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# ACID-RESISTANT *LIMOSILACTOBACILLUS FERMENTUM* ISOLATES RECOVERED FROM FERMENTED TURKISH SUCUK: SCREENING OF PROBIOTIC CHARACTERIZATION AND PHYLOGENY

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## ABSTRACT
The present study was conducted to identify acid-resistant lactic acid bacteria from fermented Turkish sucuk, detect phylogenetic affinities, and probiotic/biotechnological profiles. Samples were collected from popular fermented meat retail stores in Kayseri (n:20). The selected lactobacilli were exposed to different pH. Isolates resistant to pH2 were identified by sequencing following the 16s rRNA gene amplification and recorded in GenBank. The pH2 is distinctive for lactobacilli, as most (61.9%) of lactobacilli were inhibited ($P <0.05$). Isolates surviving at pH2 were determined to be *Limosilactobacillus fermentum*. The survival rates in bile salt, simulated gastrointestinal juices (between 97.13-106.60%), and autoaggregation, hydrophobicity, and coaggregation of isolates were statistically significant ($P <0.05$). *L. fermentum* S19 was the only isolate capable of producing exopolysaccharide; S19 had a high autoaggregation and hydrophobicity over 70%. Traditional Turkish fermented sucuk is a product with enormous potential, containing the newly isolated wild-type *L. fermentum*, which stands out for biotechnological/probiotic properties.
**Keywords**: Acid-resistant, *Limosilactobacillus fermentum,* sucuk, probiotic

# FERMENTE TÜRK SUCUKLARINDAN ELDE EDİLEN ASİDE DİRENÇLİ *LİMOSİLACTOBACİLLUS FERMENTUM*: PROBİYOTİK KARAKTERİZASYONU VE FİLOGENİNİN TARANMASI

## ÖZ
Bu çalışmada, geleneksel fermente Türk sucuğundaki aside dirençli laktik asit bakterilerini identifiye etmek, filogenetik afinitelerini ve probiyotik/biyoteknolojik profillerini tespit etmek amaçlanmaktadır. Örnekler (n:20) Kayseri'deki popüler fermente et perakende satış yerlerinden toplanmıştır. Seçilen laktobasiller farklı pH ortamlarına maruz bırakılmıştır. pH2'ye dirençli izolatlar, 16s rRNA gen amplifikasyonunu takiben dizileme yapılarak tanımlanmış ve GenBank'a kaydedilmiştir. Laktobasillerin çoğu (%61.9) inhibe edildiği için pH2, laktobasiller için ayırt edici bir

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ortamdır ($P<0.05$). pH2'de hayatta kalan izolatların *Limosilactobacillus fermentum* olduğu belirlenmiştir. *L. fermentum* izolatları arasında safra tuzu, simüle edilmiş gastrointestinal ortamlarında hayatta kalma oranları (%97.13-106.60) ve otoagregasyon, hidrofobiklik ve koagregasyon düzeyleri istatistiksel olarak anlamlı bulunmuştur ($P<0.05$). *L. fermentum* S19, ekzopolisakkarit üretme yeteneğine sahip tek izolat olduğu belirlenmiştir Ayrıca, S19 yüksek otoagregasyona sahip olup %70'in üzerinde hidrofobisite göstermiştir. Geleneksel Türk fermente sucuğu, biyoteknolojik/probiyotik özellikleriyle öne çıkan, yeni izole edilen yabani tip *L. fermentum*'u içeren, önemli potansiyele sahip bir üründür.
**Anahtar kelimeler**: Asit direnç, *Limosilactobacillus fermentum,* sucuk, probiyotik

## INTRODUCTION

Recently, there has been an increasing interest in consuming traditional fermented products produced using various raw materials, special microbiota, and production methods (Negrete-Romero et al., 2021). Kayseri province, located in Central Anatolia, is the production center of processed, fermented meat products such as sucuk and pastırma (Cevher, 2023). Sucuk is a typical dry-fermented meat product produced in Türkiye and consists of a mixture of beef and/or buffalo meat, beef fat, sheep tail fat, salt, sugar, nitrite/nitrate, and various spices such as garlic, red pepper, black pepper, cumin, and all spice (Kaban, 2010). Lactic acid bacteria (LAB) are essential in the ripening process of fermented meat products (Dincer and Kivanc, 2018). LAB, which plays a vital role in the fermentation of meat, reduces pH and produces bacteriocins that prevent the growth of pathogenic and spoilage microorganisms, thus improving the safety, stability, and shelf life of fermented meat products (Dincer and Kivanc, 2012). Some strains of the genus *Lactobacillus* are good candidates for probiotic cultures because they are natural agents of the gut microbiota and positively affect health *in vivo* (Dempsey and Corr, 2022). Using probiotics in fermented foods is a strategy that develops the functional food category from the traditional way of producing foods (Bis-Souza et al., 2019). LAB is also one of the most studied microorganism groups in developing functional foods due to its potential to prevent the formation of toxic compounds and its Generally Recognized as Safe status (GRAS) (Lorenzo et al., 2017; FDA, 2023). Although these microorganisms are associated with various health benefits, isolating new probiotic strains, characterizing them, and conducting safety assessments is necessary (Zommiti et al., 2020). Probiotic cells must resist antimicrobial factors in the stomach (low pH, gastric juice, and pepsin) and intestines (pancreatin and bile salts) (De Melo Pereira et al., 2018). To qualify as a probiotic, the potential candidate must possess certain functional and safety properties, including acid and bile salt tolerance, adhesion capacity, hemolytic activity, and antibiotic sensitivity (Xu et al., 2019). According to FAO/WHO (2006) guidelines, acid resistance is one of the commonly used in vitro tests for probiotic screening, along with tolerance to bile components. The aciduric or acidophilic properties of lactobacilli allow them to tolerate acid stress in the environment, food, and the gastrointestinal tract, including various physiological activities (Montoro et al., 2018). Highly acid-tolerant probiotic lactobacilli possess proton pumps for intracellular pH homeostasis, repair proteins for DNA damage, changes in the cellular membrane, and altered metabolism (De Angelis and Gobbetti, 2004). The present study was conducted to identify acid-resistant lactic acid bacteria isolated from fermented Turkish sucuk, determine phylogenetic affinities, and examine probiotic and biotechnological profiles.

## METHOD

### Sampling

Sucuk samples were obtained from 10 popular retail stores in Kayseri (n:20). The retail companies are grouped from RT1 to RT10. Samples from each fermented sucuk selling company were collected in June and November 2022 by visiting twice.

### pH Tolerance Profile

Resistance of selected lactic acid bacteria to pH2, pH3, and pH7 environments was determined by Yadav et al. (2016) with minor modifications. Overnight cultures were inoculated with serial dilutions into MRS broth adjusted to pH2, pH3, and pH7 with 1N HCl. The inoculums were

spread onto MRS agar for 0 and 3 hours and then incubated at 37°C for 48 hours. Plate counting evaluated cell viability, and the results were expressed as log cfu/mL.

**Identification of Acid Resistant Lactobacilli Isolates by 16S rRNA Sequencing and The Phylogenetic Relatedness**

For acid-resistant lactobacilli isolates, the 16S rRNA gene was amplified by PCR using universal target primers (27F:5'-AGAGTTTGATCCTGGCTCAG -3', 1492R: 5'-GGTTACCTTGTTACGACTT -3') (Suzuki and Giovannoni, 1996). The amplicons were sent to Macrogen (South Korea) and subjected to Sanger sequencing analysis. Paired nucleotide sequences were assembled with Geneious Prime 2020.1 (https://www.geneious.com). Paired nucleotide sequences were assembled with Geneious Prime 2020.1.1 (https://www.geneious.com). (BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi). The multiple sequences were performed using the default parameters of the ClustalW multiple alignment tool in MEGA 11. The phylogenetic analysis was performed using the neighbor-joining based on Kimura two-parameter modeling. Bootstrap values were estimated for 1000 replicates (Tavaré, 1986; Han et al, 2017).

**Bile Salt Tolerance**

For bile salt tolerance, overnight active cultures were inoculated as a serial dilution into MRS broth medium with 0.3% and 0.6% bile of bovine origin and incubated at 37°C. Inoculums at 0 and 3 hours were spread on MRS agar and incubated at 37°C for 48 hours. Plate counting evaluated cell viability, and the results were expressed as log cfu/mL. MRS broth without bile was used as a control. The survival rate indicating resistance to pH and bile salt was calculated, as stated by Yadav et al. (2016).

**Resistance to Simulated Gastric Juice (SGJ) and Simulated Intestinal Juice (SIJ)**

The in vitro model Zheng et al. (2021) mentioned was used to simulate gastric juice (SGJ) with minor modifications. Artificial gastric juice was prepared by adding 3 g/L pepsin (Sigma Aldrich, USA) into MRS broth, adjusted to pH 2.5, and sterilized with a 0.22 µm filter membrane. Plate counting evaluated cell viability, and the results were expressed as log cfu/mL. Simulated intestinal juice (SIJ) was sterilized by passing it through a 0.22 mm filter into MRS broth containing 1 mg/L pancreatin (Sigma Aldrich, USA), NaCl solution (0.5%) adjusted to pH 8.0 with 1 N NaOH (de Oliveira Coelho et al., 2019). Overnight cells were inoculated into the prepared simulated gastric juice and simulated intestinal juice by adjusting serial dilution and incubated at 37°C. The survival rate indicating resistance to SGJ and SIJ was calculated as stated by Yadav et al. (2016).

**Salt Tolerance**

For salt tolerance, overnight culture was inoculated into a sterile Brain Heart Infusion (BHI) liquid medium containing 4%, 8% and 12% NaCl for 24 hours (Bozdemir, 2021). Turbidity formation was evaluated by measuring at OD600 nm (OD:0.10-0.30 slightly turbid, positive poor development; OD:0.30-0.50 medium turbidity, good development; OD>0.60 very turbid, excellent development; OD<0.10, negative).

**Autoaggregation, Cell Surface Hydrophobicity and Coaggregation**

Autoaggregation testing was performed following the method described by Yasmin et al. (2020) with minor modifications. Overnight cells were centrifugated (4000xg, 10 min at 4°C) and washed twice with phosphate-buffered saline (PBS). Then, 2 ml of each bacterial suspension, adjusted to 0.5 ± 0.02 at 600 nm (A0) with PBS, was vortexed and incubated. Absorbance (A2) was measured at 600 nm at the third and 24th hour of incubation. It was calculated from the equation below.

$$Autoaggregation\ (\%) = [(1 - A2/(A0)]\ x\ 100$$

The overnight culture was centrifugated (4000xg, 10 min at 4°C) and washed twice with PBS for cell surface hydrophobicity testing. It was resuspended in PBS at 0 h (A0), adjusting the absorbance to 0.5 ± 0.02 at 600 nm. Then, 1 mL of hydrocarbon (xylene) was mixed with 3 mL cell suspension and pre-incubated at 37°C for 10 min.

The cell suspension and hydrocarbon mixture were vortexed for two minutes and kept at 37°C for 20 minutes for phase separation (water and hydrocarbon phase). After collecting the aqueous phase, the absorbance was measured at 600 nm (A1). Hydrophobicity was calculated from the following equation (Yasmin et al., 2020):

$$Hydrophobicity\ (\%) = [(1 - A1/(A0)]\ x\ 100$$

*Staphylococcus aureus* ATCC 25923, *Salmonella* Typhimurium ATCC 13311, *Escherichia coli* ATCC 25922, and *Listeria monocytogenes* N7144 were used as pathogenic strains for the coaggregation test. Suspensions of acid-resistant isolates and pathogenic strains made with PBS were adjusted to OD600 0.5 ± 0.02. Equal volumes (2 mL) of strains and pathogenic strains were mixed and the absorbance value (A0) of the mixture was measured and then incubated at 37°C for 24 hours. Cell suspensions of each strain were used as controls. The coaggregation percentage was calculated by the absorbance value (A2) of the mixture at the third, and 24th hours as follows (Liu et al., 2022):

$$Coaggregation\ (\%) = [(1 - A2/(A0)]\ x\ 100$$

**Agar Well Diffusion**

The antibacterial activity of the isolates against the pathogens *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 13311, *Listeria monocytogenes* N7144 and *Staphylococcus aureus* ATCC 25923 was determined by the agar well diffusion method. Each indicator pathogen was spread on Muller Hinton agar plates with a swab adjusted to 0.5 McFarland. Culture filtrate (80 µL; cell-free supernatant, CFS) was added to the wells (6 mm) opened in the medium. The plates were incubated at 37°C for 24 hours and the inhibition zone diameter of the isolate against each indicator pathogen was measured (Liu et al., 2022). After incubation, the results were expressed in mm by the arithmetic mean of the diameter of the inhibition halos around each well.

**Security Assesment**

*Antibiotic Sensitivity and Hemolytic Activity*

Antibiotic susceptibilities of acid-resistant isolates to selected antibiotics [vancomycin (VA, 30 µg; Oxoid, England), trimethoprim and sulfamethoxazole (SXT, 25 µg; Bioanalyse, Türkiye), ciprofloxacin (CIP, 5 µg; Bioanalyse, Türkiye), clindamycin (DA, 2 µg; Bioanalyse, Türkiye), tetracycline (TE, 30 µg; Bioanalyse, Türkiye), streptomycin (S, 10 mcg; Bioanalyse, Türkiye), meropenem (MEM, 10 µg; Oxoid, UK), erythromycin (E, 15 µg; Oxoid, UK) was tested. The overnight culture of the tested isolate was adjusted to 0.5 McFarland turbidity with sterile physiological saline and inoculated onto MRS agar with a swab. Antibiotic disks were placed on agar plates and incubated (at 37°C for 48 hours). After incubation, the inhibition zone was measured for each antibiotic disc. The results showed that according to the limit values recommended by the Clinical and Laboratory Standards Institute (CLSI, 2015) guidelines, isolates with an inhibition zone less than or equal to 14 mm were resistant, those with a diameter greater than 20 mm were susceptible, and isolates with a diameter of 15 and 19 mm were moderately sensitive.

Hemolytic activity was determined using blood agar containing 5% (w/v) sheep blood, and the plates were incubated at 37°C for 48 h. Hemolysis status was classified according to the lysis of red blood cells in the medium around the colonies. Green regions around the colonies on blood agar plates (α-hemolysis), transparent regions around the colonies (β-hemolysis) and no region around the colonies (γ-hemolysis) were evaluated. *S. aureus* ATCC 25923 strain was used as a positive control.

**Technological Feature Tests**

*Proteolytic Activity*

The selected isolate was assessed for proteolytic activity on skimmed milk powder agar medium. Each well was inoculated with 80 µL of bacterial culture. The plates were then incubated at 30°C for 24 hours. A transparent or opaque zone surrounding the wells indicated positive protease activity (Raveschot et al., 2020).

*Exopolysaccharide (EPS) Production*

The ruthenium red staining method was used to determine whether the strain produced EPS. It was stated whether the solid medium containing

skim milk powder, sucrose, yeast extract, and ruthenium red produced EPS based on the observation of pink and white colony formation on the plate surface. After 48 h of incubation at 30°C in an anaerobic jar, ruthenium red stains the bacterial cell wall, and the formation of pink colonies for non-ropy strains and white colonies for ropy strains was observed (Stingele et al., 1996; Dishan and Gönülalan, 2024).

**Statistical Analysis**

Statistical analysis was performed by R software for significant relationships (www.r-project.org/). Statistical significance of in vitro probiotic tests among *L. fermentum* isolates was conducted by applying variance analysis and Tukey HSD multiple comparison test. The correlation among specific properties was examined. Statistical significance was accepted when *P <0.05*.

**RESULTS**

From 20 different sucuk samples analyzed, 21 lactobacilli isolates were obtained by examining their morphology with gram staining. The pH tolerance of lactobacilli isolates obtained from sucuk is given in Table 1. The number of pH2-tolerant lactobacilli was 8 (38%). The difference between the survival rates of lactobacilli isolates obtained at different pH2, pH3, and pH7 values was statistically significant (*P <0.05*). When the sequence of the 16s rRNA gene region identified the eight isolates obtained, it was revealed that all isolates belonged to the *Limosilactobacillus fermentum* species. The *L. fermentum* isolates 16s rRNA partial sequencing has been deposited in GenBank under the accession numbers between OR768472 and OR768479.

Table 1. pH tolerance profile of lactobacilli obtained from sucuk

| Isolate Codes | pH2 | pH3 | pH7 | RT Grouping |
|---|---|---|---|---|
| S1 | $0^{f}$ | $95.1\pm0.50^{def}$ | $98.9\pm1.42^{fgh}$ | RT1 |
| S5 | $0^{f}$ | $97.3\pm2.18^{cde}$ | $103.9\pm0.86^{cde}$ | RT2 |
| S6 | $0^{f}$ | $101.1\pm1.04^{abc}$ | $104.6\pm0.33^{cd}$ | RT2 |
| S7 | $0^{f}$ | $86.7\pm1.93^{g}$ | $103.1\pm0.60^{cdef}$ | RT3 |
| S12 | $92.43\pm0.75^{a}$ | $105.5\pm0.21^{a}$ | $97.43\pm1.20^{gh}$ | RT4 |
| S13 | $0^{f}$ | $99.9\pm0.94^{abcd}$ | $95.9\pm0.23^{h}$ | RT5 |
| S14 | $73.68\pm0.07^{d}$ | $96.3\pm0.06^{cdef}$ | $114.7\pm0.09^{a}$ | RT5 |
| S16 | $75.77\pm1.16^{c}$ | $103.9\pm0.54^{ab}$ | $100.9\pm0.29^{defg}$ | RT6 |
| S18 | $0^{f}$ | $99.3\pm2.35^{bcde}$ | $99.2\pm2.74^{fgh}$ | RT7 |
| S19 | $77.86\pm0.38^{b}$ | $92.0\pm1.02^{efg}$ | $98.5\pm0.86^{gh}$ | RT7 |
| S21 | $0^{f}$ | $100.2\pm1.71^{abcd}$ | $104.3\pm2.22^{cde}$ | RT8 |
| S22 | $78.69\pm0.83^{b}$ | $96.9\pm0.70^{cde}$ | $100.3\pm0.44^{efg}$ | RT9 |
| S25 | $0^{f}$ | $98.9\pm0.69^{bcde}$ | $86.6\pm0.54^{i}$ | RT10 |
| S26 | $77.22\pm0.38^{bc}$ | $93.9\pm3.52^{ef}$ | $87.6\pm0.53^{i}$ | RT1 |
| S34 | $0^{f}$ | $0^{i}$ | $96.98\pm0.09^{gh}$ | RT4 |
| S37 | $0^{f}$ | $72.1\pm0.04^{h}$ | $109.2\pm1.61^{b}$ | RT5 |
| S41 | $0^{f}$ | $100.3\pm0.63^{abcd}$ | $95.8\pm1.15^{h}$ | RT7 |
| S45 | $0^{f}$ | $93.5\pm0.65^{ef}$ | $100.7\pm0.14^{defg}$ | RT8 |
| S47 | $73.96\pm1.04^{d}$ | $97.2\pm1.83^{cde}$ | $96.89\pm0.22^{gh}$ | RT9 |
| S49 | $57.90\pm0.47^{e}$ | $103.4\pm1.95^{ab}$ | $94.9\pm0.09^{h}$ | RT10 |
| S51 | $0^{f}$ | $90.5\pm0.10^{fg}$ | $106.6\pm0.88^{bc}$ | RT10 |

RT: Retail Stores

Means shown with different exponential letters in the same column are statistically different (*P <0.05*).

**Phylogenetic tree of Acid-tolerant *Limosilactobacillus fermentum***

The phylogenetic tree showed the genetic resemblance of eight *Limosilactobacillus fermentum* isolates based on their partial 16S rRNA gene sequence. Branches indicate the bootstrap percentage after 1000 replications. The constructed tree was divided into two main clusters: Cluster I is a large cluster including five isolates, and Cluster II consists of three isolates (Figure 1).

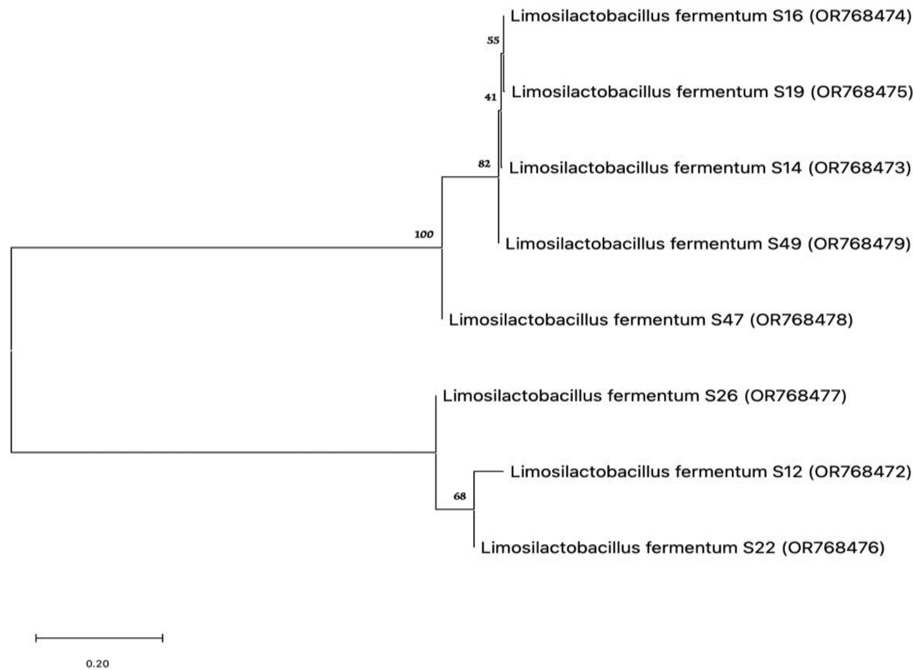


Figure 1. A phylogenetic tree was constructed by using the neighbor joining method

**Probiotic Characteristics Assessment**

Bile salt tolerance profile and viability in simulated gastric juice and simulated intestinal juice of *L. fermentum* isolates are given in Table 2. It was observed that the isolates examined were highly resistant to bile salts and simulated gastric and intestinal juice. While the effect of 0.6% bile salt concentration on the survival rate of *L. fermentum* isolates was not significant ($P > 0.05$), the effect of 0.3% bile salt concentration on the survival rate was significant ($P < 0.05$). In addition, no correlation was found between the survival values of the isolates at 0.3% and 0.6% bile salt concentrations ($P > 0.05$). The survival rates of the isolates in the simulated gastric and intestinal environment were also statistically different ($P < 0.05$).

The development of the isolate was evaluated in media containing 4%, 8%, and 12% NaCl for salt tolerance, as shown in Table 3. Poor growth was observed as the isolates formed slight turbidity in the environment containing 8% and 12% NaCl. Except for the S49 coded isolate, medium turbidity was formed in the medium without NaCl and with 4% NaCl added, and it showed good growth. *L. fermentum* S49 showed robust growth in two media and S14 in salt-free media. There is no statistical difference between the survival rates of *L. fermentum* isolates in environments with 8%, 12% NaCl, and no salt added ($P > 0.05$). However, the survival rate of the isolates in the environment with 4% salt was statistically significant ($P < 0.05$). Among the isolates, CFSs of S14, S19, S22, S47, and S49 showed antagonistic activity against all examined pathogens (Figure 2).

Table 2. Tolerance profiles of *L. fermentum* isolates

| Isolate Codes | 0.3% BS | 0.6% BS | SGJ | SIJ |
|---|---|---|---|---|
| S12 | 104.94±1.49$^{ab}$ | 98.44±0.35 | 99.23±1.25$^{ab}$ | 98.41±0.30$^{bc}$ |
| S14 | 104.00±1.17$^{abc}$ | 101.08±0.29 | 97.53±0.32$^{b}$ | 100.32±1.18$^{ab}$ |
| S16 | 101.17±0.97$^{cd}$ | 102.18±0.18 | 100.92±0.64$^{a}$ | 99.13±0.91$^{abc}$ |
| S19 | 101.08±0.16$^{cd}$ | 102.77±0.71 | 99.71±0.98$^{ab}$ | 100.01±0.58$^{ab}$ |
| S22 | 97.13±0.82$^{e}$ | 100.14±3.13 | 97.99±0.15$^{ab}$ | 101.05±0.11$^{a}$ |
| S26 | 106.60±0.19$^{a}$ | 100.03±1.18 | 100.56±0.37$^{ab}$ | 97.49±0.08$^{c}$ |
| S47 | 100.44±0.68$^{de}$ | 98.92±0.66 | 98.45±1.10$^{ab}$ | 98.11±0.55$^{bc}$ |
| S49 | 102.96±0.012$^{bcd}$ | 99.40±0.42 | 100.03±0.66$^{ab}$ | 99.67±0.07$^{abc}$ |

Table 3. Salt resistance profile of *L. fermentum* isolates

| Isolate Codes | 0% | 4% | 8% | 12% |
|---|---|---|---|---|
| S12 | 0.44±0.02 | 0.47±0.08$^{ab}$ | 0.26±0.01 | 0.21±0.02 |
| S14 | 0.57±0.06 | 0.47±0.02$^{ab}$ | 0.22±0.01 | 0.23±0.01 |
| S16 | 0.45±0.00 | 0.43±0.01$^{ab}$ | 0.22±0.03 | 0.22±0.01 |
| S19 | 0.40±0.08 | 0.41±0.03$^{ab}$ | 0.24±0.01 | 0.19±0.01 |
| S22 | 0.49±0.01 | 0.41±0.03$^{b}$ | 0.26±0.01 | 0.22±0.02 |
| S26 | 0.43±0.01 | 0.45±0.02$^{ab}$ | 0.23±0.00 | 0.20±0.04 |
| S47 | 0.41±0.04 | 0.40±0.00$^{b}$ | 0.24±0.09 | 0.19±0.05 |
| S49 | 0.56±0.20 | 0.60±0.09$^{a}$ | 0.26±0.01 | 0.21±0.06 |

Means shown with different exponential letters in the same column are statistically different ($P < 0.05$).

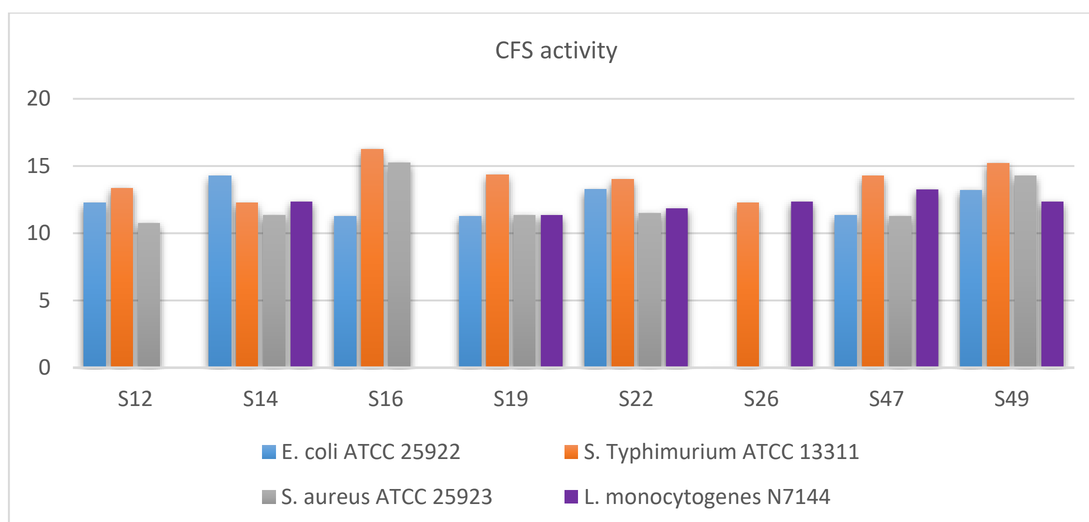


Figure 2. The average zone diameters (mm) of *L. fermentum* isolates against standard pathogens

Autoaggregation and hydrophobicity values (%) of *L. fermentum* isolates are given in Table 4. Three isolates (S12, S19, S26) showed hydrophobicity over 70%. S19 also showed high autoaggregation values. Autoaggregation values of the isolates measured at the 3rd and 24th hours contain a statistically significant difference ($P < 0.05$). Cell surface hydrophobicity percentages were statistically significant among the isolates ($P < 0.05$). However, no correlation was found between autoaggregation and hydrophobicity values ($P < 0.05$).

Table 4. Autoaggregation and hydrophobicity level of *L. fermentum* isolates

| Isolate Codes | A3 | A24 | Hydrophobicity |
|---|---|---|---|
| S12 | 3.75±0.02$^{c}$ | 28.12±0.13$^{h}$ | 75.83±0.12$^{a}$ |
| S14 | 22.67±0.34$^{b}$ | 33.37±0.35$^{f}$ | 36.46±0.28$^{f}$ |
| S16 | 18.05±0.55$^{b}$ | 29.38±0.07$^{g}$ | 47.04±0.25$^{d}$ |
| S19 | 46.89±3.95$^{a}$ | 79.06±0.26$^{a}$ | 73.90±0.11$^{b}$ |
| S22 | 19.67±0.17$^{b}$ | 37.30±0.03$^{d}$ | 17.86±0.02$^{h}$ |
| S26 | 3.16±0.18$^{c}$ | 39.70±0.07$^{c}$ | 72.61±0.21$^{c}$ |
| S47 | 44.75±0.43$^{a}$ | 56.61±0.15$^{b}$ | 37.49±0.04$^{e}$ |
| S49 | 18.18±0.63$^{b}$ | 35.00±0.03$^{e}$ | 29.47±0.03$^{g}$ |

A3 and A24: Autoaggregation value in the third hour and 24th hour
Means shown with different exponential letters in the same column are statistically different ($P < 0.05$).

Coaggregation values of *L. fermentum* isolates against the examined pathogens are given in Table 5. Coaggregation values against each pathogen were statistically different in the 3rd and 24th-hour measurements ($P < 0.05$).

Table 5. Coaggregation level of *L. fermentum* isolates against standard pathogens

| Isolate Codes | EC3 | EC24 | SA3 | SA24 | ST3 | ST24 | LM3 | LM24 |
|---|---|---|---|---|---|---|---|---|
| S12 | 1.23±0.22$^{e}$ | 39.63±0.04$^{f}$ | 27.32±0.10$^{b}$ | 58.89±0.03$^{b}$ | 24.87±0.10$^{c}$ | 54.70±0.02$^{b}$ | 30.78±0.09$^{c}$ | 64.46±0.07$^{b}$ |
| S14 | 8.20±0.35$^{c}$ | 46.12±0.01$^{d}$ | 14.86±0.02$^{f}$ | 40.45±0.06$^{e}$ | 16.15±0.11$^{f}$ | 40.33±0.03$^{c}$ | 16.55±0.10$^{f}$ | 46.76±0.08$^{d}$ |
| S16 | 18.17±0.08$^{a}$ | 43.53±0.02$^{e}$ | 42.29±0.07$^{a}$ | 66.82±0.11$^{a}$ | 41.30±0.07$^{a}$ | 65.96±0.29$^{a}$ | 50.07±0.10$^{a}$ | 76.89±0.37$^{a}$ |
| S19 | 5.49±0.49$^{d}$ | 10.26±0.05$^{g}$ | 14.15±0.03$^{f}$ | 22.30±0.15$^{h}$ | 29.77±0.07$^{b}$ | 55.81±0.12$^{b}$ | 35.33±0.27$^{b}$ | 61.97±0.27$^{c}$ |
| S22 | 10.04±0.36$^{c}$ | 47.29±0.01$^{c}$ | 16.56±0.29$^{e}$ | 42.90±0.48$^{d}$ | 15.18±0.02$^{g}$ | 36.77±1.10$^{d}$ | 17.88±0.57$^{e}$ | 42.13±0.57$^{e}$ |
| S26 | 5.60±1.63$^{d}$ | 46.09±0.14$^{d}$ | 20.29±0.32$^{c}$ | 28.50±0.03$^{g}$ | 20.22±0.03$^{d}$ | 30.57±0.05$^{e}$ | 20.15±0.11$^{d}$ | 24.90±0.13$^{g}$ |
| S47 | 8.13±0.01$^{c}$ | 48.79±0.45$^{b}$ | 18.99±0.05$^{d}$ | 45.38±0.11$^{c}$ | 16.52±0.02$^{e}$ | 39.87±0.18$^{c}$ | 19.67±0.05$^{d}$ | 47.51±0.07$^{d}$ |
| S49 | 14.33±0.02$^{b}$ | 83.13±0.44$^{a}$ | 14.83±0.27$^{f}$ | 33.89±0.04$^{f}$ | 14.70±0.14$^{h}$ | 31.27±0.04$^{e}$ | 16.68±0.28$^{f}$ | 34.27±0.04$^{f}$ |

EC3 and EC24: Coaggregation against *E. coli* ATCC 25922 in the third and 24th hour
SA3 and SA24: Coaggregation against *S. aureus* ATCC 25923 in the third and 24th hour
ST3 and ST24: Coaggregation against *S.* Typhimuriu*m* ATCC 13311 in the third and 24th hour
LM3 and LM24: Coaggregation against *L. monocytogenes* N7144 in the third and 24th hour
Means shown with different exponential letters in the same column are statistically different ($P < 0.05$).

**Biotechnological Assessment**

The zone diameter formed as a result of the protease activity of the isolates is given in Figure 3. It was determined that all isolates had protease activity. The EPS-forming ability of *L. fermentum* isolates was determined according to the color of the colony formed in the medium containing ruthenium red, and isolate S19 gave white colonies, indicating EPS formation. No EPS-producing feature was found phenotypically in other isolates.

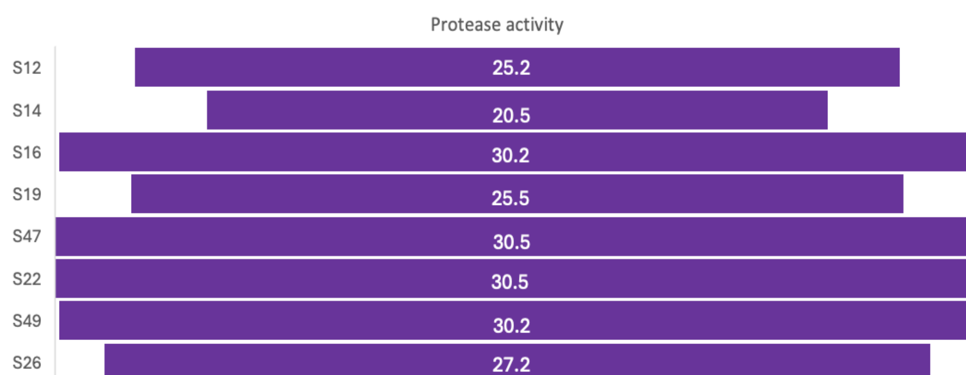


Figure 3. The average protease activity (mm) of *L. fermentum* isolate isolates

**Safety Assessment**

*L. fermentum* isolates showed different antibiotic resistance profiles. While all the isolates were resistant to VA, they were sensitive to DA, MEM, and E (Table 6, Figure 4). Also, none of the isolates showed hemolytic activity.

Table 6. Antibiotic resistance profiles of *L. fermentum* isolates

| Isolate Codes | S | DA | MEM | CIP | E | VA | TE | SXT |
|---|---|---|---|---|---|---|---|---|
| S12 | S | S | S | R | S | R | S | S |
| S14 | S | S | S | I | S | R | S | I |
| S16 | S | S | S | R | S | R | S | R |
| S19 | S | S | S | R | S | R | I | R |
| S22 | R | S | S | R | S | R | I | R |
| S26 | I | S | S | R | S | R | S | R |
| S47 | R | S | S | R | S | R | S | R |
| S49 | R | S | S | R | S | R | I | R |

(S: Susceptible, R: Resistant, I: Intermediate)
(S: Streptomycin, DA: Clindamycin, MEM: Meropenem, CIP: Ciprofloxacin, E: Erythromycin, VA: Vancomycin, TE: Tetracycline, SXT: Trimethoprim/Sulfamethoxazole)

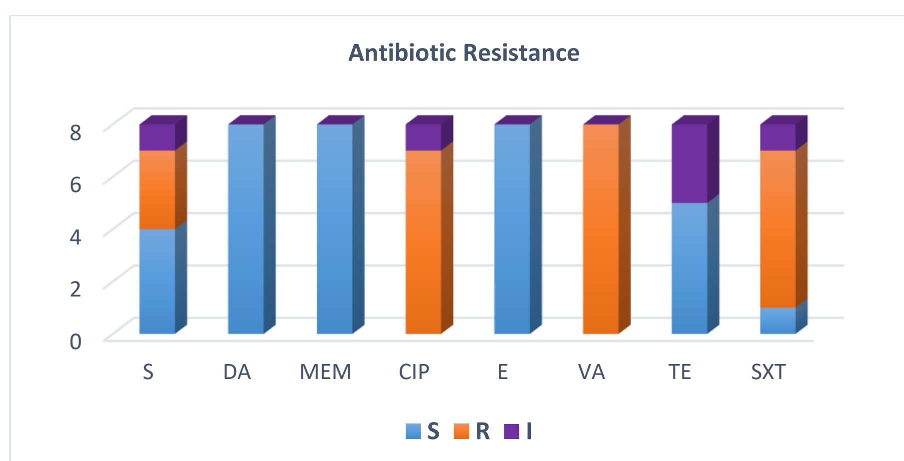


Figure 4. Distribution rate of antibiotic susceptibilities among isolates
(S: Susceptible, R: Resistant, I: Intermediate)
(S: Streptomycin, DA: Clindamycin, MEM: Meropenem, CIP: Ciprofloxacin, E: Erythromycin, VA: Vancomycin, TE: Tetracycline, SXT: Trimethoprim/Sulfamethoxazole)

**Discussion**

It was concluded that most of the lactobacilli (61.9%) did not survive in the pH2 environment, and it is noteworthy that pH2 is distinctive for lactobacilli (*P <0.05*). It is generally accepted that there is a decrease in the total number of probiotics when exposed to pH1.5 and pH3 environments (Sahadeva et al., 2011). Although these bacteria were expected to survive and reach the intestines due to the buffer effect at the pH of the stomach environment when taken via food (Soares et al., 2019), high acid tolerance may be vital for various gastric diseases (Lambert and Hull, 1996). D'ambrosio et al. (2022) reported that *L. fermentum* from buffalo milk replaced *Helicobacter pylori* in the gastric epithelial cell model. The acid tolerance of bacteria is desirable for withstanding gastric stress and its use as a dietary supplement, allowing strains to survive longer on high acid carrier foods without further inhibition (Shehata et al., 2016). Similarly, Bozdemir et al. (2022) and Asan-Ozusaglam and Gunyaktı (2019) reported that *L. fermentum* isolates survived at pH2. Kesmen et al. (2012) reported that the majority of the lactic acid biota of sucuk belongs to *Lactobacillus sakei, L. plantarum, L. curvatus, L. brevis, L. farciminis, L. alimentarius*, and minorly contains *Leuconostoc* and *Weisella*. No specific study has been conducted for acid-tolerant sucuk isolates. Hitherto, there have been many studies on the health-beneficial effects of *L. fermentum* (Rodríguez-Sojo et al., 2021; Paulino do Nascimento et al., 2022; Phujumpa et al., 2022). This study revealed that traditional fermented Turkish sucuk contains acid-tolerant *L. fermentum*, and probiotic properties characterize those.

Bile salt tolerance is essential for bacteria colonization and metabolic activity in the host's small intestine (Shehata et al., 2016). Suwannaphan (2021) considered lactobacilli's 75% survival rate after two hours of incubation in simulated gastric juice and bile salt as the cut-off level. *L fermentum* isolates with high acid tolerance have high bile salt tolerance in gastric and intestinal juice (*P <0.05*). Masco et al. (2007) reported that it was associated with gastric transit ability in isolates with enhanced acid tolerance. It is crucial to identify how well the cells in a probiotic product can survive in the gastrointestinal tract (GIT) and thus be able to mediate the desired health benefit while passing through the human body (Wendel, 2022). Collado and Sanz (2007) reported that acid-resistant strains showed better growth ability in the presence of bile salt and NaCl (6-10%).

Similarly, all *L. fermentum* isolates could grow in 12% salt. The resistance and survival to extreme osmotic resistance is one of the specific requirements of beneficial bacteria after the technological processes for their inclusion in probiotic formulas (Silva et al., 2019). Hydrophobicity is one of the critical properties improving the first contact between bacteria and host cells (Krausova et al., 2019). Autoaggregation indicates lactobacilli's capacity to bind to intestinal epithelial cells' surface, protecting the host from invading pathogens. Coaggregation of lactobacilli with pathogens also demonstrates their ability to bind to pathogens *in vivo* and create a microenvironment where their antimicrobial metabolites can inhibit pathogens (An et al., 2000; Potočnjak et al., 2017). Similar to the current study, Krausova et al. (2019) found no correlation between autoaggregation and hydrophobicity (*P >0.05*). Strikingly, our results showed that the S19-coded isolate, demonstrated to produce EPS, had an elevated level of autoaggregation and hydrophobicity. QingWu et al. (2022) reported that EPS and EPS proteins' hydrophobic interaction contributes to aggregation and hydrophobicity. The coaggregation spectrum shown by the isolates was also broad (1.23-83.13%). Compared to measurements at the third hour, the fastest coaggregation increase was shown against *E. coli*, especially in the isolate coded S49. However, high coaggregation generally belongs to the isolate S16 from the first measurement. Considering the antimicrobial activity of CFSs, S16 is not effective against *L. monocytogenes*. Likewise, Thayalan et al. (2021) did not observe zone formation in the antagonistic activity of *L. salivarus*, which has coaggregation properties against some pathogens. In addition, the antagonistic activities of *L. fermentum* isolate from cheese by Tulumoglu et al. (2014) against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 are

compatible with the current study. Owusu-Kwarteng et al. (2015) reported that *L. fermentum* isolates did not have an antagonistic effect against *E. coli* O157 882364 and *S.* Enteritidis ATCC 13076, but they observed an effect on 25% of the isolates against *L. monocytogenes* NCTC 10527.

Proteases synthesized by bacteria have a significant industrial potential due to the biochemical diversity of bacteria and their genetic manipulability (Zhang et al., 2019). Beganović et al. (2013) reported the protease activity of *L. helveticus* M92 as 18 mm. Raveschot et al. (2020) found the average protease activity values in *L. delbruecki* isolates to be 32 mm, emphasizing the importance of a fermentation starter for initiating proteolysis in yogurt production. In meat products, hydrolysis products from proteins are important flavor precursors in fermented sausage, producing a variety of oxidized volatile and non-volatile flavor compounds that contribute to the final flavor profile of the product (Candogan and Acton, 2004). The current study emphasizes that *L. fermentum* isolates, with their high proteolytic activity, will contribute to final products with ideal biotechnological properties in traditional fermented meat products. *Lactobacillus* species have been reported to be intrinsically resistant to vancomycin, streptomycin, ciprofloxacin, and sulfamethoxazole-trimethoprim (Ammor et al., 2007; Gueimonde et al., 2013; Campedelli et al., 2019). However, acquired resistance can be transferred from one bacterium to another, contributing to a greater and more widespread risk than intrinsic resistance (Li et al., 2020). Among the antibiotics examined, *L. fermentum* isolates from sucuk are not characterized by acquired resistance.

In conclusion, this study showed that traditional fermented Turkish sucuk harbors the newly isolated wild-type *L. fermentum*, regarding biotechnological, safety, and probiotic properties with exciting potential. Further confirmation of efficiency in experimental animal models is remarkable in elucidating its potential health benefits. Additionally, examining these isolates more comprehensively within the framework of omics approaches will allow them to be evaluated in all health-promoting studies.

## CONFLICT OF INTEREST

The authors declare no competing financial interest.

## ETHICAL APPROVAL

No ethical approval was required as no live animals were used in this study.

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## DATA AVAILABILITY

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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