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Partial characterization and antioxidant activity of exopolysaccharides produced by lactic acid bacteria isolated from "Çökelek" a traditional cheese in Türkiye

Türkiye'nin geleneksel peynirlerinden Çökelek'ten izole edilen laktik asit bakterileri tarafından üretilmiş ekzopolisakkaritlerin kısmi karakterizasyonu ve antioksidan aktiviteleri

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Abstract In this study, 13 different traditionally produced "Çökelek" cheese samples were collected from the plateaus of Gümüşhane province, Turkiye. A total of 35 different strains were isolated from these samples. As a result of genotypic characterization, 9 isolates were determined to be lactic acid bacteria (LAB) strains according to the 16S rRNA gene sequence. It was determined that 9 LAB strains were *Lactobacillus brevis* (2), *Lactobacillus plantarum, Lactobacillus kefiri, Lactobacillus paracasei, Lactococcus garvieae, Lactococcus lactis* ssp. *lactis*, *Enterococcus casseliflavus* and *Pediococcus acidilactici* strains. 5 strains of 9 LAB strains were selected as EPS producer LAB according to the literature and by observing colony morphology. It was determined that all EPS samples are heteropolymeric structures consisting of glucose, galactose and fructose monosaccharides. Antioxidant characteristics of EPS samples were determined by ABTS and DPPH radical scavenging activity tests. As a result of the antioxidant activity tests, it was determined that EPS samples had between 41.20% and 59.13% DPPH scavenging activity, while the EPS sample with the highest DPPH scavenging activity produced by *Lactobacillus brevis* LS13. According to ABTS scavenging assay, EPS sample produced by *Lactobacillus kefiri* LS15 showed the highest antioxidant activity among EPS samples (71,43%). This was followed by *Lactobacillus brevis* LS13 (70,27%), *Lactobacillus paracasei* LM13 (68,81%), *Pediococcus acidilactici* LS6 (63,28%) and *Lactobacillus plantarum* LS11 (60,82%) samples, respectively.

Keywords: Çökelek, Lactic acid bacteria, Exopolysaccharide, Antioxidant activity

Özet Bu çalışma kapsamında Türkiye'nin Gümüşhane ili yaylalarında geleneksel olarak üretilmiş 13 farklı Çökelek peyniri örneği toplanmıştır. Bu örneklerden toplam 35 farklı suş izole edilmiştir. Genotipik karakterizasyon sonucunda 9 izolatın 16S rRNA gen dizilimine göre laktik asit bakteri suşu olduğu belirlenmiştir. Bu suşların iki tanesinin *Lactobacillus brevis*; diğerlerinin ise *Lactobacillus plantarum*, *Lactobacillus kefiri, Lactobacillus paracasei, Lactococcus garvieae, Lactococcus lactis ssp. lactis, Enterococcus casseliflavus, Pediococcus acidilactici* suşları olduğu tespit edilmiştir. Bu 9 suşun arasından 5 tanesi mevcut literatür ve koloni morfolojisi dikkate alınmak sureti ile EPS üreticisi LAB olarak seçilmiştir. Seçilen LAB suşlarının ürettikleri EPS örneklerinin tamamının glukoz, galaktoz ve fruktoz monosakkaritlerinden oluşan heteropolimerik yapıda olduğu belirlenmiştir. EPS örneklerinin antioksidan aktivite özellikleri ABTS ve DPPH radikal süpürücü aktivite testleri ile belirlenmiştir. Sonuç olarak ilgili EPS'lerin DPPH süpürme aktivitelerinin %41,20 ile %59,13 arasında değiştiği ve en yüksek değerin ise *Lactobacillus brevis* LS13 örneğine ait olduğu ortaya çıkmıştır. ABTS yönteminde ise en yüksek antioksidan aktivite değerinin (%71,43) *Lactobacillus kefiri* LS15 tarafından üretilen EPS numunesine ait olduğu ve bunu *Lactobacillus brevis* LS13 (%70,27), *Lactobacillus paracasei* LM13 (%68,81), *Pediococcus acidilactici* LS6 (%63,28) ve *Lactobacillus plantarum* LS11 (%60,82) örneklerinin izlediği tespit edilmiştir.

Anahtar Kelimeler: Çökelek, Laktik asit bakterileri, Ekzopolisakkarit, Antioksidan aktivite

1. INTRODUCTION

Nowadays, consumer expectation is for foods to have acceptable taste, appearance and texture, as well as be additive-free and natural. As the perception of healthy eating has evolved, many consumers have intensified the search for functional foods to improve their diets. Fewer calories, more fiber, plant protein, less salt and fewer additives have become consumer demands for a healthier diet (Nionelli *et al.* 2018; Roman *et al.* 2017). This situation has led researchers to search for ways to increase the textural properties and positive effects of food without adding any additives to the food.

Lactic acid bacteria have a long and safe history of use and consumption in the production of fermented foods and beverages (Obis *et al.* 2019; Leroy and De Vust, 2004; Caplice and Fitzgerald, 1999; Ray, 1992; Wood, 1997; Wood and Holzaphel, 1995). The most important functions of lactic acid bacteria in fermented foods are to produce organic acids, especially lactic acid. In addition to organic acid production, other secretions of LABs such as ethanol, aroma compounds, bacteriocin, exopolysaccharide, and enzymes can provide positive effects on human health as well as prolong the shelf life of food and improve its properties (Leroy and De Vust, 2004).

EPS production by LAB has attracted the attention of researchers for the last two decades. It has been reported that *Streptococcus thermophilus*, *Lactococcus lactis* and more than thirty *Lactobacillus* species produce EPS and these polymers are used in food industry as a thickener, stabilizer, emulsifier, oil replacer and other important subjects (Badel *et al.* 2011, Broadbent *et al.* 2003).

In addition to the important physicochemical properties that EPS brings to food, it has been revealed that EPS has many important physiological and biological activities for human health (Zhou *et al.* 2019) such as antioxidant activity (Zhang *et al.* 2013), antibacterial (Trabelsi *et al.* 2017), cholesterol-lowering (Korcz *et al.* 2018), immunoregulatory function (Dinic *et al.* 2018), antitumor (Wang *et al.* 2015), anticoagulant (Hussain *et al.* 2017) and antiviral activity (Nacher-Vazquez *et al.* 2015).

An important feature of EPS, which has been determined recently, is its antioxidant activity. Reactive oxygen species (ROS) are constantly produced in our body due to various metabolic activities. As with oxidative stress, it represents an imbalance between ROS production and the biological system's ability to repair the resulting damage. As a result, accumulation of ROS in organisms causes degradation of DNA, carbohydrates, lipids and proteins. Antioxidants play an important role in neutralizing these ROS. Recently, microbial exopolysaccharides have attracted attention in terms of their antioxidant properties. Many studies have demonstrated the antioxidant activities of these polysaccharides (Yıldız and Karataş 2018).

The feature that makes important the lactic acid bacteria produce EPS is improving the texture of the food and its beneficial properties without adding additives to fermented foods.

Numerous studies have shown that domestic microorganisms in fermented foods may improve safety, technological and sensory properties, and also shelf-life of dairy products (Domingos-Lopes *et al.* 2017; Moraes *et al.* 2013; Abriouel *et al.* 2008). On the other hand, continuous use of the same commercial starter culture poses a risk for bacteriophage in production. Therefore, it is very important to isolate and identify wild lactic acid bacteria from traditional fermented foods. In addition, finding EPS production capabilities of the isolated strains is extremely important in terms of improving the structural, organoleptic, and health-beneficial properties of food without adding additives.

In this study, lactic acid bacteria were isolated from "Çökelek", a traditional cheese produced in the pastures of Gümüşhane, one of the virgin regions of Turkiye, and the structures and antioxidant properties of EPS produced by selected EPS producer LAB were determined.

2. MATERIAL and METHOD

2.1. Isolation of lactic acid bacteria from Çökelek

Çökelek samples *(n = 13)* were collected from pastures in Gümüşhane. Samples were stored at 4°C and analyzed within 24 h. 10 g of Çökelek samples were taken aseptically and transferred to sterile bags and suspended in 90 ml buffer. After homgenization serial dilutions were performed from these suspensions. Aliquots of these dilutions were plated to MRS (de Man, Rogosa and Sharpe) and M17 agars and incubated at 37°C for 48 h. Potential different isolates were randomly selected from MRS and M17 agar and tested for Gram stain. Gram positive strains further subcultured in MRS and M17 broths and these isolates were then stored at -80°C in glycerol (40% v/v).

2.2. Genotypic characterization by Rep-PCR analysis

Rep-PCR analysis were performed for the discrimination of LAB strains isolated from Çökelek samples at strain level according to Dertli *et al.* 2016. A commercial isolation kit was used for the isolation of genomic DNA (EcoPURE, Türkiye). Extractions were prepared according to manufacturer's protocol. Rep-PCR analysis was conducted with primer $(GTG)_5$ (5'-GTGGTGGTGGTGGTG-3'). PCR reactions were prepared containing 0,3 µl DNA template, 1,25 µl 5 x PCR buffer for Taq polymerase (Promega), 1 µl dNTPs (Bioline), 0.5 µl 20 mM primer $(GTG)_5$, 0,03125 µl Taq polymerase and 9,41875 µl of sterile H_2O. PCR was performed using a thermal cycler (Benchmark, TC9639) with the following program: Initial denaturation for 10 min at 95°C, 35 cycles at 94°C for 1 min, 40°C for 1 min, and 65°C for 8 min; then final step of 65°C for 16 min. The PCR products were separated with electrophoresis on %1,6 (w/v) agarose gels at 90 V for 1,5 h and band

patterns were visualized by ethidium bromide staining and photographed under UV illumination.

2.3. Bacterial identification by 16S RNA gene sequencing

16S rRNA sequencing was applied to identify LAB strains as described previously (İspirli *et al.* 2016). The 1.5 kb 16S rRNA genes of isolates were amplified using primers AMP_F (5'- GAGAGTTTGATYCTGGCTCAG -3') and AMP_R (5'- AAGGAGGTGATCCARCCGCA -3') (Baker *et al.* 2013). PCR reactions were prepared containing 1 µl DNA template, 5 µl 5 x PCR buffer for Taq polymerase (Promega), 4 µl dNTPs (Bioline), 1 µl 20 mM primers AMP_F and AMP_R, 0,125 µl Taq polymerase and 37,875 µl of sterile H_2O. PCR was performed with the following program: Initial denaturation for 2 min at 95°C, 25 cycles at 95°C for 30 s, 55°C for 20 s, and 72°C for 30 s; then final step of 72°C for 5 min. PCR products were run on a gel to check the amplication and PCR products were sent to Medsantek (Türkiye) for sequencing. Sequences obtained were interrogated with the NCBI database using the BLAST algorithm with a similarity criterion of 97-100%.

2.4. Isolation of EPSs

Exopolysaccharides (EPS) were isolated and purificated from bacterial strain using the method described previously (Dertli, 2015). Modified BHI medium containing sodium acetate 5 g/L, peptone casein 5 g/L, peptone meat 5 g/L, Tween 20 1 g/L, and magnesium sulfate 0,2 g/L was used as substrate. Isolates were grown in 500 ml modified BHI culture, inoculated at %1 (v/v) with an overnight culture then incubated at 37°C for 2 d. Following the growth of the strains in the medium, the bacterial precipitate was separated by centrifugation at 6000 × *g* (4°C for 30 minutes). After by adding an equal amount of chilled ethanol (4°C) to the collected supernatant, EPS was precipitated by keeping it at 4°C for 1 night. Subsequently, the precipitated EPS was recovered by centrifugation at 10000 × g for 30 minutes at 4°C. The EPS precipitate was then dissolved in water (at 50°C) and kept at 4°C for one more night by adding 2 times as much chilled ethanol. EPS was centrifuged again and dissolved in H_2O. After this process, pure EPS was obtained by drying the EPS solution at 50°C for 10 hours.

2.5. Monosaccharide composition of EPSs by HPLC analysis

EPS was hydrolysed as described previously (Dertli, 2018). The monosaccharide composition was analysed using high performance liquid chormatograpgh (HPLC) with a CARBOSep CHO 87C Column (Chrom-tech, USA) and RID-10A refractive index detector. The mobile phase consisted of H_2O, flow rate 0,7 mL/min and column temperature of 25°C. Standard monosaccharides were glucose, galactose, fructose and maltose.

2.6. FTIR Spectroscopy analysis of EPS

Determination of the main structural groups in purified EPS was performed with a FTS 175C Digilab FT-IR spectrometer (Bio-Rad, USA) equipped with a MCT detector and single-reflection diamond ATR sampling accessory (GoldenGate, Specac). The spectra were recorded in the region of 4000-500 cm^{-1} at 4 cm^{-1} resolution.

2.7. Antioxidant activity of purified EPS

2.7.1. DPPH radical scavenging activity

The 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) radical scavenging capacity of purified EPS was determined according to the method described by Aburas *et al.* (2020) with some modification. Various concentrations of EPS samples (1, 2, 5 and 10 mg/mL) were prepared and 1 mL of these solutions were mixed with 0,3 mL DPPH-ethanol solution (0,288 mM). Then, the mixture solution reacted in the dark for 30 min. The absorbance of the solutions was measured at 517 nm. The following formula was used to determine the scavenging of DPPH radical;

$$DPPH\ (\%) = [(A_0 - A_I) / A_0] \times 100$$

A_I: Absorbance value of the sample with known concentration

A_0: Absorbance value of the control sample

2.7.2. ABTS radical scavenging activity

The 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging acticity of EPS samples was determined as the method described by Xu *et al.* (2019). It was formed by the reaction between 7 mM ABTS solution and 2.45 mM potassium persulfate solution, and kept in the dark for 16 h at room temperature until a dark blue color was formed, and ABTS solution was obtained. Before the analysis, the ABTS radical was diluted with ethyl alcohol to have an absorbance of 0,7 ± 0.025 at 734 nm. 4 mL of ABTS solution was added to 2 ml of EPS extract at different concentrations (1, 2,0, 5,0 and 10 mg/mL) and after incubation in the dark for 5 min, the absorbance was read at 734 nm in the UV-spectrophotometer. The % inhibition values were calculated according to the following equation.

$$ABTS\ (\%) = [(A_0 - A_I) / A_0] \times 100$$

A_I: Absorbance value of the sample with known concentration

A_0: Absorbance value of the control sample

2.8. Statistical analysis

All the experiments were carried out in triplicate. The relative standard deviation and the mean values were calculated using Microsoft Excel 2016 software.

3. RESULTS and DISCUSSION

In this study, EPS producing lactic acid bacteria isolated from Çökelek samples (*n=13*) produced traditionally in pastures of Gümüşhane, Turkiye. Additionally, some characteristics of EPS's produced by these isolates were determined. Microbiological analysis revealed that LAB_{MRS} and LAB_{M17} numbers were ranged between 4,03±0.01 and 7,71±0.01, 4,53±0.02 and 7,95±0.01 log CFU/g, respectively. LAB counts were in a similar range with the previous observations (Öksüztepe *et al.* 2007; Kavaz *et al.* 2012). A total of 35 different colonies with typical slimy characteristics were selected from MRS and M17 agar plates (Table 1).

Table 1. LAB counts of Çökelek samples

Çökelek sample	LAB_{MRS} log CFU/g	LAB_{M17} log CFU/g	Number of isolates
A	6,18±0.03	6,26±0.01	3
B	7,17±0.01	7,95±0.01	3
C	6,17±0.01	4,53±0.02	3
D	5,01±0.02	5,23±0.01	1
E	4,03±0.01	5,12±0.03	3
F	4,62±0.01	4,97±0.02	1
G	5,51±0.04	5,86±0.01	2
H	6,08±0.02	6,28±0.01	3
I	5,70±0.02	5,46±0.01	5
J	7,42±0.01	7,42±0.02	3
K	6,18±0.04	5,65±0.03	2
L	7,71±0.01	7,52±0.02	2
M	7,61±0.01	7,77±0.01	4

As a result of genotypic characterization, 9 isolates were determined to be lactic acid bacteria (LAB) strains according to the 16S rRNA gene sequence. It was determined that 9 LAB strains were *Lactobacillus brevis* (2), *Lactobacillus plantarum, Lactobacillus kefiri, Lactobacillus paracasei, Lactococcus garvieae, Lactococcus lactis* ssp. *lactis*, *Enterococcus casseliflavus* and *Pediococcus acidilactici* strains were presented in Çökelek cheese samples (Table 2).

Table 2. LAB strains isolated in this study

Çökelek sample	Isolates
I	*Lactobacillus brevis* LS10
L	*Lactobacillus brevis* LS13
J	*Lactobacillus plantarum* LS11
M	*Lactobacillus kefiri* LS15
I	*Lactobacillus paracasei* LM13
E	*Lactococcus garvieae* LM9
M	*Lactococcus lactis* ssp. *lactis* LM18
J	*Enterococcus casseliflavus* LS12
G	*Pediococcus acidilactici* LS6

Numerous studies have reported the presence of these strains in different traditional cheese samples (Herreros *et al.* 2003; Callon *et al.* 2004; Veljovic *et al.* 2007; González *et al.* 2007; Abriouel *et al.* 2008; Colombo *et al.*, 2009; Franciosi *et al.* 2009; Domingos-Lopes *et al.* 2017). Similar to our findings, *lactobacilli* dominate the non-starter lactic acid bacteria in most cheeses (NSLAB) population. (Bouton *et al.* 1998; Fitzsimons *et al.* 1999; Swearingen *et al.* 2001). Based on the slimy colony formation on MRS and M17 agar, 5 different strains (*Pediococcus acidilactiic* LS6, *Lactobacillus plantarum* LS11, *Lactobacillus brevis* LS13, *Lactobacillus kefiri* LS15, *Lactobacillus paracasei* LM13) were selected from 9 different LAB strains isolated and identified in this study.

The monosaccharide compositions of the purified EPSs were determined by HPLC. Accordingly, it was determined that all of the EPS produced by the 5 isolates consisted of a heteropolymeric structure consisting of glucose, galactose and fructose (data not shown). As is known, most EPSs produced by LAB are heteropolysaccharides containing three to eight repeating units composed of two or more monosaccharides (Sanlibaba and Çakmak 2016). The FTIR spectrums of EPS samples are shown in Fig. 1. All peaks in the spectrum diagram were in agreement with the typical absorption peaks of a polysaccharide (Wang *et al.* 2014). A broad and intensely stretched peak was observed at around 3262,9-3277,2 cm^{-1} due to hydroxyl groups (O-H) and confirms the polysaccharide characteristic of the samples (Taylan *et al.* 2019). Weak peaks were observed at around 2918,6-2936,7 cm^{-1} corresponding to methyl groups as well as methylene groups (Wang *et al.* 2010). Broad peaks were observed at around 1625,4-1644,8 cm^{-1} due to the bound water and indicated the characteristic IR absorption of polysaccharides (Singh *et al.* 2011; Park, 1971). The bands at around 1547,9 and 1237,7 cm^{-1} were attributed to the symmetrical stretching vibration of carboxyl groups (Rani *et al.* 2017). The clear bands at around 1020,4-1050,9 cm^{-1} were attributed to the stretch of C-O which ascertain the existence of polymer (Beć *et al.* 2020; Rani *et al.* 2017). The peaks observed around 812 cm^{-1} were characteristic peaks of heteropolysaccharides (Sirajunnisa *et al.* 2016), which is consistent with our data obtained from the HPLC analysis.

DPPH radical scavenging test was performed to determine the antioxidant activity of EPS samples. While it was determined that EPS samples had radical scavenging activities between 37,2% and 59,1%, it was observed that *Lactobacillus brevis* LS13 strain produced the EPS sample with the highest antioxidant activity (Fig 2). In a previous study, the DPPH radical scavenging activity of EPS samples was found to be between 26,55 and 37,48%, and it was determined that these results were lower than ascorbic acid used as a standard substance (Xu *et al.* 2019). In the study by Aburas *et al.* (2020), DPPH radical scavenging activity was found to be 35,35% at a

concentration of 8 mg/mL. These results were found to be similar to the results of our analysis.

The DPPH radical, which is used to determine the free radical scavenging activities of antioxidants, is a free nitrogen radical. Zhang *et al.* (2013) determined that EPS produced by *L. plantarum* C88 has 52,23% DPPH free radical scavenging activity at 4 mg/mL EPS concentration in vitro. These results are quite similar to our results.

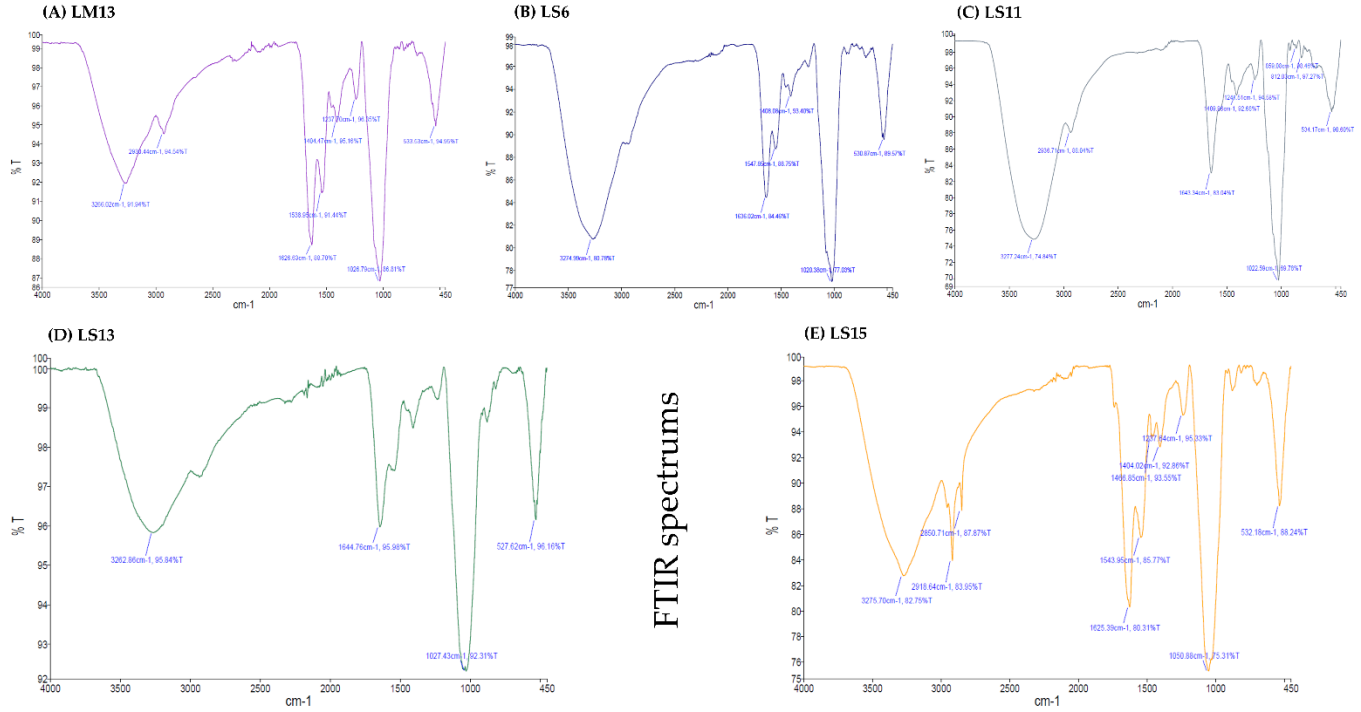

Figure 1. FTIR spectra of the EPS samples (in the range of 400–4000 cm^{-1})

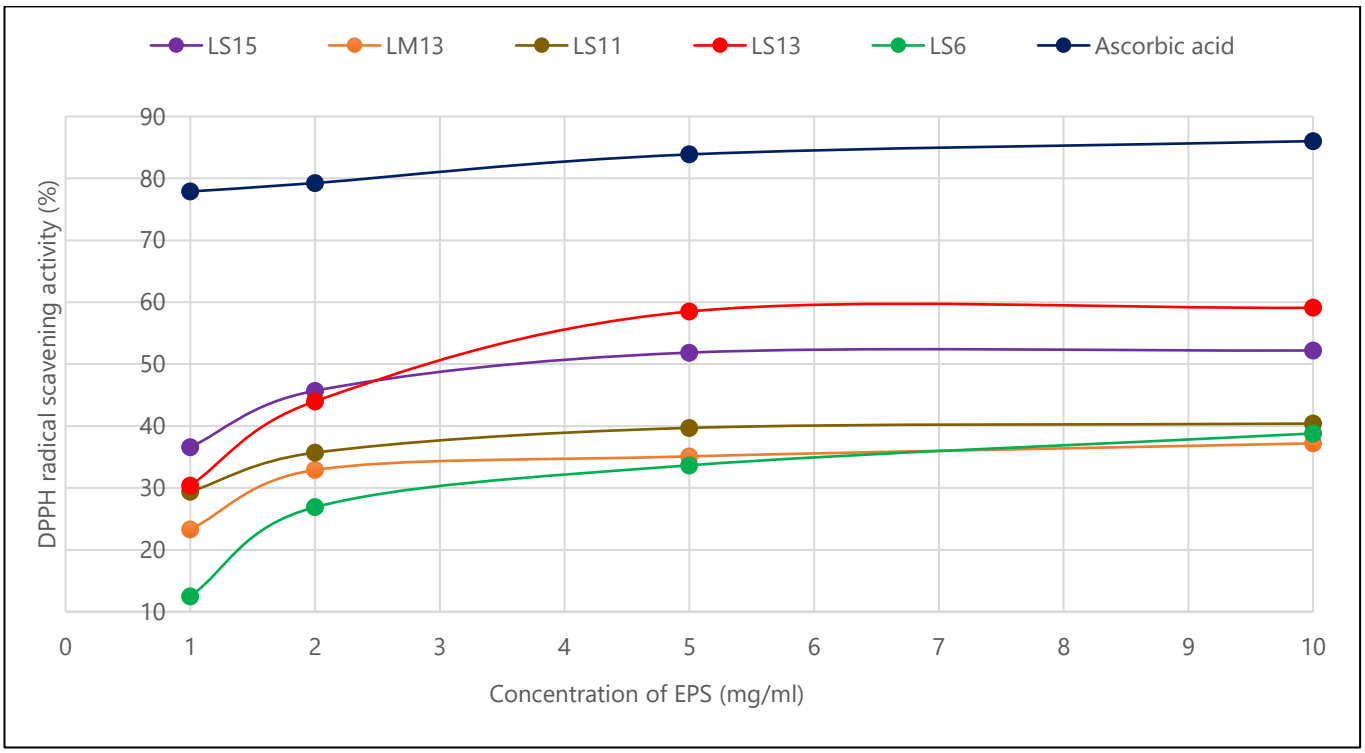

Figure 2. DPPH radical scavenging activity of EPS samples

According to ABTS method results, LS15 sample showed the highest antioxidant activity among EPS samples (71,43%). This was followed by LS13 (70,27%), LM13 (68,81%), LS6 (63,28%) and LS11 (60,82%) samples, respectively (Fig. 3). The antioxidant activity of ascorbic acid used as a standard was determined as 90,01%. According to a study, the antioxidant activity in MED 17 EPS samples in the ABTS method was found to be 59,76%

at the highest concentration of 8 mg/ml (Aburas *et al.* 2020). In a study by Xu *et al.* (2019), the highest antioxidant activity in EPS was determined as 74,35% according to the ABTS method.

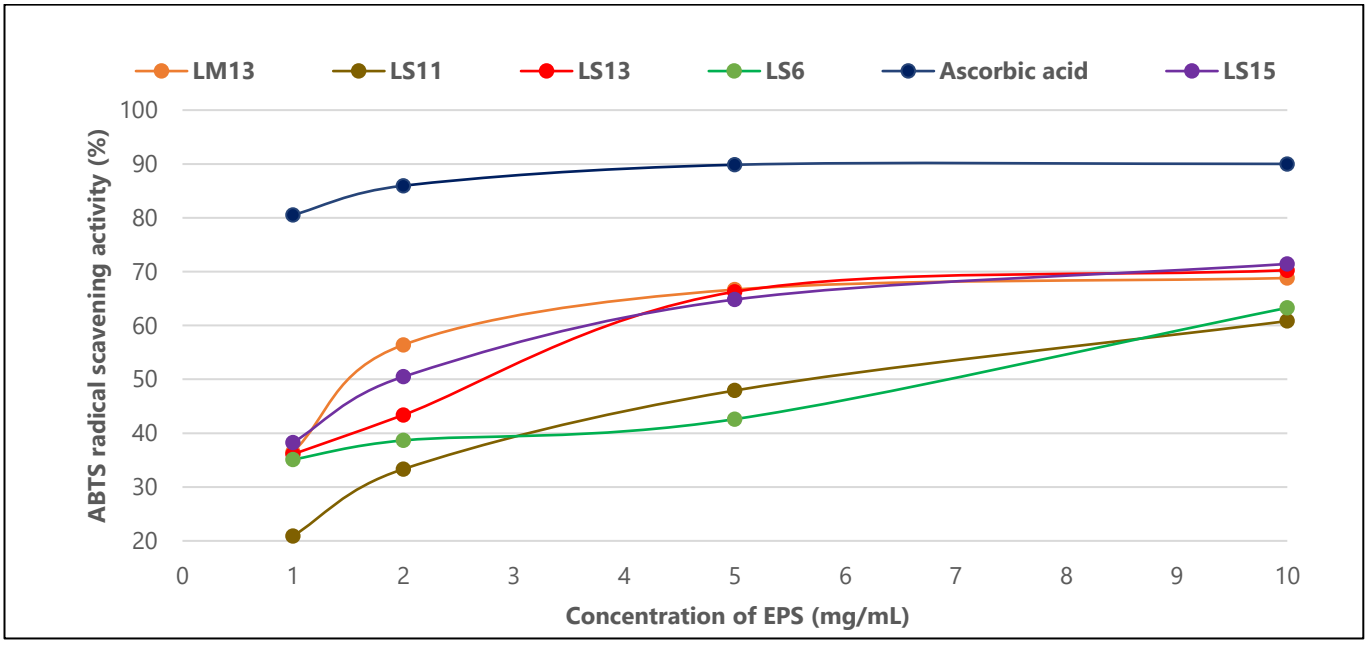

Figure 3. ABTS radical scavenging activity of EPS samples

4. CONCLUSIONS

A total of 9 distinct LAB strains were isolated and identified from the Çökelek samples. Partial characterization of EPSs of 5 distinct isolates were investigated. It was determined that all EPS produced by 5 LABs were in heteropolysaccharide structure containing glucose, galactose and fructose. According to the antioxidant activity test performed on EPS, it was determined that especially *Lactobacillus brevis* LS13 and *Lactobacillus kefiri* LS15 had significant antioxidant activity. In conclusion, it was observed that the EPS producing lactic acid bacteria isolated in this study have significant potentials. These findings clearly reveal that Çökelek isolates have crucial technological properties and future studies will be required to determine their roles under *in situ* and *in vivo* environments.

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