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Research Paper / Araştırma Makalesi

Isolation of Distinct *Lactobacillaceae* spp. with Functional Characteristics from Traditional Sourdough Samples

Hümeyra İspirli[1], Enes Dertli[2]

[1]Central Research Laboratory, Bayburt University, Bayburt, Turkey
[2]Department of Food Engineering, Faculty of Chemical and Metallurgical Engineering, Yıldız Technical University, İstanbul, Turkey

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Corresponding author (Yazışmalardan Sorumlu Yazar): humeyra_ispirli@hotmail.com (H. İspirli)
+90 458 333 20 33 +90 458 211 11 58

ABSTRACT

Lactic Acid Bacteria (LAB) were isolated from traditional sourdough samples, and functional properties of selected LAB species were determined. Five distinct strains *Limosilactobacillus fermentum* LR1, *Lacticaseibacillus rhamnosus* LR2, *Levilactobacillus senmaizukei* CE37, *Lactiplantibacillus plantarum* CE48 and *Limosilactobacillus reuteri* KD44A were identified following the genotypic discrimination of hundred isolates. Presence of bacteriocin genes in identified species was determined by PCR, and sourdough isolates have been shown to carry bacteriocin genes although they vary according to species. Exopolysaccharide (EPS) production tests showed that the amount of EPS production varies in different media and different incubation conditions. Antimicrobial effects of 5 different LAB species were determined against *Bacillus cereus* BC 6830, which cause rop problems, and other pathogen species and also molds isolated from bread. Amylolytic activities of isolates, which are important in terms of technological roles, were determined genotypically and phenotypically. Finally, the phytase and phosphatase activities of these species were studied. This study was aimed to unveil the functional sourdough LAB species to be used for industrial purposes.

Keywords: Traditional sourdough, Lactic acid bacteria (LAB), Phytase activity, Antifungal activity

Geleneksel Ekşi Hamur Örneklerinden Fonksiyonel Özelliklere Sahip Farklı *Lactobacillaceae* spp. İzolasyonu

ÖZ

Bu çalışmada geleneksel ekşi hamur örneklerinden Laktik Asit Bakterileri (LAB) izole edilerek tanımlanmış ve tanımlanan LAB türlerinin fonskiyonel nitelikleri tespit edilmiştir. Ekşi hamur örneklerinden toplamda 100 adet izolat elde edilerek izolatların genotipik ayrımı yapılmış ve sonuç olarak *Limosilactobacillus fermentum* LR1, *Lacticaseibacillus rhamnosus* LR2, *Levilactobacillus senmaizukei* CE37, *Lactiplantibacillus plantarum* CE48 ve *Limosilactobacillus reuteri* KD44A suşları tanımlanmıştır. Bu suşlarda bakteriyosin genlerinin varlığı PCR metodu ile test edilmiş ve suşlarda farklı bakteriyosin genlerinin varlığı açığa çıkarılmıştır. Ekzopolisakkarit (EPS) üretim testleri, bu suşların farklı koşullarda farklı oranda EPS üretim kabiliyetine sahip olduğunu göstermiştir. Yapılan antimikrobiyal testleri sonucunda test edilen suşların rop etmeni *Bacillus cereus* BC 6830 dahil farklı patojenlere ve ekmek küflerine karşı antimikrobiyal etkiye sahip olduğu ortaya konmuştur. Ekmek teknolojisi açısından bir diğer önemli teknolojik fonksiyon olan amilolitik aktivite bu suşlarda genotipik ve fenotipik olarak belirlenmiş ve son olarak suşların fosfotaz ve fitaz aktiviteleri değerlendirilmiştir. Bu çalışmada fonksiyonel etkiye sahip ekşi hamur orjinli LAB suşlarının endüstriyel olarak değerlendirilebilirliklerinin test edilmesi amaçlanmıştır.

Anahtar Kelimeler: Geleneksel ekşi hamur, Laktik Asit Bakterileri (LAB), Fitaz aktivitesi, Antifungal aktivite

INTRODUCTION

Sourdough is an intermediate product in which LAB species perform the fermentation process in dough. Use of sourdough technology in bread production results in the formation of bread with increased structure, nutritive, taste and shelflife characteristics. LAB and yeasts are essential elements of the microbial flora of the sourdough and generally LAB / yeast ratio is expected to be 100:1 in sourdough [1]. Unlike other fermented products, generally heterofermantative LAB species are predominant in sourdough [2]. Together with lactic acid, acetic acid produced by heterofermentative species has an important effect on sourdough's unique taste [3]. Following the acidification of dough by the LAB and yeast fermantation, particularly cereal-derived proteases are activated and formation of peptides and free amino acids occurs whihc results in the development of nutritional and functional properties of bakery products [4, 5]. Additionally, acidification activates phytases in cereals and increases the availability of nutrients, thereby improving the nutritional value of bread [6]. Other metabolic activities of sourdough LAB on bread quality comprise formtation of antimicrobial agents that affect bread shelf life, production of exopolysaccharides (EPS) affecting the structure of bread, their amylolytic activities that positively affect the dough's workability and the swelling of bread. The antimicrobial activity of LAB species generally originates from bacteriocins, which are antimicrobial peptides produced by these species [7], and sourdough LAB were shown to inhibit foodborne pathogenin bacteria including *Bacillus cereus* BC 6830 [8]. It has been reported that the antifungal activity of LAB is a complex process which can be caused by many different compounds such as phenyllactic acid, 4 hydroxyphenyllactic acid [9], and proteinaceous antifugal compounds [10] produced by these species. Amylolytic LAB species have been shown to be extremely important in the processing of starch-based products such as sourdough and in obtaining the final product with the desired structure and characteristics [11]. One of the important properties of sourdough LAB species is EPS production [12] and technological properties of sourdough and sourdough bread can be improved with *in situ* EPS production [13]. Another important function of sourdough LAB species is the phytase and phosphatase activities, and thanks to these activities, they increase the bioavailability of minerals and amino acids in cereal products by breaking down the components such as phytic acid which binds the elements such as protein and minerals in the cereals [14].

In this study, LAB species were isolated from traditional sourdoughs collected from Trabzon province of Turkey where famous sourdough breads are produced and isolates were then genotypically characterized which was followed by their techno-functional characterisation in terms of antimicrobial activity, EPS production and phytase, phosphatase and amylase activities.

MATERIALS and METHODS

Isolation of LAB Starins from Turkish Sourdough

Sourdough samples (n=6) were collected from the bakeries producing bread using traditional sourdough in Trabzon province of Turkey. Sourdough samples were brought to the laboratory within the day for the analysis. For the isolation of LAB, 10 g of representative sourdough samples were diluted in 0.85% NaCl solution and homogenized. Serial dilutions were performed from these suspensions and plated on modified MRS agar containing 10 g maltose, 5 g fructose, 0.5 g cysteine-HCl, 1 mL/L vitamin mix and 0.1 g/L cycloheximide in 1000 mL distilled water in addition to its standard formula. Plates were then incubated at 30°C for 48 h and colonies with distinct morphologies were selected and further analysed for Gram stain, cell morphology, and catalase reaction. Following these tests, 100 isolates were selected for genotypic characterization.

Genotypic Characterization by RAPD-PCR Analysis

RAPD-PCR analysis was applied to differentiate potential LAB strains. Genomic DNA was isolated by phenol – chloroform methodology and was used as a template for PCR amplification. RAPD-PCR analysis was performed with primer M13 (5'-AGGGTGGCGGTTC-3'). PCR reactions were prepared containing 1 µl DNA template, 5 µl 10X DreamTaq Buffer for DreamTaq DNA Polymerase (Thermo Fisher), 0.4 µl dNTPs (Bioline), 2 µl 20 mM primer M13, 0.125 µl 5 U DreamTaq DNA Polymerase and up to 50 µl of sterile H_2O. PCR was performed using a thermal cycler (Benchmark, TC9639) with the following program: 35 cycles of 94°C for 1 min, 40°C for 20 s, then final step of 72°C for 2 min. The PCR products were separated on a 1% (wt/vol) agarose gel at 90 V for 1 h and band patterns visualised by ethidium bromide staining and photographed under UV illumination [15].

Bacterial Identification by 16S rRNA Gene Sequencing

Following RAPD-PCR anaysis distinct strains were identified by 16S rRNA sequencing and 16S rRNA PCR analysis was conducted with primers AMP_F (5'-GAGAGTTTGATYCTGGCTCAG-3') and AMP_R (5'-AAGGAGGTGATCCARCCGCA-3') [16]. The PCR mix and reaction conditions were performed as previously described [15].

Determination of Antibacterial Activity and Detection of Bacteriocin Coding Genes of Sourdough Isolates

Antimicrobial effects of sourdough strains were determined by well diffusion method as previously described [17]. Five different pathogenic bacteria; *Staphylococcus aureus* ATCC 25923, *Yersinia enterocolitica* ATCC 27729, *Salmonella typhimurium* RSSK 95091, *Escherichia coli* BC 1402 and *Bacillus*

cereus BC 6830 were used for the antibacterial activity tests and inhibition zones (expressed in millimeters) formed around the wells after 24 hours of incubation at 37°C were visualised.

The genes encoding curvacin, sakacin, plantaricin and nisin were determined by PCR analysis in the sourdough isolates using the primer pairs given in Table 1. The amplicon lengths targeted for each gene and PCR conditions used were given in Table 1.

Table 1. Primers used for the detection of bacteriocin genes

Primer	Sequence (5′→3′)	Expected Amplicon (bp)	PCR Conditions
curA_F curA_R	GTAAAAGAATTAAGTATGACA TTACATTCCAGCTAAACCACT	180	95°C (2 min) 35 cycles of 95°C (30 s), 50°C (1 min), 72°C (1 min) 72°C (5 min)
sakQ_F sakQ_R	ATGCAAAATACAAAAGAACTAA CGCTTGTTTAGAGACACCCGTT	200	95°C (2 min) 35 cycles of 95°C (30 s), 50°C (1 min), 72°C (1 min) 72°C (5 min)
plnA_F plnA_R	GTACAGTACTAATGGGAG CTTACGCCAATCTATACG	450	95°C (2 min) 35 cycles of 95°C (30 s), 53°C (1 min), 72°C (1 min) 72°C (5 min)
NisF NisR	CTATGAAGTTGCGACGCATCA CATGCCACTGATACCCAAGT	608	95°C (2 min) 35 cycles of 95°C (30 s), 55°C (1 min), 72°C (1 min) 72°C (5 min)

Antifungal Activities of Sourdough Isolates

Antifungal effects of LAB strains were tested against *Penicillium chrysogenum, Botrytis cinerea, Aspergillus parasiticus, Fusarium oxysporum, Aspergillus niger* and *Alternaria alternata*. Spores of molds tested were grown on PDA agar plates to obtain 1 × 10^6 spores / mL. LAB stains were grown and plated to MRS plates which were then incubated at 30°C for 48 h under aerobic conditions. Then, malt extract soft agar (0.7% agar) containing 1 × 10^6 spores / mL each fungus was added to the petri dishes and plates containing both LAB and fungi were then incubated at 30°C for 36 h and inhibition zones were determined according to previously described scale [8].

Antibiotic Susceptibility of Sourdough Isolates

Antibiotic diffusion discs were used for the antibiotic susceptibility tests. Chloramphenicol (C, 30 µg), ampicillin (AM, 10 µg), kanamycin (K, 30 µg), streptomycin (S, 10 µg), erythromycin (E, 15 µg), tetracycline (TE, 30 µg), penicillin G (P, 10 units) and oxytetracycline (T, 30 µg) were the antibiotics tested in this study. The selected species were activated and their concentrations were adjusted to 10^7 cfu / mL and were inoculated at 100 ul by spreading method on MRS agar plates. Corresponding antibiotic disks were placed to the placaes for each strain and plates were incubated at 37°C for 24-48 h and zone of inhibitions around disks were recorded in centimetres (cm).

Isolation of EPS from Sourdough Isolates and Determination of EPS Production Levels

LAB strains were activated in 10 mL MRS broth and inoculated into 100 mL of modified BHI broth containing sucrose and incubated at 30°C for 2 d aerobically. Following the incubation, culture supernatant was obtained by centrifugation followed by 10% TCA application and this mixture as then shaken with rocker for 4-5 hours. This solution was then centrifuged at 4000 × g for 10 minutes and supernatant was obtained and an equal volume of chilled ethanol was added to the supernatant which was left at 4°C for 1 d. The precipitate was obtained and ethanol precipitation was applied for 2 more times and at final stage, partially purified EPS was obtained which was then dissolved in distilled water to determine its level by phenolsulphuric acid methodology [19].

Determination of Amylase Gene Presence and Amylase Activity

The presence of amylase gene in LAB strains was determined by PCR using the primer pair Amy10F (GTTGCTCAAGCGGATAGTGA) and Amy10R (GACGCGCTATTTCCAACTTT). PCR conditions were; 95°C for 1 min, 33 cycles of 95°C for 35 s, 57°C for 1.15 min, and 65°C for 1.15 min and 65°C for 5 min final extension and products were checked by agarose gel electrophoresis. The amylase production potentials of sourdough isolates were tested by making some modifications in the aforementioned method [20]. The selected strains were inoculated at 1% in 10 mL MRS broth to determine their amylase production potential and were activated at 37°C for 24 hours. Activated cultures were spotted on modified starch medium (30 g L^{-1} starch, 5 g L^{-1} yeast extract, 10 g L^{-1} peptone from

casein, 10 g L^{-1} NaCl, 15 g L^{-1} agar-agar, pH: 7) and incubated for 2-5 d at 37°C. At the end of incubation, starchy agar was coated with iodine solution (14 mM KI and 10 mM I_2) and the reaction was evaluated.

Determination of Extracellular and Intracellular Phytase Activities of LAB Species

The phytase activity of sourdough LAB isolates were determined according to previously described methodology [21].

Determination of Phosphatase Activities of Sourdough Isolates

For the determination of phosphatase activity of sourdough isolates, cells were harvested following overnight incubation by centrifugation at 12.000 g for 15 minutes at 4°C and washed twice with Tris-HCl (pH 6.5) solution and cells were suspended in 50 mM sodium acetate (pH 5.0) solution and used for enzyme activity tests [14]. Reaction mixture was prepared to contain 62.5 uL of 50 mM sodium acetate-acetic acid containing 6 mM p-nitrophenyl-phosphate and the same amount of cell suspension. The reaction mixture was incubated for 15 min at 50°C and the reaction was stopped by the addition of 125 uL of 1 M NaOH. Similarly, negative controls were prepared by adding NaOH to the starting samples. The release of p-nitrophenol was determined at 405 nm. One unit of phosphatase activity (U) was expressed as the amount of enzyme producing 1 µmol p-nitrophenol per hour at 50°C.

RESULTS and DISCUSSION

In total, 100 LAB strains were tested during RAPD-PCR analysis for genotypic discrimination. A represantative image of the RAPD-PCR profile of the tested strain is given in Figure 1 and in total five distinct strains were obtained for identification.

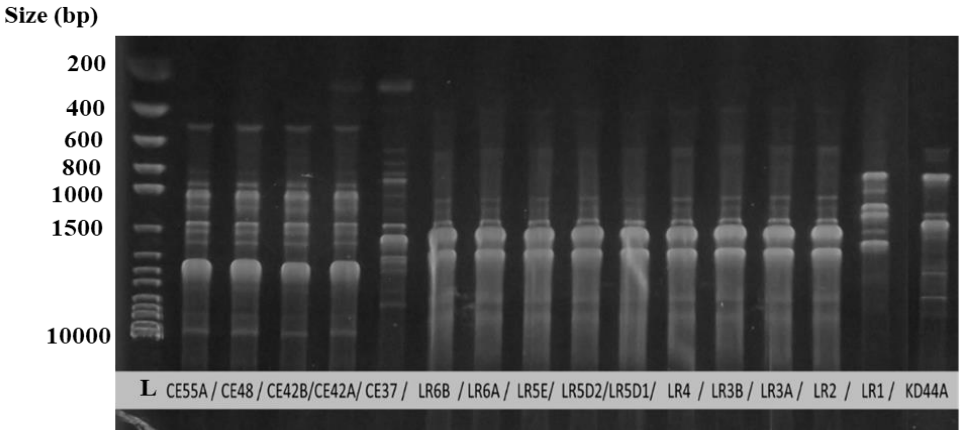

Figure 1. RAPD-PCR patterns of sourdough LAB strains

The 16S rRNA sequence of these LAB isolates revealed the presence of 5 distinct species belonging to *Limosilactobacillus fermentum* LR1, *Lacticaseibacillus rhamnosus* LR2, *Levilactobacillus senmaizukei* CE37, *Lactiplantibacillus plantarum* CE48 and *Limosilactobacillus reuteri* KD44A.

Similar to our findings, *Limosilactobacillus fermentum*, *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum* and *Limosilactobacillus reuteri* species are among the predominant LAB species reported to be found in traditional sourdoughs [3, 15, 22-24]. Apart from these strains *Levilactobacillus senmaizukei* was not reported as a sourdough isolate but isolated from different fermented products [25], and to the best of our knowledge, this LAB strain as a sourdough isolate has not been reported previously in sourdough environment. Functional characteristics of these five strains in terms of inhibition of pathogens, production of bacteriocin(s), antifungal effects, antibiotic susceptibility, EPS production levels, detection of amylase gene and amylolytic activity phytase and phosphatase activities and were determined.

One of the important functional characteristics of LAB is their antibacterial activity and antibacterial activity of five sourdough LAB strains against important pathogens such as *Salmonella typhimurium* RSSK 9509, *Yersinia enterocolitica* ATCC 27729, *Esherichia coli* BC 1402, *Bacillus cereus* BC 6830, 1, and *Staphylococus aureus* ATCC 25923 was tested in this study. As can be seen in Table 2, all isolates showed antibacterial effects at different rates on tested pathogens exceptthe effect of *L. reuteri* KD44A on *Escherichia coli* BC 1402 which did not show antibacterial activity in this case.

The inhibitory effects of LAB strains to different pathogenic strains are a well-known activity for LAB strains [26, 27]. In order to determine whether this antibacterial activity in sourdough isolates was originated from proteinaceous substances such asbacteriocins the culture fluids were treated at 100°C for 10, 20, 30 and 60 minutes and antibacterial activity was tested. It has been found that antibacterial activity was not significantly affected by temperature treatments. However, loss of activity was observed as a result of treatment with proteolytic enzymes such as trypsin, proteinase K and pepsin. This result shows that the antibacterial activity might be originated from bacteriocin production. Since all LAB species exhibited antibacterial activity potentially due to the bacteriocin production, the presence of the genes responsible for bacteriocin

production in these isolates was screened by PCR. Table 3 shows the results of the screening of the presence of curvacin, sakacin, nisin and plantaricin genes in the genomes of tested sourdough isolates. Among the tested bacteriocin genes curvacin and nisin genes were not detected fort he isolates tested in this study. The presence of the sakacin gene was detected in *L. rhamnosus* LR2 and *L. plantarum* CE48 whereasthe other isolates were not positive for the presence of this gene. Sakacin that belongs to the class IIa bacteriocin group is a bacteriocin produced by *L. sakei* [28, 29]. However, *L. curvatus* has also been reported to produce sakacin [30]. The presence of sakacin P gene within *L. rhamnosus* and *L. plantarum* strains could be related with some similarities of the origins of the bacteriocin related genes as previously suggested [31]. Importantly, the plantaricin gene was only detected in *L. plantarum* CE48 as can be expected and this finding migh reflect that this strain can be a potential plantaricin producer.

Table 2. Antibacterial activities of sourdough isolates against tested pathogens*

Microorganisms	*E. coli* BC 1402	*B. cereus* BC 6830	*S. typhimurium* RSSK 95091	*Y. enterocolitica* ATCC 27729	*S. aureus* ATC 25923
L. fermentum LR1	+	+	+	+	+
L. rhamnosus LR2	++	+	+	++	+
L. senmaizukei CE37	+	+	+	+	+
L. plantarum CE48	+	+	+	+	+
L. reuteri KD44A	-	+	+	++	+

*: Results are expressed as diameters of the inhibition zone and standard deviations in mm. ++ inhibition zone 2 mm, + inhibition zone 1 mm, – no inhibiton zone

Table 3. Testing the genes related with nisin, curvacin A, sakacin P, and plantaricin A bacteriocin production tested strains*

Microorganisms	Curvacin A	Sakacin P	Nisin	Plantaricin A
L. fermentum LR1	-	-	-	-
L. rhamnosus LR2	-	+	-	-
L. senmaizukei CE37	-	-	-	-
L. plantarum CE48	-	+	-	+
L. reuteri KD44A	-	-	-	-

*: The presence and abundance of each characteristic within the corresponding species were represented as + and - respectively.

One of the important factors that cause the spoilage of bakery products is mold growth [32]. Sourdough LAB strains might have strong antifungal effects and it has been reported that they could increase the shelf life of bakery products by delaying the mold growth [33]. The antifungal functionality of LAB strains has a very complex mechanism that can be related with distinct metabolites produced by them and the synergistic effect between these metabolites [34]. The antifungal effect of sourdough LAB species was tested against five different mold speciesincluding *Penicillium*, *Botrytis*, *Aspergillus*, *Fusarium* and *Alternaria* species (Table 4). In general, the tested sourdough LAB species showed promising antifungal activities against all mold genera.

Table 4. Antifungal activities of sourdough LAB species against *F. oxysporum, P. chrysogenum, B. cinerea, A. parasiticus, A. niger* and *Al. alternate**

Microorganisms	*Penicillium chrysogenum*	*Botrytis cinerea*	*Aspergillus parasiticus*	*Fusarium oxysporum*	*Aspergilus niger*	*Alternaria alternata*
L. fermentum LR1	+	+	+++	+	-	+
L. rhamnosus LR2	++	+++	+++	+++	++	+++
L.senmaizukei CE37	++	++	++++	++++	+	+++
L. plantarum CE48	++	+++	++++	+++	+	+++
L. reuteri KD44A	-	-	+	+	-	+

*: The antigunfal activity was detected in the following state; (++++) extensive inhibition of spore formation and mycelial growth with definite clear zones around colonies, (+++) spore formation delayed with good clear zone around the colony, (++) spore formation delayed with a small clear zone around the colony, (+) spore formation delayed but no clear zone and (-) no inhibition.

Figure 2 shows the representative images for the antifungal effect of the tested LAB strains against corresponding mold species. *L.rhamnosus* LR2*, L. senmaizukei* CE37 and *L. plantarum* CE48 showed significant antifungal activity against all mold species, whereas *L. fermentum* LR1 did not show any antifungal activity against *Aspergilus niger* and also *L. reuteri* KD44A did not show any antifungal activity against *Penicillium chrysogenum*, *Botrytis cinerea* and *Aspergilus niger*. Previous studies also demonstrated antifungal activities of *L. rhamnosus* strains to mycotoxigenic fungi including *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* [35]. Importantly, *L. plantarum* CE48 demonstrated significant inhibitory activity to tested mold species. Previously, antifungal activity of *L. plantarum* strains was also demonstrated by other researchers [36, 37].

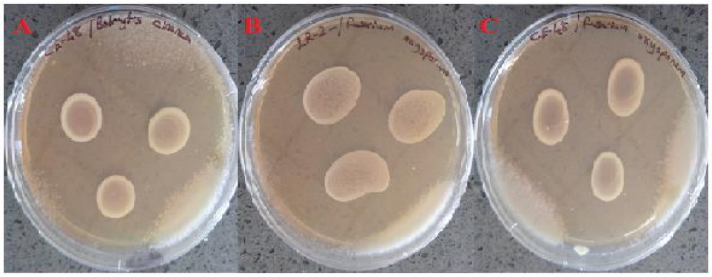

Figure 2. Representative images showing the inhibitory zones of *L. plantarum* CE48 to *B. cinerea* (A) and *F. oxysporum* (C) and *L. rhamnosus* LR2 against *F. oxysporum* (B)

Microbial resistance to clinically important antibiotics should not be present in bacterial species in the food chain. Probiotic bacteria might also transfer antibiotic resistance genes in food products which can is a major concern in food industry. In this respect, potential resistance of LAB strains to ampicillin, chloramphenicol, erythromycin, kanamycin, oxytetracycline, penicillin, streptomycin and tetracycline was tested (Table 5). All LAB strains tested were resistant to kanamycin. Although *L. fermentum* LR1, *L. senmaizukei* CE37 and *L. plantarum* CE48 were resistant to streptomycin, *L. rhamnosus* LR2 and *L. reuteri* KD44A were appeared to be sensitive to streptomycin (Table 5). Similar to our findings, LAB strains were shown to have high level of resistance to kanamycin and streptomycin as aminoglycoside antibiotics [38]. Apart from these results, LAB strains were found to be sensitive to other antibiotics tested in this study.

Table 5. Resistance of sourdough LAB species against antibiotics (inhibition zone, cm)*

Microorganisms	Inhibition zone (cm)							
	AM	C	E	K	T	P	S	TE
L. fermentum LR1	1.7±0.1	1.1±0.2	1.0±0.02	-	1.0±0.2	1.5±0.02	-	0.8±0.01
L. rhamnosus LR2	1.2±0.02	1.1±0.1	1.2±0.02	-	1.2±0.1	1.5±0.1	0.1±0.01	1.2±0.2
L. senmaizukei CE37	1.1±0.01	1.3±0.01	0.9±0.01	-	0.4±0.01	1.0±0.02	-	0.4±0.02
L. plantarum CE48	1.3±0.1	1.4±0.1	1.0±0.1	-	0.7±0.02	1.3±0.01	-	0.9±0.01
L. reuteri KD44A	2.2±0.1	1.9±0.2	1.4±0.02	-	1.3±0.2	2.2±0.1	0.1±0.01	1.5±0.1

*: AM: ampicillin; C: chloramphenicol; E: erythromycin; K: kanamycin; T: oxytetracycline; P: penicillin G; S: streptomycin; TE: tetracycline. —: no inhibition zone.

Exopolysaccharide (EPS) production is one of the important technological functions of sourdough LAB species. In this respect, we determined the EPS production characteristics of five distinct LAB species depending on their incubation temperatures and growth medium conditions. Selected isolates were grown in MRS and modified BHI mediums at 30°C, 37°C and 45°C and EPS production levels were determined. Figure 3 shows the amount of EPS produced by sourdough isolates under these conditions. In general, these isolates were capable of producing different amount of EPS between species and at different temperature parameters. The highest EPS production was observed in *L. plantarum* CE48 and *L. senmaizukei* CE37, respectively. EPS production levels of LAB can be affected by medium, fermentation conditions and genetic factors [39].

Amylolytic activity is an important activity in LAB species of sourdough origin that results in the breakdown of amylose which increases the level of free sugar in dough environment. The amylolytic activity of LAB species might increase the technological properties of bread. Accordingly, the presence of the amylase gene in LAB strains was determined. The expression products in *L. plantarum* CE48 and *L. rhamnosus* LR2 species were observed at the molecular level (Table 5). Additionally, the degradation of amylose by sourdough isolates in starch-containing medium was investigated. As an indication of amylase activity, the presence of starch hydrolysis zone was evaluated (Table 6). Amylase activity was observed in all strains tested except for *L. reuteri* KD44A. Similar to our results previous studies have shown that *L. plantarum* and *L. fermentum* strains are among the LAB that can exhibit amylolytic activity [40].

Phytic acid, which is an important component of wheat kernel, is considered to be an antinutritional factor due to its strong chelator properties to Ca^{2+}, Mg^{2+} and Fe^{2+} [41]. It has been suggested that an effective reduction of phytate content can be achieved by addition of phytase active LAB [42]. Therefore, in this study sourdough isolates were tested for their phytase activities. Extracellular and intracellular phytase activities were determined in tested LAB species and it was found that extracellular phytase activity was higher in all strains than intracellular phytase activity (Table 7). The extracellular and inracellular phytase activities were different among distinct species. The highest extracellular and inracellular phytase activity were observed in *L. reuteri* KD44A with values of 603.79 U/mL and 110.20 U/mL, respectively. The lowest extracellular and lowest intracellular phytase activity were observed in *L. fermentum* LR1 and *L. plantarum* CE48, respectively. The extracellular phytase activities

of sourdough isolates were significantly ($p < 0.05$) altered in these strains. However, intracellular phytase activities of isolates were significantly ($p < 0.05$) altered in these strains except *L. fermentum* LR1 and *L. rhamnosus* LR2 that showed same levels of activities. Previously, phytase activities of *Pediococcus pentosaceus*, *L. panis*, *L. reuteri* and *L. fermentum* of strains were found to be different between among species and strains [43].

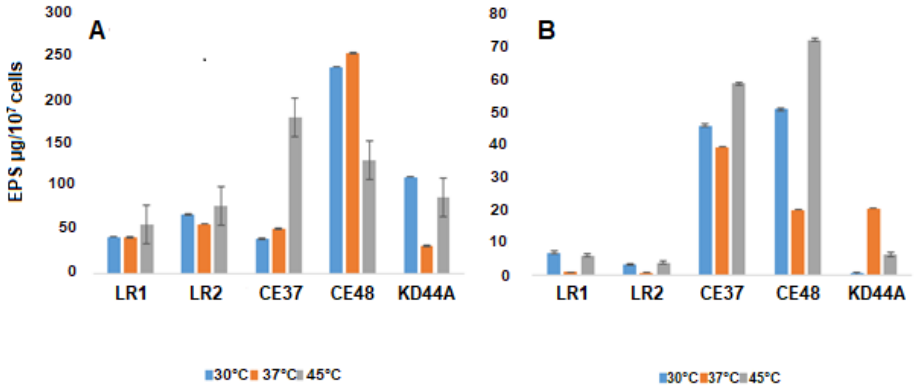

Figure 3. The level of EPS production of sourdough LAB species (*L. fermentum* LR1, *L. rhamnosus* LR2, *L. senmaizukei* CE37, *L. plantarum* CE48, *L. reuteri* KD44A) at 30-37 and 45°C. EPS were isolated from stationary-phase cultures of LAB strains grown in MRS (A) and mBHI (B) medium. (ca. 10^7 cells)

Table 6. Screening of amylase gene among the sourdough LAB strains and presence of hidrolysis zone obtained by determination of amylase activity of sourdough isolates with iodine solution using %3 starch-containing medium

Microorganisms	Amylase Gene	Amylase Activity
L. fermentum LR1	-	+
L. rhamnosus LR2	+	+
L. senmaizukei CE37	-	+
L. plantarum CE48	+	+
L. reuteri KD44A	-	-

*: + and - represent the presence and the abundance of each characteristic within the corresponding species).

Table 7. Extracellular and intracellular phytase activities of sourdough isolates

Microorganisms	Extracellular phytase activity (U/mL)	Intracellular phytase activity (U/mL)
L. fermentum LR1	295.22 ± 1.58^{E}	83.20 ± 2.98^{B}
L. rhamnosus LR2	405.12 ± 2.96^{C}	81.95 ± 2.94^{B}
L. senmaizukei CE37	501.97 ± 4.06^{B}	39.11 ± 2.93^{C}
L. plantarum CE48	348 ± 1.386^{D}	18.94 ± 1.65^{D}
L. reuteri KD44A	603.79 ± 4.41^{A}	110.20 ± 1.73^{A}

$^{A-E}$Within the column, different superscript uppercase letters show differences between the strains ($p < 0.05$)

Finally, sourdough isolates were tested for phosphatase activities which were found to be altered between 6.73 U and 1.03 U (Table 8). When the phosphatase activities of the isolates were compared, it was determined that there was a significant difference between the results except for *L. fermentum* LR1 and *L. plantarum* CE48 ($p<0.05$) and the strongest enzymatic activity belonged to the *L. reuteri* KD44A strain. Results of this study revealed that the phosphatase activity of sourdough isolates varied which can be related with strain dependent phosphatase activity of LAB as previously suggested [44].

Table 8. Phosphatase activity of sourdough isolates

Microorganisms	Phosphatase activity (U)*
L. fermentum LR1	2.4 ± 0.07^{BC}
L. rhamnosus LR2	2.1 ± 0.17^{C}
L. senmaizukei CE37	4.02 ± 0.07^{B}
L. plantarum CE48	1.03 ± 0.06^{C}
L. reuteri KD44A	6.73 ± 0.98^{A}

*1 Uof enzyme is the level of enzyme that can perform the production of 1 µmol p-nitrophenol in 1 h at 50°C. $^{A-C}$Within the column, different superscript uppercase letters show differences between the strains ($p < 0.05$).

CONCLUSION

The sourdough technology is becoming more important in the world and Turkey is one of the countries trying to adopt this technology to bread technology. In this study five distinct LAB strains were identified (*L. fermentum* LR1, *L. rhamnosus* LR2, *L. senmaizukei* CE37, *L. plantarum* CE48 and *L. reuteri* KD44A) and their technological potential in terms of sourdough technology was determined. All strains demonstrated significant antibacterial and antifungal activities against tested pathogenic bacteria and detrimentl fungi. All strains were EPS producers although the EPS production levels altered depending on strain specific conditions. Finally, amylolytic, phytase and phosphatase activites of the distinct strains were determined and promising results were obtained for these activities. This study revealed the functional characteristics of distinct LAB species isolated from traditional Turkish sourdough.

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