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ORIGINAL ARTICLE

Evaluation of metabolic activities and probiotic characteristics of two *Latilactobacillus sakei* strains isolated from pastırma

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Abstract

The current study was carried out to investigate metabolic activities and main probiotic characteristics of two *Latilactobacillus sakei* strains (8.P1 and 28.P2) isolated from pastırma, a highly seasoned, air-dried cured beef. Both strains showed antimicrobial activity against important foodborne pathogens like *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and so forth. For the characterization of antimicrobial activity, the effect of various enzymes, temperature and pH were tested. The results of the tests demonstrated that the antimicrobial activity of strains was based on the production of protein-structured compounds such as bacteriocin or bacteriocin like peptides. In metabolic activity studies, amounts of the lactic acid, proteolytic activity and hydrogen peroxide produced by the 8.P1 and 28.P2 were found to range between 16.09 and 17.32 mg/mL, 0.24 and 0.04 mg/mL and 0.98 and 0.04 µg/mL, respectively. It was also observed that neither strain could produce exopolysaccharide. Both strains were found susceptible to vancomycin, chloramphenicol, gentamicin, tetracycline, and netilmicin sulfate. When the strains are evaluated with respect to their probiotic potential, 28.P2 could tolerate acidic conditions, but 8.P1 showed sensitivity. The survival rate of the strains in the simulated gastric juice and their adhesion abilities were found suitable to stay alive in the gastrointestinal tract and to proliferate in the intestine. The evaluation of all the features of both strains demonstrated that both strains had the potential to be used as a protective culture. In addition, it was observed that 8.P1 and 28.P2 were more suitable as a starter culture and a probiotic candidate respectively.

Keywords Lactic acid bacteria · *Latilactobacillus sakei* · Microbial preservative · Probiotic culture · Starter culture

Introduction

Probiotics, which were first discovered in the early 1900s, are among the popular research topics of the 2000s, especially in the last 10 years of the 2000s. They have gained popularity due to their positive effects on human health. Since the importance of intestinal microbiota on human health and the use of probiotics to modulate the microbiota was realized, studies in the field of probiotics have increased a great deal. Probiotic microorganisms are generally derived from lactic acid bacteria (LAB) and bifidobacteria, which are the main components of the intestinal microbiota in humans (Özen and Dinleyici 2015; Puebla-Barragan and Reid 2019; Trush et al. 2020). In today's world, some probiotic microorganisms have been commercialized, and probiotics have become an industrially legitimate research area with a certain global market share. Between 2005 and 2015, more than 500 new foods and beverages enriched with probiotic microorganisms were offered for sale worldwide. Current analyses show that probiotic sales are growing steadily all over the world and it is estimated that the probiotic market will increase up to 69.3 billion USD by 2023 (Trush et al. 2020).

The industrial importance of LAB, a probiotic which comes to mind first, has actually been known for nearly over a century. They are already used as starter culture, adjunct culture, or protective culture in the food industry. In the first place, LAB attracted attention because they increase the acidity due to their lactic acid production capabilities in the food production process. Since the effect of these bacteria on many important characteristics of food such as taste, odor and nutritional properties when used as starter culture was realized, their importance has increased. In addition, LAB are a salient group with their antimicrobial agent production

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capabilities. In parallel with consumers' increasing interest in foods that do not contain chemical preservatives, studies on the possibilities of using LAB as preservatives in foods have attracted more attention and thus gained importance due to the antimicrobial activity of LAB against pathogenic microorganisms with various antimicrobial compounds, especially bacteriocins (e.g. antimicrobial peptides synthesized in ribosomes). Finally, since the probiotic concept and its importance were realized, LAB have been much more popular (Özen and Dinleyici 2015; Puebla-Barragan and Reid 2019; Trush et al. 2020).

With the recognition of the importance of probiotic microorganisms, there has been a significant increase in studies in which the different probiotic strains are investigated. Up till now, a wide variety of sources such as plants, soil, food, and the gastrointestinal systems of animals have been used in probiotic research (Bhat and Bajaj 2019). In such studies, at the first stage, potential probiotic candidates are isolated from natural sources and identified. Then they are evaluated in vitro in line with the probiotic selection criteria. The probiotic selection criteria basically include characteristics associated with survival in the human digestive system, ability to proliferate in the intestines and safety requirements in the case of consumption. These studies are generally performed based on the "Guidelines for the Evaluation of Probiotics in Food" published by the Food and Agriculture Organization/World Health Organization (FAO/WHO) in 2002. Among the selection criteria in the aforementioned guideline, are characteristic features such as acid and bile resistance, antimicrobial activity, resistance to digestive enzymes, adhesion to human epithelial cells or mucus layer, and antibiotic sensitivity (FAO/WHO 2002; Hill et al. 2014).

Since LAB can be found naturally in foods, many food types are considered as good sources for research on probiotics. There are a lot many studies reporting that potential probiotic LAB strains are isolated from various foods such as meat products, dairy products, fish, pickles or fermented fruit juices (Bhat and Bajaj 2019). Despite all the studies and commercial probiotics available in the market, the demand for potential probiotic strains is increasing in the light of the growing body of knowledge and developing technologies. Therefore, studies on new probiotic microorganisms continue without slowing down. In this context, studies on traditional foods attract interest (Ahire et al. 2021). One of these traditional products is Pastırma is produced in many parts of the world including Middle Asian, Middle Eastern, some European and some Mediterranean countries like Turkey (Abd-Elghany et al. 2020; Aksu et al. 2020; Da Silva Cardoso et al. 2020). It is obtained by the technological processing of parts properly separated from healthy bovine carcass meat and dried after the permitted additives (nitrate or nitrite or mixture of both) are added. Then it is coated with çemen, a paste of ground fenugreek seeds, and dried again. In short, pastırma includes dry-cured muscle as a raw material and it is produced without heating or smoking. This very popular product is consumed without cooking especially for breakfast (Topçu et al. 2020).

By this time, various studies have been carried out on the production, and physicochemical and microbiological quality of pastırma. It is also known that LAB constitute the dominant microflora of this ready-to-eat dry meat product, but almost no studies are available on the probiotic potential of LAB found naturally in this ready-to-eat meat product (Aksu et al. 2020; Topçu et al. 2020). Moreover, although it is a dominant Lactobacillus species present in the meat products, no data are available about any *Latilactobacillus sakei* (updating the species name was proposed by Zheng et al. (2020) which was formerly called *Lactobacillus sakei*) strain isolated from pastırma. The characteristic properties of L. *sakei* strains obtained from pastırma have never been investigated before. In the current study aims to determine the possibility of two *L. sakei* strains to be used as starter or probiotic culture.

Materials and methods

Strains, chemicals and cell culture

Two *L. sakei* strains that were identified previously (with ribotyping systems) from pastırma samples by Dincer and Kivanc (2012) were used in this study. Nine type culture (*Bacillus cereus* NRRL B–3711, *Proteus vulgaris* NRRL B-123, *Pseudomonas aeruginosa* ATCC 27,853, *Escherichia coli* NRRL B–3704, *Salmonella typhimurium* NRRL B-4420, *Enterococcus feacalis* ATCC 29,212, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* NRLL B-744, *Listeria monocytogenes* ATCC 7644) and two isolates from our own laboratory collection (*Klebsiella pneumoniae* and *Yersinia enterocolitica* strains) were used as indicators in the antimicrobial activity study. All microorganisms were stored at − 80 °C in in 20% glycerol (v/v) and were pre-cultivated twice before they were used. *L. sakei* strains were propagated in Man, Rogosa and Sharpe (MRS) broth at 30 °C for 18 h. Indicator strains were propagated in Brain Hearth Infusion (BHI) broth at 30 or 37 °C for 18 h depending on the species. While growth mediums and chemicals were obtained from Merck, Turkey, enzymes, antibiotic discs and human colorectal adenocarcinoma Caco-2 cell line (Accession Number: 98,052,301) were obtained from Sigma-Aldrich, Turkey, Oxoid-Hemakim, Turkey and the Republic of Turkey Ministry of Food Agriculture and Livestock, Foot & Mouth Disease Institute respectively.

Antimicrobial activity and partial characterization

Cell free supernatant (CFS) preparation

To be used in antimicrobial activity studies, 10-fold condensed CFS of *L. sakei* strains were prepared using the method proposed by Bennik et al. (1997). For this purpose, strains were grown in 10 mL MRS broth for 48 h at 30 °C and 800 µL inoculum was taken here and transferred in MRS broth (final volume is 80 mL) and incubated for 24 h at 30 °C. After the incubation, the supernatant was collected through centrifugation at 11.000 *g* for 30 min, and pH was adjusted as 6.0 ± 0.2. Then, it was lyophilized using a lyophilizer (Christ Alpha 1–4 Freeze Dryer, Malvern UK) for two days, dissolved in 8 mL sterile distilled water and sterilized using a syringe filter with a 0.22 µm pore size.

Antimicrobial activity – its spectrum and nature

The antimicrobial activity of the strains was evaluated by using the agar well diffusion method (Tagg and McGiven 1971). In the first stage of our study, to determine the spectrum of antimicrobial activity and whether antimicrobial activity was induced by the production of bacteriocin, the antimicrobial activity of pure CFS against 11 indicator microorganisms (mentioned in section ''strains, chemicals and cell culture'') was detected. Afterwards, the same work was repeated with samples of CFS treated with catalase and proteinase K to determine whether the antimicrobial activity was due to the production of bacteriocin (Zhu et al. 2000).

Characterization of antimicrobial activity

After the results of the first stages our study was evaluated, four indicator microorganisms to be used in the characterization studies of antimicrobial activity were selected. At this stage, the effects of enzymes (trypsin, α-chymotrypsin, lysozyme, pronase, α-amylase) on antimicrobial activity were determined. Then, the effects of temperature and pH on antimicrobial activity were investigated. To detect the effect of temperature, CFS samples were held at temperatures ranging between 50 and 110 °C (at 10-degree intervals) for 30 min and 120 °C for 20 min and then the residual antimicrobial activity was determined. To detect the effect of pH, CFSs were adjusted to pH 1–13 (at 2-degree intervals) using 1 N NaOH or 1 N HCl and incubated at 37 °C for 24 h. At the end of the incubation periods, pH of samples was adjusted again as 6.0 ± 0.2 and residual antimicrobial activity was determined (Zhu et al. 2000).

In the standard manner, in all antimicrobial activity studies, after the 1% (v/v) volume freshly prepared cell suspension of indicator microorganism (McFarland standard No: 0.5 adjusted) was taken and transferred to the nutrient agar medium, it was scattered into the petri dishes. Wells 0.8 cm in diameter were made, and 80 µL CFS sample was used all the time. Used enzymes were added to CFS at relevant concentrations (1 mg/mL for lysozyme, α-amylase and pronase, 2 mg/mL for trypsin, 5 mg/mL α-chymotrypsin) after they were dissolved in 0.05 M phosphate buffered saline (PBS), incubated at 37 °C for 4 h and used for analysis forthwith. All the assays were performed twice and the average of two repetitions was used for calculations.

Determination of the important metabolic characteristics

Acidification and lactic acid production

To determination of acidification, strains were grown in MRS broth at 30 °C for 18 h, 90 µl inoculum were taken here and transferred into 9 ml MRS broth adjusted the pH 6.5. After the incubation at 30 °C for 24 h, the pH of the medium was recorded (Banwo et al. 2012). The amount of lactic acid was determined by using the method described by Demirci and Gündüz (1994). For analysis strains were grown in MRS broth at 30 °C for 18 h, 50 µl inoculum were taken from here and transferred into MRS broth (Merck, 69,966) adjusted to pH 6.2 ± 0.2 for the optimum growth (final volume is 5 mL) and incubated for 48 h at 30 °C. Measurements of the optical density values were recorded at 400 nm using a spectrophotometer and were turned into the mg lactic acid /mL, according to the standard curve prepared with lactic acid solutions in MRS broth at concentrations ranging from 1 to 16 mg/mL.

Hydrogen peroxide production

For analysis strains were grown in MRS broth at 30 °C for 18 h, 50 µl inoculum were taken from here and transferred into MRS broth (Merck, 69,966) adjusted to pH 6.2 ± 0.2 for the optimum growth (final volume is 5 mL) and incubated for 48 h at 30 °C. The amount of hydrogen peroxide was determined by using the method described by Patrick and Wagner (1949). Measurements of the optical density values were recorded at 350 nm using a spectrophotometer and were turned into the µg hydrogen peroxide/mL, according to the standard curve prepared with hydrogen peroxide solutions in distilled water at concentrations ranging from 1 to 10 µg/mL.

Proteolytic activity

For analysis strains were grown in MRS broth at 30˚C for 18 h, 50 µl inoculum were taken from here and transferred into sterilized skim milk (Oxoid, LP0031) adjusted to pH 6.5 ± 0.2 (final volume is 5 mL) and incubated for 48 h at

30 °C. Proteolytic activity was determined with the modified Hull method proposed by Citti et al. (1963). Measurements of the optical density values were recorded at 650 nm using a spectrophotometer and were turned into the mg tyrosine/mL, according to the standard curve prepared with tyrosine in MRS broth at concentrations ranging from 0.02 to 1 mg/mL.

Exopolysaccharide (EPS) production

EPS production was determined by using four different MRS agar forms with modified carbon contents (containing glucose, sucrose, fructose and lactose as carbon source). Other ingredients were used, unchanged, in the amounts specified in the MRS agar (Merck, 1.10660) formulation. For the study, after the 18-hour active cultures of strains were grown at 30 °C for 24–48 h onto each form of the MRS agar plates, the colony morphology of strains was assessed. Strains have ropy or mucoid phenotypes was accepted as a potential EPS producer (Ruas-Madiedo and De Los Reyes-Gavilán 2005).

To determine metabolic characteristics, all the assays were performed three times and the average of three repetitions was used for calculations.

Evaluation of probiotic characteristics in vitro

Acid tolerance

To determine the tolerance of strains to acidic environmental conditions,18-hour active cultures of strains were centrifuged (10.000 ***g*** 10 min) and rinsed twice using PBS. After the densities of strains were adjusted according to McFarland standard No: 0.5 using the same buffer solution, 1-mL samples were taken and transferred to 9 mL MRS broth adjusted to pH 2.5 to mimic the acidic environment of the stomach., and then they were incubated at 37 °C. Viable cell counts were determined initially and at 3 and 6 h of incubation (Thirabunyanon et al. 2009; Won et al. 2020).

Survival in the simulated gastric environment

Survival of strains in simulated gastric environment was evaluated by using the method proposed by Corcoran et al. (2005). For the preparation of gastric juice, 3.5 g glucose, 2.05 g NaCl, 0.60 g KH_2PO_4, 0.11 g $CaCl_2$ and 0.37 g KCl was dissolved 1000 mL distilled water. After adjusted to pH 2.0 using 1 M HCl, solution was sterilized by autoclaving at 121 °C for 15 min. Prior to analysis, lysozyme (0.1 g/L^{-1}), porcine bile (0.05 g/L), and pepsin (13.3 mg/L) were added as stock solutions. For the analysis, after the 18-hour active cultures of strains were centrifuged (7000 ***g*** 10 min) and rinsed using ringer solution were transferred into prepared gastric juice and incubated at 37 °C. After that, viable cell counts were detected initially and 10, 30, 60, 90 min of incubation.

Adhesion assay

Adhesion properties of strains were determined using Caco-2 cell line (10^6 cells/well) in 6 well tissue culture plates by the method proposed by Thirabunyanon et al. (2009). The Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) F-12, including 10% fetal bovine serum (FBS), 1% (v/v) L-glutamine, 1% (v/v) penicillin-streptomycin solution, and 7.5% NaHCO3. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 and 95% air, and sub-cultivated at 70–80% confluence (Er et al. 2015). For the analysis, the 18-hour active cultures of strains were centrifuged and rinsed twice by using PBS. After the densities of strains were adjusted as 10^8 CFU/mL in non-supplemented DMEM, 0.5 mL samples were taken and transferred to Caco-2 cells well. Caco-2 cells plates were incubated at 37 °C in 5% CO_2 for 1 h for attachment and at the end of the incubation periods they were rinsed three times using PBS to eliminate the incohesive bacterial cells. Afterward, 0.1% (v v^{-1}) Triton X-100 was transferred to each well and incubated at 37 °C for 5 for 5 min for lysis, the serial dilution was prepared and viable cell counts were detected.

In all the assays conducted to evaluate probiotic characteristics, viable cell count was performed in MRS agar plates after 48 h of incubation at 30 °C and the results were evaluated by the following formula:

$$\left(\log \text{CFU } N_1 / \log \text{CFU } N_0\right) \times 100, \quad (1)$$

N_1: Number of viable cells after the analysis, N_0: number of viable cells before the analysis. All the assays were performed three times and the average of three repetitions was used for calculations.

Safety assessment

Determination of the antibiotic resistance patterns

Antibiotic resistance pattern of 8.P1 and 28.P2 was detected using the disk-diffusion method also known as the Kirby-Bauer test (Bauer et al. 1966). In the present study, 14 different commercially available antibiotics were used. The study and the interpretation of measured zone diameters were performed with reference to the guidelines formed by Clinical and Laboratory Standards Institute (2013).

Results

Antimicrobial activity and partial characterization

In our study, we determined that antimicrobial activity of two *L. sakei* strains. We also determined the nature of the antimicrobial activity. As a results of the studies conducted on antimicrobial activity spectrum, 8.P1 was found effective against 11 indicator microorganisms, and 28.P2 against 10 indicator microorganisms. In addition, although antimicrobial activity of strains against small number indicator microorganism *was partially decreased at a negligible level*, it was observed that the antimicrobial activity in general was not affected by the addition of catalase enzyme, whereas it was highly affected by the addition of Proteinase K. The results are given in Table 1 in detail.

The results of the studies conducted on the characterization of antimicrobial activity are summarized in Table 2. Although the results differ depending on the indicator microorganism used, the antimicrobial activity of both strains partially or wholly disappeared due to the proteolytic enzymes like trypsin and pronase. Additionally, the activity was completely inactive at pH 1, 9, 11, and 13, the activity was observed only at pH 3, 5 and 7. The antimicrobial activity of the strains was not affected by temperature changes against indicator microorganisms other than *S. aureus*. Even when the CFS of strains was kept at 120 °C for 20 min, there was no change in antimicrobial activity.

Determination of the other important metabolic characteristics

Important metabolic characteristics like lactic acid and hydrogen peroxide production or proteolytic activity and EPS production ability of strains are presented in Table 3. As a result of the studies carried out, EPS production was not detected in both strains. Also, it was observed that 28.P2 strain had almost no hydrogen peroxide production potential and had very low proteolytic activity, while the 8.P1 strain produced low amount of hydrogen peroxide and showed proteolytic activity. On the other hand, the acidification capacities and lactic acid production potentials of the strains were found to be similar.

Evaluation of probiotic characteristics in vitro

The results of all the assays conducted to evaluate probiotic characteristics are given in Table 4. In brief, 8.P1 lost viability completely and 28.P2 lost viability greatly at pH 2.5 three hours after, because of acidity. After 90 min of incubation in the simulated gastric juice, strains kept the viability partially. Both strains showed quite high adhesion ability.

Safety assessment

To determine the antibiotic resistance/susceptibility patterns of strains, different antibiotic groups including aminoglycosides, broad-spectrum antibiotics, fluoroquinolones, cephalosporin, macrolides, β–lactams and glycopeptides were used. Both strains were susceptible to vancomycin,

Table 1 Antimicrobial activity spectrum of not treated cell free supernatants and nature of inhibitory agent

	8.P1			28.P2		
	Non-treated	*5 µg/mL Catalase added*	*1 mg/mL Proteinase K added*	*Non-treated*	*5 µg/mL Catalase added*	*1 mg/mL Proteinase K added*
P. vulgaris NRRL B-123	++	++	–	–	–	–
B. cereus NRRL B–3711	+++	+++	–	+++	++	–
E. coli NRRL B–3704	++	++	–	++	++	+
B. subtilis NRLL B-744	++	++	–	++	++	+
K. pneumoniae	+	+	–	+	+	+
S. typhimurium NRRL B-4420	++	–	–	+	+	+
E. faecalis ATCC 29,212	++	++	–	++	+	–
Y. enterocolitica	+++	+++	–	+	+	–
S. aureus ATCC 6538	++	++	–	++	+	–
L. monocytogenes ATCC 7644	+++	+++	–	++	++	–
P. aeruginosa ATCC 27,853	++	++	–	++	++	–

The diameter of inhibition zones (mm) was measured, the zone diameter of the wells was subtracted and data are given as the average of two repetitions

-: Absence of inhibition zone, +: zone $<$ 4 mm, ++: zone 4–5 mm, +++: zone 6–7 mm, ++++: zone $\geq$ 8 mm

Table 2 Effect of enzymes and pH and temperature on antimicrobial activity

		L. monocytogenes ATCC 7644	*E. faecalis* ATCC 29212	*S. aureus* ATCC 6538	*P. aeruginosa* ATCC 27853
8.P1	*None treated*	+++	++	++	++
	2 mg/mL Trypsin	-	-	-	++
	5 mg/mL α-chymotrypsin	-	-	++	++
	1 mg/mL Pronase	-	-	-	-
	1 mg/mL Lysozyme	+	-	+	++
	1 mg/mL α-amylase	++	-	-	-
	pH 1	-	-	-	-
	pH: 3	-	-	-	+
	pH: 5	+++	++	-	++
	pH: 7	++	+	-	-
	pH 9	-	-	-	-
	pH 11	-	-	-	-
	pH 13	-	-	-	-
	50 °C 30 min.	+++	++	-	++
	60 °C 30 min.	+++	++	-	++
	70 °C 30 min.	+++	++	-	++
	80 °C 30 min.	+++	++	-	++
	90 °C 30 min.	+++	++	-	++
	100 °C 30 min.	+++	++	-	++
	110 °C 30 min.	+++	++	-	++
	120 °C 20 min.	+++	++	-	++
28.P2	*None treated*	++	++	++	++
	2 mg/mL Trypsin	++	-	-	-
	5 mg/mL α-chymotrypsin	++	-	-	++
	1 mg/mL Pronase	-	-	-	-
	1 mg/mL Lysozyme	-	-	-	++
	1 mg/mL α-amylase	++	-	-	-
	pH 1	-	-	-	-
	pH: 3	-	-	-	+
	pH: 5	++	++	-	++
	pH: 7	++	++	-	-
	pH 9	-	-	-	-
	pH 11	-	-	-	-
	pH 13	-	-	-	-
	50 °C 30 min.	++	++	-	++
	60 °C 30 min.	++	++	-	++
	70 °C 30 min.	++	++	-	++
	80 °C 30 min.	++	++	-	++
	90 °C 30 min.	++	++	-	++
	100 °C 30 min.	++	++	-	++
	110 °C 30 min.	++	++	-	++
	120 °C 20 min.	++	++	-	++

The diameter of inhibition zones (mm) was measured, the zone diameter of the wells was subtracted and data are given as the average of two repetitions

-: Absence of inhibition zone, +: zone < 4 mm, ++: zone 4–5 mm, +++: zone 6–7 mm, ++++: zone ≥ 8 mm

chloramphenicol, gentamicin, tetracycline, and netilmicin sulfate, but resistant to kanamycin, lomefloxacin and ceftriaxone. On the other hand, whereas 8.P1 were susceptible or tolerant to streptomycin, amikacin, penicillin-G and erythromycin, 28.P2 were resistant to these antibiotics. The results are given in detail in Table 5.

Table 3 EPS production, proteolytic activities and other important metabolic characteristics of strains

	pH	Lactic acid (mg/mL)	Proteolytic activity (mg tyrosine/mL)	Hydrogen peroxide (µg/mL)	EPS production
8.P1	4,18	16,09 ± 0,002	0,240 ± 0,014	0,984 ± 0,095	Not detected
28.P2	4,27	17,32 ± 0,01	0,043 ± 0,01	0,046 ± 0,065	Not detected

All the assays were performed three times and data are given as mean ± standard deviation of the three repetitions

Table 4 Survival rates under simulated gastrointestinal conditions and adhesion ability of strains

	Survival rate at pH 2.5 (%)		Survival rate simulated gastric environment (%)		Adhesion ability (%)
8.P1			10 min	90.07 ± 7.03	72.5 ± 0.55
	3 h	Not detected	30 min	85.95 ± 4.59	
	6 h	Not detected	60 min	85.9 ± 4.64	
			90 min	76.46 ± 3.99	
28.P2			10 min	86.84 ± 2.52	66.01 ± 5.56
	3 h	21.17 ± 0.48	30 min	58.13 ± 3.01	
	6 h	7.05 ± 0.38	60 min	39.74 ± 2.67	
			90 min	36.43 ± 4.29	

All the assays were performed three times and data are given as mean ± standard deviation of the three repetitions

Discussion

Antimicrobial activity and partial characterization

Antimicrobial activity, is one of the main features that LAB strains should have in terms of both their use in food industry as preservative or their evaluation as probiotics (Gomes et al. 2012). In the present study, antimicrobial activity of 2 *L. sakei* strains against 11 indicator microorganisms was detected by agar well diffusion method and has been observed that both strains have a broad spectrum of antimicrobial activity. LAB are responsible for the natural fermentation of various products. They produce lactic acid as the main fermentation product, create an acidic environment by lowering the pH of the environment, and this has an inhibitory effect on various microorganisms. While investigating whether LAB strains have antimicrobial activity due to bacteriocin production, acidity-induced antimicrobial activity needs to be eliminated. Therefore, the pH value of the CFS samples used in our studies was adjusted to pH 6.02 during sample preparation, which means that it is known that the antimicrobial activity of both strains is not due to acidity. In addition, as is seen in Table 1, the antimicrobial activity of the strains was not affected by the addition of catalase enzyme, whereas it was highly affected by the addition of Proteinase K. These results eliminate the possibility of antimicrobial activity through hydrogen peroxide production and show that the antimicrobial activity is induced by the protein-structured compounds such as bacteriocin or bacteriocin like peptides.

According to the most accepted bacteriocin classification system, bacteriocins produced by *L. sakei* strains include sakacins and lactocin S. Sakacins are classified in Class II bacteriocins (They generally belong to Class IIa subclass), and there are many types such as sakacin A, sacacin P, sakacin G, and sakacin D98. Sakacins are generally heat- and pH-stable bacteriocins with strong anti-listerial activity. Lactocin S is classified into lantibiotics (i.e. Class I bacteriocins), it has lower heat stability compared to sakacins and it is considered to be moderately heat-stable bacteriocins. It is also known to be effective in various foodborne pathogens like *L. monocytogenes* and *S. aureus*. The comparison with the existing literature data demonstrated that, both of our strains have broad spectrum of antimicrobial activity (Schillinger and Lücke 1989; Sawa et al. 2013; Barbosa et al. 2014; Mogoşanu et al. 2017). On the other hand, it should be noted that the antimicrobial agent is concentrated 10-fold before analysis and this process may increase the antimicrobial activity.

Table 5 Antibiotic resistance/susceptibility patterns of strains

	C	VA	K	GAT	SH	LOM	E	TE	AK	CIP	CN	P	NET	CRO
8.P1	S	S	R	R	S	R	I	S	S	I	S	S	S	R
28.P2	S	S	R	I	R	R	R	S	R	S	S	R	S	R

C chloramphenicol (30 µg), *VA* vancomycin (30 µg), *K* kanamycin (30 µg), *GAT* gatifloxacin (5 µg), *SH* streptomycin (10 µg), *LOM* lomefloxacin (10 µg), *E* erythromycin (15 µg), *TE* tetracycline (30 µg), *AK* amikacin (30 µg), *CIP* ciprofloxacin (5 µg), *CN* gentamicin (10 µg), *P* penicillin-G (10 U), *NET* netilmicin sulfate (30 µg), *CRO* ceftriaxone (30 µg)

R resistant, *I*: Intermediate, *S* susceptible

In the present study, for the in vitro partial characterization of bacteriocin or bacteriocin-like peptides produced by 8.P1 and 28.P2 strains studied in our study, the effect of various enzymes and physicochemical parameters on antimicrobial activity was investigated. Because of their protein-structure, they lose their activity in the presence of proteolytic enzymes, which is an expected result for all bacteriocins including sakacins (Todorov et al. 2013). Therefore, in our study, partial or complete abolition of antimicrobial activity of both strains by trypsin and pronase was associated with bacteriocin production. The sakacins produced by *L. sakei* strains are generally heat stable. Our results about the heat stability are consistent with data published by other authors (Schillinger and Lücke 1989; Todorov et al. 2013; Mogoşanu et al. 2017).

In addition to its thermostable properties, sakacins are known to be relatively less affected by pH changes. Some researchers reported that sakacins were stable in a wide pH range like 4–10, while others reported they were stable in a relatively narrow pH range like 2–6. As a result, pH stability varies according to the type of sakacins (Todorov et al. 2013; Barbosa et al. 2014). Similarly, in our study, the antimicrobial activity of both strains was observed at pH 3, 5 and 7 (Table 2). Although these results indicate that 8.P1 and 28.P2 strains studied in our study are potential producers of sakacin, unexpectedly, it was observed that the antimicrobial activities of the strains were also inactivated by α-amylase, and that in the presence of this enzyme, only antimicrobial activity against *L. monocytogenes* was unchanged. This result indicate that our strains produce antimicrobial compounds possess carbohydrate moiety in its active part. Current literature data show that bacteriocins in glycoprotein structure have α-amylase sensitivity and that conventionally, these type bacteriocins are considered complex bacteriocins and are classified into Class IV. However, amylase sensitivity can also be observed, though rarely, in bacteriocins not belonging to Class IV. In a small number of studies, it is reported that amylase sensitivity in Class II bacteriocins are produced by Enterococcus or Leuconostoc strains (Lewus et al. 1992; Seo et al. 2014), but to our knowledge, this is the first report indicating that amylase sensitivity in bacteriocins are produced by *L. sakei* strains. In conclusion, when all findings are evaluated together, it is thought that the strains produce not only bacteriocin like the classical sakacin A (especially considering the antimicrobial activity results against *L. monocytogenes*) but also a second bacteriocin or bacteriocin-like peptide in glycoprotein structure (especially considering the sensitivity of α-amylase and antimicrobial activity results against *S. aureus*). However, to make an exact definition, more studies should be conducted on topics such as determining protein sizes or searching for bacteriocin genes.

Determination of the important metabolic characteristics

L. sakei strains are known to be well adapted to the meat environment. Through the metabolic activities, they are widely used as starter culture or protective culture in meat fermentation, especially for production of sausage (Champomier-Vergès et al. 2001; Barbieri et al. 2020). Just like other LAB, these strains produce various organic acids, mostly lactic acid and also cause acidification of the environment. Tolerance of strains to acidification is a key characteristic of the adaptation to fermentation conditions to exhibit other properties. Organic acid production is important both in the elimination of undesirable microorganisms and the formation of organoleptic characteristics of meat products. Especially, lactic acid plays a crucial role in meat fermentation (Champomier-Vergès et al. 2001; Zagorec and Champomier-Vergès 2017; Montanari et al. 2018). In our study, the acidification capacities and lactic acid production potentials of 8.P1 and 28.P2 were similar (Table 3). When compared with the literature data, the amount of lactic acid produced by our strains was found to be slightly higher than other LAB species isolated from dairy products. Yüksekdağ et al. (2004) investigated the organic acids production potential of lactococci isolated from kefir and reported lactic acid levels were 2.3–9.9 mg/ml. Kıvanc et al. (2011) reported that the amount of lactic acid produced by LAB strains isolated Boza varies in the range between 0.16 and 7.79 mg/ml. On the other hand, our results more consistent with the report of study involving other *L. sakei* strains (Montanari et al. 2018). Since the lactic acid production capacity of our strains is not at extreme values (such as very high or very low) and they are already isolated from the meat product, it is thought that when used as a starter culture in the production of meat products, it will not adversely affect the organoleptic qualities of the product.

Another compound produced by LAB strains is hydrogen peroxide. The limited amount of hydrogen peroxide produced in the initial stage of fermentation creates a selective environment and plays an important role in the inhibition of undesirable microorganisms in food products. Therefore, when LAB strains are evaluated as starter or protective culture in meat products, even if hydrogen peroxide production capability is not one of the main selection criteria, it is considered as an advantageous trait. On the other hand, the amount of hydrogen peroxide produced by LAB is also important, as excessive hydrogen peroxide accumulation can cause fat rancidity and discoloration in meat products. Thereby, hydrogen peroxide production ability can also be considered as a disadvantageous trait depending on the amount of hydrogen peroxide produced (Batdorj et al. 2007; An et al. 2010). In the light of this information, the fact that 8.P1 strain produces a lower amount of hydrogen

peroxide compared to other LAB strains in the literature (Zalán et al. 2005; Batdorj et al. 2007) and that the 28.P2 strain has almost no hydrogen peroxide production potential (Table 3) is not evaluated as a negative feature. On the contrary, regarding the data that strains studied in our study can produce other antimicrobial compounds such as bacteriocin and lactic acid, they are considered to have the potential to be used as a preservative culture without causing any adverse effects on the product structure.

Taste and flavor formation in fermented meat products is a very complex system and is affected by many factors. Proteolysis of proteins is one of the main factors in the formation of the organoleptic characteristic of a product. Literature data show that *L. sakei* strains, along with other microorganisms such as coagulase negative cocci and yeasts found in meat products, also contribute to flavor formation through their proteolytic activities (Champomier-Vergès et al. 2001). In this context, the very low proteolytic activity of 28.P2 strain is evaluated as a feature that may limit the possibility of its use as a starter culture. On the other hand, 8.P1 shows proteolytic activity, although it is not very high (Table 3). This strain was found to be similar to other LAB strains isolated from dairy or meat products in terms of proteolytic activity (Yuksekdag and Aslım 2010; Celik et al. 2021).

Another compound produced by LAB strains and important to the food industry is EPSs. These compounds are natural polymers associated with textural and biochemical properties in foods especially in dairy products. On the other hand, EPS production by LAB is not always a desired feature. It is also considered as an undesirable feature in meat products in various situations such as slime production. Compared to other LAB strains, fewer studies have been performed on EPS production in *L. sakei* strains. Even so, it is known that the EPS production capabilities of this species are weak (Champomier-Vergès et al. 2001; Dertli et al. 2016). Consequently, the lack of EPS production abilities of 8.P1 and 28.P2 strains studied in our study is an expected result, and it is not evaluated negatively.

Evaluation of probiotic characteristics

Current studies indicate that fermented foods are possible sources of LAB for the gut microbiome (Ahire et al. 2021; Zagorec and Champomier-Vergès 2017; Mulaw et al. 2019). In this sense, studies in which *L. sakei* strains isolated from various foods were evaluated as probiotics are available in the literature (Gomes et al. 2012; Lim et al. 2020). In order for a probiotic microorganism to exhibit its possible benefits in humans, it must first stay alive in the gastrointestinal tract and proliferate in the intestine. Therefore, resistance to acidity and other unfavorable conditions in the stomach environment is regarded as basic criteria in the evaluation of probiotic candidates (FAO/WHO 2002; Hill et al. 2014; Won et al. 2020).

In the current study, whether our *L. sakei* strains can be used as probiotics was also investigated in vitro. For this purpose, tolerance of 8.P1 and 28.P2 strains studied in our study to simulated stomach environment besides acidic circumstances were determined. In addition, their adhesion ability was analyzed and the results are summarized in Table 4. Although with a significant increase after food-intake, the pH of the stomach is generally around 2.5 to 3.5. Thus, pH values ranging from 1 to 5 have been widely considered to evaluate in vitro the acid tolerance of probiotic candidates (Hosseini et al. 2009). In our study, as in various studies (Thirabunyanon et al. 2009; Won et al. 2020), the resistance of the strains to acidic conditions was determined at pH 2.5 to mimic the acidic environment of the stomach. Also, resistance to other unfavorable conditions like bile salt, proteolytic enzymes was detected by preparing simulated gastric juice which consists of various indigents, like pepsin, lysozyme and porcine bile and so forth. The comparison of the results obtained in our study with those obtained in the literature demonstrates that 8.P1 and 28.P2 strains studied in our study can tolerate adverse conditions in the stomach environment as do other *L. sakei* strains in the literature, but they are less resistant to acidic conditions (Gomes et al. 2012; Won et al. 2020; Kim et al. 2021). In particular, it is an unexpected result that 8.P1 survives at a rate of 76.46% under simulated gastric conditions (pH 2) although cannot survive at pH 2.5. During the analysis process, strains exposed to pH 2.5 for 180 min while they exposed to gastric juice for 90 min. Therefore, this result is likely to be related to the duration of exposure to the simulated gastric conditions.

The adhesion ability of the LAB strains is another important criterion used to evaluate probiotic candidates. This ability which reflects the proliferation possibilities of candidate strains in the intestine is generally determined in vitro by various methods (either aggregation and co-aggregation assays or in tissue culture using cell lines like Caco2, HT29 and so forth other epithelial cells) in the preliminary characterization studies of probiotics (Ouwehand and Salminen 2003). In the current study, adhesion ability of the two *L. sakei* strains was detected in the tissue culture using Caco2 cell line and observed that the adhesion abilities of the strains were quite high (Table 4). While our results regarding the adhesion characteristics of *L. sakei* strains are consistent with those reported by some authors (Gomes et al. 2012; Kim et al. 2021), they are much higher than were those reported by some other authors (Won et al. 2020).

Safety assessment

Although approximately 35 Lactobacillus species, including *L. sakei*, are considered non-pathogenic and have qualified presumption of safety (QPS) status according to the European Food Safety Authority (EFSA); in studies the use of LAB strains must be evaluated in terms of safety. In this respect, especially it is necessary to determine the resistance or susceptibility characteristics of the strains to antibiotics (Campedei et al. 2019). According to the directives of authorities such as EFSA and The International Scientific Association for Probiotics and Prebiotics (ISAPP), a LAB strain must not have transferable antibiotic resistance factors to be considered safe for human consumption. Lactobacillus species generally have intrinsic resistance to aminoglycosides like kanamycin, amikacin or gentamicin and this type resistance is presumed to present a low risk of horizontal spread. On the other hand, lots of Lactobacillus show acquired resistance against some broad spectrum antibiotics such as chloramphenicol or tetracycline and some macrolides such as erythromycin. Because of this type resistance can be passed horizontally, it can limit the use of LAB strains (Ammor et al. 2007; Hill et al. 2014; Campedelli et al. 2019). When evaluated from this point of view, the sensitivity of 8.P1 and 28.P2 strains studied in our study particularly to chloramphenicol and tetracycline can be considered as an advantage, their tolerance or resistance to erythromycin can be considered as a disadvantage. In the literature, some authors have also reported the resistance to erythromycin. Zonenschain et al. (2009) have reported erythromycin resistance in 7 of 24 *L. sakei* strains. Similar to our results, Dušková et al. (2020) reported that all 6 *L. sakei* strains of food origin sensitive to tetracycline and chloramphenicol but 4 of them resistant to kanamycin. In the same study while all strains resistant to gentamycin and streptomycin, our both strains were sensitive to gentamicin. At this point, it should be noted that the determination of antibiotic susceptibility by disc diffusion technique for LAB gives only general idea. It is more appropriate to determine the minimum inhibitory concentration (MIC) of antibiotics with the standard microdilution method according to the guidelines and criteria published by the EFSA (2018). Therefore, to reach a definite conclusion, MIC values should be determined and resistance genes should be investigated at the molecular level.

Conclusion

LAB has been a part of human life for centuries and has been used for a wide variety of purposes. In the light of the studies carried out on the issue, the importance of these microorganisms is being realized more and more every day. In this context, it is necessary to isolate LAB strains from different sources and to determine their unique properties. In the present study, when all the determined features are evaluated together, it can be said that both strains have the potential for use in the food industry. Furthermore, the use of both strains as a preservative is appropriate since, coming from the food itself, it does not modify its organoleptic qualities, probably preserved by the strong seasoning that the food itself carries. When the results are discussed in more detail, based on the bacteriocin production, antibiotic resistance spectrum and metabolic characteristics, both strains can be assumed to have the potential to be used as a protective culture. In addition, the data obtained also indicate that the 8.P1 strain is more likely to be used as a starter culture in meat fermentation compared to the 28.P strain. When strains are evaluated in terms of their probiotic potential, although the survival potential in the simulated gastric juice and adhesion abilities of both strains are promising, the sensitivity of 8.P1 strain to acidic conditions is considered as a limiting factor for its use as a probiotic. 28.P2 can be considered as a more suitable probiotic candidate. However, use of 28.P2 as a probiotic agent still presents certain caution that must be studied, since it has only been proven theoretically without having verified how it affects the balance of the intestinal microbiota and whether it displaces other families of beneficial bacteria or favors the growth of other undesirable families.

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Data availability All data generated or analysed during this study are included in this article.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Consent to participate This study has been approved by all authors for their participation.

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