supports their candidacy as probiotics. Similarly, many researchers have reported the absence of the gelatinase and DNase activity and virulence factor genes in *L. plantarum* strains [47–49].

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Table 2: Zone diameter (mm) against antibiotic discs, antibiotic resistance pattern and MAR index of the Surk strains[a].

Strains	Antibiotics									MAR
	C	K	E	DA	CN	VA	AM	S	TE	
P1	$19.00^{c} \pm 0.00$ (I)	0.00 ± 0.00 (R)	$18.50^{a} \pm 0.71$ (I)	$20.50^{a} \pm 0.71$ (S)	$6.50^{cd} \pm 0.00$ (R)	0.00 ± 0.00 (R)	$19.50^{de} \pm 0.71$ (I)	6.50 ± 0.71^{a} (R)	$12.50^{abcd} \pm 0.71$ (R)	0.56
P2	$19.50^{bc} \pm 0.71$ (I)	0.00 ± 0.00 (R)	$15.00^{de} \pm 0.00$ (R)	$0.00^{c} \pm 0.00$ (R)	$7.00^{bc} \pm 0.00$ (R)	0.00 ± 0.00 (R)	$20.25^{d} \pm 0.35$ (S)	0.00 ± 0.00^{d} (R)	$8.50^{f} \pm 0.71$ (R)	0.78
P3	$19.50^{bc} \pm 0.71$ (I)	0.00 ± 0.00 (R)	$15.75^{cd} \pm 0.35$ (R)	$0.00^{c} \pm 0.00$ (R)	$5.25^{ef} \pm 0.35$ (R)	0.00 ± 0.00 (R)	$23.50^{ab} \pm 0.71$ (S)	0.00 ± 0.00^{d} (R)	$11.50^{bcde} \pm 0.71$ (R)	0.78
P4	$19.00^{c} \pm 1.41$ (I)	0.00 ± 0.00 (R)	$16.50^{bcd} \pm 0.71$ (I)	$20.50^{a} \pm 0.71$ (S)	$5.00^{f} \pm 0.00$ (R)	0.00 ± 0.00 (R)	$25.00^{a} \pm 1.41$ (S)	0.00 ± 0.00^{d} (R)	$13.50^{ab} \pm 0.71$ (R)	0.56
P5	$19.50^{bc} \pm 0.71$ (I)	0.00 ± 0.00 (R)	$14.00^{e} \pm 1.41$ (R)	$0.00^{c} \pm 0.00$ (R)	$6.00^{def} \pm 0.00$ (R)	0.00 ± 0.00 (R)	$21.25^{cd} \pm 0.35$ (S)	0.00 ± 0.00^{d} (R)	$9.50^{ef} \pm 0.71$ (R)	0.78
P6	$22.50^{a} \pm 0.71$ (S)	0.00 ± 0.00 (R)	$16.50^{bcd} \pm 0.71$ (I)	$0.00^{c} \pm 0.00$ (R)	$5.75^{def} \pm 0.35$ (R)	0.00 ± 0.00 (R)	$22.50^{bc} \pm 0.71$ (S)	0.00 ± 0.00^{d} (R)	$13.00^{abc} \pm 1.41$ (R)	0.67
P7	$21.25^{ab} \pm 1.06$ (S)	0.00 ± 0.00 (R)	$16.00^{bcd} \pm 0.00$ (I)	$0.00^{c} \pm 0.00$ (R)	$7.75^{ab} \pm 1.06$ (R)	0.00 ± 0.00 (R)	$21.00^{cd} \pm 1.41$ (S)	0.00 ± 0.00^{d} (R)	$10.50^{def} \pm 0.71$ (R)	0.67
P8	$20.25^{bc} \pm 1.06$ (S)	0.00 ± 0.00 (R)	$16.00^{bcd} \pm 0.00$ (I)	$19.50^{b} \pm 0.71$ (I)	$5.75^{def} \pm 0.35$ (R)	0.00 ± 0.00 (R)	$24.50^{a} \pm 0.71$ (S)	4.00 ± 0.00^{c} (R)	$13.75^{a} \pm 0.35$ (R)	0.56
P9	$19.75^{bc} \pm 0.35$ (I)	0.00 ± 0.00 (R)	$17.25^{abc} \pm 0.35$ (I)	$0.00^{c} \pm 0.00$ (R)	$6.25^{cde} \pm 0.35$ (R)	0.00 ± 0.00 (R)	$18.00^{e} \pm 0.00$ (I)	4.00 ± 0.00^{c} (R)	$11.00^{cde} \pm 0.00$ (R)	0.67
P10	$19.00^{c} \pm 0.00$ (I)	0.00 ± 0.00 (R)	$17.50^{ab} \pm 0.71$ (I)	$0.00^{c} \pm 0.00$ (R)	$8.25^{a} \pm 0.35$ (R)	0.00 ± 0.00 (R)	$19.50^{de} \pm 0.71$ (I)	5.00 ± 0.00^{b} (R)	$10.00^{ef} \pm 1.41$ (R)	0.67
Sig.	*	ns	**	***	***	ns	***	***	**	

Note: Sig.: degree of statistical significance, ns: not statistically significant. MAR: multiple antibiotic resistance index, C: chloramphenicol (30 μg/disc), K: kanamycin (30 μg/disc), E: erythromycin (15 μg/disc), DA: clindamycin (2 μg/disc), CN: gentamicin (10 μg/disc), VA: vancomycin (30 μg/disc), AM: ampicillin (20 μg/disc), S: streptomycin (10 μg/disc), TE: tetracycline (30 μg/disc), S: sensitive, I: intermediate, R: resistant. Lowercase letters represent statistical differences in the same column.

[a]All results are expressed as zone diameter (mm) and mean ± standard deviation.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

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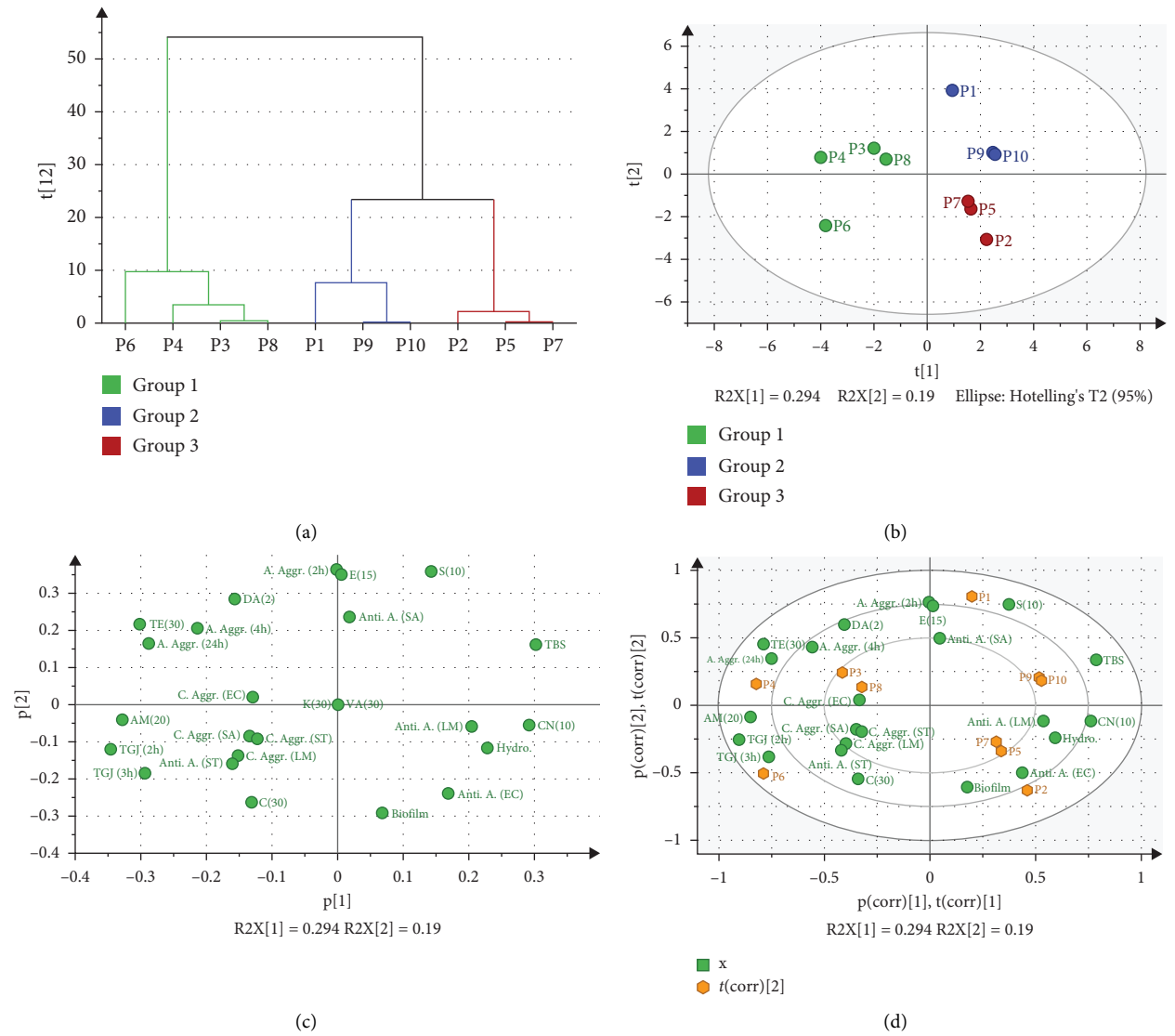


FIGURE 4: Hierarchical cluster analysis (a), score scatter plot (b), loading scatter plot (c) and biplot (d) of the principal component analysis. R2X[1] and R2X[2] in the principal component analysis are the variations explained by the PC1 and the PC2, respectively, together explaining 48.4% of the variation. TBS: tolerance to bile salts, TGJ: tolerance to gastric juice, A. Aggr.: auto-aggregation, C. Aggr.: co-aggregation, Hydro.: cell surface hydrophobicity, biofilm: biofilm formation, Anti. A.: antimicrobial activity, C: chloramphenicol, K: kanamycin, E: erythromycin, DA: clindamycin, CN: gentamicin, VA: vancomycin, AM: ampicillin, S: streptomycin, TE: tetracycline.

3.6. Biofilm Formation Ability. In a favourable environment, planktonic bacteria migrate along the surface and attach to it by forming microcolonies. This attachment is initially reversible, but becomes permanent through bacterial proliferation and exopolysaccharide production. In addition, bacteria surrounded by exopolysaccharides in the biofilm layer gain resistance to adverse environmental conditions, such as cold, extreme heat, chemicals, high salinity and disinfectants [50]. This is considered an indirect indicator of the adhesion and colonisation potential of microorganisms. Biofilm formation is an important feature for probiotics to adhere to the gastrointestinal mucosa, persist on the host and compete with potential pathogens [51]. In this study, the ability of the biofilm formation of the Surk strains was defined, and the results are shown in Figure 3. *Pseudomonas aeruginosa* ATCC 9027, used as a positive control, formed a moderate biofilm ($OD_{570} = 0.187$), while MRS broth without microorganisms, used as a negative control, formed a non-/weak biofilm ($OD_{570} = 0.059$). However, *L. acidipiscis* P1 and P3, *L. plantarum* P5 and P8, and *C. alimentarius* P10 had non-/weak biofilm formation ability with OD_{570} values of 0.112 and 0.087, 0.083 and 0.092, and 0.077, respectively; *L. acidipiscis* P4 had moderate biofilm formation ability with OD_{570} value of 0.147; and *L. acidipiscis* P2, *L. plantarum* P6 and P7, and *C. alimentarius* P9 had strong biofilm formation ability with OD_{570} values of 0.391, 0.315 and 0.441, and 0.281, respectively. There was also a statistical difference between the biofilm formation ability of the Surk isolates, with *L. plantarum* P7 having the statistically highest biofilm formation ability ($p < 0.001$). The same strains had different characteristics in biofilm formation and other parameters [52], indicating that some of these isolates have very high

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adhesion and colonisation potential. In addition, the strains with high biofilm formation ability in this study are thought to have an important property in terms of adhesion to the gastrointestinal mucosa and competition with potential pathogens [51].

3.7. Antibiotic Susceptibility. Complex relationships and interactions between humans, medicines and the environment can sometimes lead to the emergence of bacterial resistance to antibiotics. For example, different environmental factors contribute to differences in human antibiotic resistance between geographical regions. Antibiotic resistance occurs naturally in microorganisms but can also be acquired through horizontal gene transfer [53]. For this reason, it is very important to determine the antibiotic resistance profiles of microorganisms to be used for starter cultures, cocultures, etc., especially probiotics [54]. In this study, the susceptibility status of the Surk strains to different antibiotics is presented in Table 2. All the strains were resistant to kanamycin, gentamicin, vancomycin, streptomycin and tetracycline. The findings also demonstrated that the majority of strains were resistant to multiple antibiotics and that isolates differed significantly from one another. Resistance to vancomycin and kanamycin is considered a natural trait of many *Lactobacillus* species and is generally known to be of chromosomal origin [55]. However, widespread resistance to antibiotics, such as gentamicin, streptomycin and tetracycline, is also observed, indicating a multiantibiotic resistance profile in some strains. In particular, *L. acidipiscis* P2 and P3 and *L. plantarum* P5 were resistant to 7 of 9 antibiotics, and their MAR index was calculated as 0.78. This value is an important indicator for the possibility of exposure to environmental sources contaminated with antibiotics and requires further genetic investigation of such isolates. Other strains had safer antibiotic resistance profiles, and they showed moderate susceptibility to some antibiotics, with relatively low MAR index (range 0.56–0.67). In conclusion, the antibiotic susceptibility profiles of the Surk strains vary on a strain basis. This study again highlights the importance of antibiotic susceptibility analysis in the safety assessment of strains derived from traditional fermented products [54, 56].

3.8. Principal Component Analysis. All of the data collected for this investigation were subjected to principal component analysis. Figure 4 displays the principal component analysis results, which include a biplot, score scatter plot, loading scatter plot and hierarchical cluster analysis. When all the data were analysed, the Surk strains fell into three distinct groups (Figures 4(a) and 4(b)). The loading scatter plot is presented in Figure 4(c), and PC1 accounts for 29.40% of the variation and PC2 accounts for 19.00% of the variation, totalling 48.40% variation. There was a clear separation between *L. acidipiscis* P3 and P4 and *L. plantarum* P6 and P8 (group 1); *L. acidipiscis* P1 and *C. alimentarius* P9 and P10 (group 2); and *L. acidipiscis* P2 and *L. plantarum* P5 and P7 (group 3). The separation between group 1 and the others was driven by the inhibition zone of the ampicillin and tetracycline, tolerance to simulated gastric juice and autoaggregation ability (Figure 4(d)). In addition, the separation between group 2 and the others was driven by the inhibition zone of streptomycin and tolerance to bile salts (Figure 4(d)). However, the separation between group 3 and the others was driven by the antimicrobial activity against *Listeria monocytogenes* ATCC 7644 (Figure 4(d)).

4. Conclusion

The results revealed that all the Surk strains could tolerate simulated gastric juice and bile salts. The DNase and gelatinase activity and virulence factors were not detected in the strains, and this indicated that they can be safe for potential probiotic use. Moreover, *L. acidipiscis* P1 and P4; *L. plantarum* P6, P7 and P8; and *C. alimentarius* P9 and P10 stood out as the most suitable probiotic candidates due to their survivability, antimicrobial activity, adhesion and overall safety. This study showed that the Surk, an important traditional cheese for the Eastern Mediterranean region, may contain important probiotic microorganisms, but unfortunately it may have MAR. Finally, due to the resistance of the strains to various antibiotics, further studies are needed to transfer the resistance genes of all isolates to these antibiotics to other microorganisms. Once this situation is clarified, the usability of these isolates as starter and/or probiotic cultures can be investigated on a product basis.

Data Availability Statement

Data will be made available upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Yusuf Esen: data curation, investigation, methodology, roles/writing–original draft, and writing–review and editing.

Haktan Aktaş: data curation, investigation, methodology, resources, software, validation, visualisation, roles/writing–original draft, and writing–review and editing.

Bülent Çetin: conceptualisation, formal analysis, methodology, project administration, resources, software, supervision, and writing–review and editing.

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