

## Isolation and Characterization of Exopolysaccharide-Producing *Lactobacillus plantarum* SKT109 from Tibet Kefir

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*Lactobacillus plantarum* SKT109 was isolated and identified from Tibet Kefir, and the exopolysaccharide (EPS)-producing properties of the strain were evaluated. Growth of strain SKT109 in a semi-defined medium at 37°C increased the viscosity of the medium, corresponding to production of an EPS (58.66 mg/L). The EPS was isolated and purified, and it was shown to consist of fructose and glucose in an approximate molar ratio of 3:1, with an average molecular weight of  $2.1 \times 10^6$  Da. The aqueous solution of EPS at 1% (w/v) exhibited shear thinning behavior. Microstructural studies of the EPS demonstrated a highly compact structure with a smooth surface, facilitating formation of film by the polymer; the EPS was composed of many different sizes of spherical lumps with tendency to form molecular aggregates. Studies on the milk fermentation characteristics of *L. plantarum* SKT109 showed that the strain survived well in fermented milk with counts about 8.0 log cfu/g during 21 days of storage at 4°C. The use of the EPS-producing strain improved the rheology of the fermented milk without causing post-acidification during storage. Particularly, *L. plantarum* SKT109 improved the fermented milk flavor by increasing the concentration of characteristic flavor compounds and eliminating those with disgusting flavors. The results of the present study indicated that EPS-producing *L. plantarum* SKT109 could serve as a promising candidate for further exploitation in fermented foods.

### INTRODUCTION

Lactic acid bacteria (LAB) are able to produce exopolysaccharides (EPSs) in the surrounding medium as a slime or on the surface of bacterial cells to form a capsule [Ramchandran & Shah, 2009]. The EPS-producing LAB strains have increasingly been used for manufacturing fermented products due to their capability of improving rheology, texture and mouthfeel, and reducing thermal and physical shock and syneresis of the products [Behare *et al.*, 2010; Prasanna *et al.*, 2013; Güler-Akin *et al.*, 2009; Yang *et al.*, 2010]. Since bacterial EPSs are generally of protective nature, production of EPS may increase the resistance of the bacterial cells against unfavorable environmental factors, *e.g.* resistance to high acidity and bile salts [de los Reyes-Gavilán *et al.*, 2011]. EPSs from LAB could promote the colonization of LAB to intestinal mucosa, and thus enhance the immunity of host [Górska *et al.*, 2010]. Bacterial EPSs had the biosorption ability on the trace metal ions [Ye *et al.*, 2014; Wang *et al.*, 2014a]. EPS-producing LAB strains may provide other physiological benefits including antitumor, immunomodulation, lowering cholesterol and antioxidant activities [Li *et al.*, 2014a; Shao *et al.*, 2014; Zhang *et al.*, 2013a; Zhang *et al.*, 2013b].

EPS-producing LAB are typical functional starter cultures due to their contribution to the consistency and rheology of fermented products. Incorporation of EPS-producing LAB in various fermented products, such as fermented milk, cheese and fermented beverage, has become a recent trend [Li *et al.*, 2012a; Lynch *et al.*, 2014]. In recent years, different EPS producing species of LAB have been used in fermented milk to prevent syneresis and to replace stabilizers [Kailasapathy, 2006]. Streptococci [Purohit *et al.*, 2009], lactobacilli [Folkenberg *et al.*, 2006], lactococci [Ayala-Hernández *et al.*, 2009] and bifidobacterium [Prasanna *et al.*, 2013] are some EPS-producing bacterial species which have been successfully used to produce fermented milk with varying improvement of physicochemical and biological properties. The EPS-producing *Lactobacillus plantarum* H2 was shown to increase the viscosity of stirred, fermented milks to a similar extent as the EPS-producing *Streptococcus thermophilus* strain [Salazar *et al.*, 2009]. Another EPS-producing strain of *S. thermophilus* 1275 was capable of improving the consistency, but reducing the firmness and spontaneous whey separation of low-fat yogurt, and the EPS showed a protective effect on the survival of *L. delbrueckii* ssp. *bulgaricus*. [Ramchandran & Shah, 2009]. In addition, *L. delbrueckii* subsp. *bulgaricus* 291 was found to produce an EPS with linear structure that increased the apparent viscosity, firmness and whey retention of fermented milks [Gentès *et al.*, 2011].

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Among EPS-producing LAB, *L. plantarum* exists in a wide range of food niches including fermented vegetable or plant products, and fermented dairy and meat products. Recently, some EPS-producing *L. plantarum* strains isolated from traditional Chinese sauerkraut [Yu *et al.*, 2013; Wang *et al.*, 2014b], Inner Mongolia “Hurood” cheese [Zhang *et al.*, 2014; Zhang *et al.*, 2013b] and Tibet Kefir [Li *et al.*, 2012b; Wang *et al.*, 2010] have been evaluated on their probiotic and functional properties. However, so far few studies have been performed on characterization of EPS-producing *L. plantarum* strains and their use as functional starters in fermented milk manufacturing. Therefore, the present study was carried out to isolate, identify and characterize an EPS-producing *L. plantarum* strain from Tibet Kefir, and the milk fermentation characteristics of the strain were also evaluated.

## MATERIALS AND METHODS

### Isolation of EPS-producing strains

Isolation of LAB strains from 14 Kefir grain samples collected from Tibet of China was performed according to a procedure described by Smitinont *et al.* [1999]. The isolated strains were maintained as frozen ( $-80^{\circ}\text{C}$ ) stocks in MRS broth supplemented with 20% (v/v) glycerol. To screen the strains for EPS quantification, the isolated strains were inoculated (2%, v/v) into 100 mL of sterile semi-defined medium (SDM). The SDM contained (per 100 mL): 1 g of bactocastone (Difco), 0.5 g of yeast nitrogen base (Difco), 0.2 g of ammonium citrate, 0.5 g of sodium acetate, 0.01 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005 g of  $\text{MnSO}_4$ , 0.2 g of  $\text{K}_2\text{HPO}_4$ , 2 g of glucose and 0.1 mL of Tween 80, adjusted to pH 6.6 with 1 mol/L acetic acid [Kimmel & Roberts, 1998].

After incubation at  $37^{\circ}\text{C}$  for 24 h, trichloroacetic acid (TCA) was added to the culture to a final concentration of 4% (w/v), and the mixture was stirred for 30 min at room temperature. Cells and precipitated proteins were removed by centrifugation at  $10,000 \times g$  for 20 min at  $4^{\circ}\text{C}$ , and the supernatant was precipitated with two volumes of chilled ethanol and held at  $4^{\circ}\text{C}$  for 24 h. The precipitated crude EPS was collected by centrifugation at  $10,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ . The pellet was dissolved in deionized water and dialyzed against distilled water at  $4^{\circ}\text{C}$  for 24 h and then lyophilized. The EPS yield (expressed as mg/L) was determined by phenol-sulfuric acid method using glucose as a standard.

### Identification of EPS-producing strain

The EPS-producing strain was primarily identified based on Gram reaction, catalase tests and cell morphology. The strain was further identified to the species level by API 50 CHL test (Bio-Mérieux, France) and 16S rDNA sequencing analysis. Total chromosomal DNA was extracted using a DNA isolation kit (Dingguo Biotech. Ltd., China). Two primers: A27F (5'-AGCGGATCACTTCACACAGGACTACGGC-TACCTTGTTACGA-3') and A1495R (5'-GCAGAGTTCTC-GGAGTCACGAAGAGTTTGATCCTGGCTCAG-3'), were provided by China Agricultural University. DNA fragments were amplified by a Bio-Rad thermal cycler (T100, Singapore) set as follows: initial denaturation at  $95^{\circ}\text{C}$  for 3 min, followed by 30 cycles consisting of denaturation at  $95^{\circ}\text{C}$  for 30 s, an-

nealing at  $55^{\circ}\text{C}$  for 30 s, extension at  $72^{\circ}\text{C}$  for 1 min and final extension step at  $72^{\circ}\text{C}$  for 10 min. PCR product was checked with 1% (w/v) agarose gel electrophoresis, and sequenced (Dingguo Biotech. Ltd., China). The nucleotide sequences were compared with standard strains for the sequence similarity through BLAST (<http://www.ncbi.nlm.nih.gov/blast>).

### Analysis of bacterial growth and EPS production

The EPS-producing strain was inoculated in 2000-mL Erlenmeyer flasks containing 1000 mL of the SDM broth as mentioned above and incubated at  $37^{\circ}\text{C}$ . Samples (50 mL) were withdrawn at different time intervals from 0 to 56 h. The pH was determined with a pH meter (FE20, Mettler Toledo, Switzerland). The bacterial growth was determined by dilution plating with MRS agar medium incubated at  $37^{\circ}\text{C}$  for 48 h. EPS yield was estimated by phenol-sulfuric acid method using glucose as a standard. The viscosity was determined by a Brookfield DV-III viscometer (Brookfield Engineering Lab Inc., Stoughton, MA) with a SC4-18 spindle at 80 rpm/min. The viscosity measurement was over a continuous period of 2 min required to collect 50 data points, and the average value of the 50 data points was regarded as the viscosity of the sample.

### Purification of EPS

The crude EPS solution (20 mg/mL, 5 mL) was fractionated with an anion exchange chromatography on a DEAE-cellulose column ( $26 \times 40$  cm), eluted with deionized water, subsequently with 0.2 and 0.5 mol/L NaCl solutions at a flow rate of 1 mL/min. Fractions (5 mL) were collected automatically and the carbohydrate content was determined by phenol-sulfuric acid method. The fractions containing polysaccharides were pooled, dialyzed and lyophilized for further purification by gel permeation chromatography on a Sepharose CL-6B column ( $25 \times 50$  cm). Elution was done with 0.9% (w/v) NaCl at a flow rate of 0.5 mL/min, and the fractions containing the EPS were pooled, dialyzed and lyophilized to obtain the purified EPS sample.

The purity of the purified EPS sample was checked by Ultraviolet-visible (UV-vis) spectroscopy using a UV-vis spectrophotometer (U-3900, Hitachi Ltd., Japan). The EPS solution was prepared by suspending the sample in distilled water for UV-vis measurement in the wave-length range of 190–550 nm.

### Physicochemical characterization of EPS

Number and weight average molecular weights ( $M_n$  and  $M_w$ , respectively), as well as the polydispersity index ( $M_w/M_n$ ) of the purified EPS were obtained by gel-permeation chromatography (GPC). The GPC system consisted of a Shodex SB-806m-HQ  $13 \mu\text{m}$ ,  $300 \times 8.0$  mm column, connected with a SB-G  $10 \mu\text{m}$ ,  $50 \times 6.0$  mm guard column. The EPS were detected using a refractive index detector (RI) (Optilab Wyatt, USA) and a multi angle laser-light scattering detector (MALLS) (DAWN HELEOS-II Wyatt, USA), at an internal temperature of  $40^{\circ}\text{C}$ . The column was eluted with 0.1 mol/L  $\text{NaNO}_3$  solution at a flow rate of 0.5 mL/min, and the injection volume of sample was 200  $\mu\text{L}$ , and  $dn/dc$  of 0.147 as a refractive index increment was used for polysac-

charides solution. Data processing were performed with Wyatt Astra software (Version 5.3.4.14, Wyatt Technology, USA).

For monosaccharide analysis, the purified EPS (5 mg) was hydrolyzed with 2 mL of 2 mol/L trifluoroacetic acid (TFA) at 120°C for 2 h. After hydrolysis, TFA in the sample was removed by decompressing evaporation. Methanol was added into the dry sample and evaporated by decompression. The hydrolysate was then subjected to aldonitrile acetate precolumn-derivatization gas chromatography (GC) for determination of the monosaccharide composition. GC was performed on an Agilent 7890A GC fitted with a flame ionization detector (FID) and a DB-WAX column (30 m length  $\times$  0.25 mm inner diameter  $\times$  0.25  $\mu$ m film thickness; J & W Scientific, Folsom, CA). The carrier gas used was ultra-high purity helium with a flow rate of 1.0 mL/min. The temperature program was set as follows: starting at 100°C for 5 min, increasing at 5°C/min to 150°C and holding for 5 min, then increasing at 5°C/min to 240°C and holding for 2 min. The temperatures of the injector and detector were 250°C. The injection volume was 1 mL. Sugar identification was done by comparison with standard rhamnose, arabinose, galactose, glucose, mannose and fructose.

The rheological behavior of the purified EPS was studied in a Brookfield DV-III Ultra programmable rheometer (Brookfield Engineering Laboratories Inc., Stoughton, Massachusetts, USA) with a No. SC4–18 spindle that rotated in chamber equipped with temperature control system. For this, the lyophilized EPS or xanthan gum was dissolved in deionized water at 1% (w/v). The experiments were carried out at 20°C by measuring the viscosity as a function of shear rate from 10 to 300 s<sup>-1</sup>.

The microcosmic morphology of the purified EPS was analyzed by observation with scanning electron microscopy (SEM). The lyophilized samples of the purified EPS (5 mg) were fixed to the SEM stubs with double sided tape, then coated with a layer of gold, – 10 nm thick. The samples were observed in a scanning electron microscope (S-4800, Hitachi Ltd., Japan) at an accelerating voltage of 3.0 kV.

The surface topography of the purified EPS was determined using atomic force microscopy (AFM) (Bruker Instruments Co., Germany) in tapping mode. A stock solution (1 mg/mL) was prepared by adding the purified EPS into deionized H<sub>2</sub>O. The aqueous solution was stirred for about 1 h at 40°C in a sealed bottle under N<sub>2</sub> stream so that EPS dissolved completely. After cooling to room temperature, the solution was continuously diluted to the final concentration of 0.01 mg/mL. About 5  $\mu$ L of diluted EPS solution was dropped on the surface of a mica sample carrier, and allowed to dry at a room temperature.

### Milk fermentation characteristics

Raw milk (protein 3.1%, fat 3.5%) was heated at 95°C for 10 min, followed by cooling to 42°C using a cold water bath. Two types of fermented milk were prepared: a) control fermented milk, inoculated with 0.002% (w/v) YO-MIX commercial fermented milk starter culture (*Streptococcus thermophilus* and *L. delbrueckii* subsp. *bulgaricus*); b) fermented milk inoculated with 0.002% (w/v) of YO-MIX starter culture together with 2% (w/v) of the adjunct culture containing the EPS-

producing strain (7 log cfu/mL), prepared by subculturing of the strain for 3 times in milk before use. All the samples were incubated at 42°C to reach final pH of 4.5 and immediately cooled, stored at 4°C for 12 h to finish maturation, and further stored at 4°C for 21 days.

### Changes in EPS content, pH, viable cell counts and water holding capacity during storage of fermented milk

The EPS content of fermented milk was estimated by phenol-sulfuric acid method using glucose as a standard. The pH of fermented milk was measured using a pH meter (FE20 pH meter, Mettler Toledo, Switzerland) at room temperature. Viability of the EPS-producing strain in fermented milk was determined using a spread plate technique. One gram of each fermented milk sample was serially diluted in 9 mL of 0.09% (w/v) sterile saline solution. The EPS-producing strain (*L. plantarum*) was enumerated on selective media under aerobic incubation at 37°C for 72 h [Bujalance *et al.*, 2006]. The water-holding capacity (WHC) of fermented milk was determined as described by Doleyres *et al.* [2005] with slight modifications. The fermented milk sample (15 g) was centrifuged at 4000 $\times$ g for 10 min at 4°C. The whey expelled (WE) was removed and weighed. The WHC was expressed as follows:

$$\text{WHC (\%)} = (m_1/m_2) \times 100.$$

where  $m_1$  is the mass of precipitate after centrifugation (g) and  $m_2$  is the mass of the fermented milk sample (g).

### Rheological analysis of fermented milk

The rheological behavior of the fermented milk at 21 d was studied with an AR-1500ex rheometer (AR-1500ex, TA Instruments Ltd., USA). All measurements were carried out at 25°C. The fermented milk sample was subjected to a frequency sweep, and the frequency was varied from 0.1 to 10 Hz at a 0.1% strain. The elastic modulus ( $G'$ ) and storage modulus ( $G''$ ) were recorded as a function of frequency.

### Analysis of volatile compounds in fermented milk

Fermented milk samples (5 mL) were placed in 20 mL SPME vials and then 1 g NaCl was added. The mixtures were stirred for 20 min at 40°C to accelerate the equilibrium of headspace volatile compounds. Then, volatile compounds were extracted by placing a single 1 cm  $\times$  50/30  $\mu$ m divinylbenzene/carboxen/polydimethylsiloxane SPME fibre (Supelco, Bellefonte, PA) into the vial and exposing it to the headspace above the samples for 30 min at 40°C. The analysis of absorbed volatile compounds was performed on a GC 7890A gas chromatograph coupled to a Triple Quad 7000B MS (both Agilent, Palo Alto, CA, USA). Separations in GC were performed on DB-WAX (30 m length  $\times$  0.25 mm inner diameter  $\times$  0.25  $\mu$ m film thickness; J & W Scientific, Folsom, CA). The carrier gas used was ultra-high purity helium with a flow rate of 1.2 mL/min. The oven temperature was programmed at 40°C for 3 min, increased at 5°C/min to 230°C, then increased at 15°C/min to 260°C and held for 3 min. The temperatures of the injector and the GC-MS transfer line were 250°C and 280°C, respectively. Electron-impact mass spectra were generated at 70 eV, with an m/z scan range from

35 to 550 amu. The ion source temperature was 230°C. Compounds were identified according to NIST 2.0 mass spectra libraries installed in the GC-MS equipment.

### Statistical analysis

The results are presented as the mean  $\pm$  SD and were analyzed by one-way analysis of variance (ANOVA) using SPSS version 16.0. The values of  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$  were used to identify statistically significant differences. All measurements were performed in triplicate.

## RESULTS AND DISCUSSION

### Isolation and identification of strain SKT109

Total 53 bacterial strains were isolated from Tibet Kefir, and they were initially identified as lactic acid bacteria based on their Gram-positive, catalase-negative properties, and on the morphology of colonies that appeared to be milky, smooth and rosy. Among these strains, twelve were found to produce EPSs, and one of the strains, namely SKT109, produced EPS with a yield of 58.66 mg/L in SDM medium. Identification of strain SKT109 by API 50 CHL showed a fermentation profile with 99.7% identity to that of *L. plantarum* species. Further identification by analysis of 16S rDNA gene sequence similarity through BLAST showed that strain SKT109 gave 99% similarity with *L. plantarum* M10-1, *L. plantarum* WCFS1, *L. plantarum* AP1, *L. plantarum* MP-10, *L. plantarum* KW30 and *L. plantarum* JDM1. So the strain was named as *L. plantarum* SKT109, with the Genebank access number of KJ764641.

### Production and purification of EPS

The time courses of viable counts, pH, viscosity and accumulation of EPS during the growth of *L. plantarum* SKT109 in SDM medium at 37°C are shown in Figure 1.

Strain SKT109 exhibited a fast growth after incubation for 8 h, and reached a maximal viable cell count of 9.75 log cfu/mL at the beginning of the stationary phase (16 h), with a sharp decrease in the late stationary phase to about 7.10 log cfu/mL at 56 h. Correspondingly, there was a rapid decrease in pH to about 4.10 during the first 16 h of growth, then gradually lowering to about pH 3.80 at 56 h. Growth of strain SKT109 in SDM medium also increased the viscosity of the medium up to 15.84 cp at 16 h, and simultaneously there was production of EPS with a maximal amount of 58.66 mg/L at 16 h. During the late stationary phase, EPS production (about 50 mg/L) by strain SKT109 and the viscosity of the medium (about 13 cp) were relatively stable, indicating no obvious degradation of the EPS molecules. However, there were reports about decreased EPS production during stationary phase of growth by different LAB strains in different media, e.g. *L. plantarum* C88 in SDM media [Zhang *et al.*, 2013b], *L. plantarum* 70810 in MRS-glucose media [Wang *et al.*, 2014b], and *L. helveticus* MB2-1 in whey media [Li *et al.*, 2014b]. This decrease in EPS production after prolonged incubation was attributed to the possible presence of glycohydrolases in the culture that catalyzed the degradation of polysaccharides [Degeest *et al.*, 2002].

The crude EPS obtained from the treatment with TCA and subsequently with cold ethanol of the growth medium supernatant of *L. plantarum* SKT109 was fractionated on an anion-exchange chromatography column of DEAE-52 cellulose (Figure 2A). One major peak was eluted with distilled water, and the corresponding fractions containing the neutral polysaccharide were collected for further purification by Sepharose CL-6B gel permeation chromatography (Figure 2B). The fractions corresponding to the single elution peak of the EPS were collected, concentrated, dialyzed and lyophilized to obtain the purified form of EPS, which was used for further physicochemical analysis.

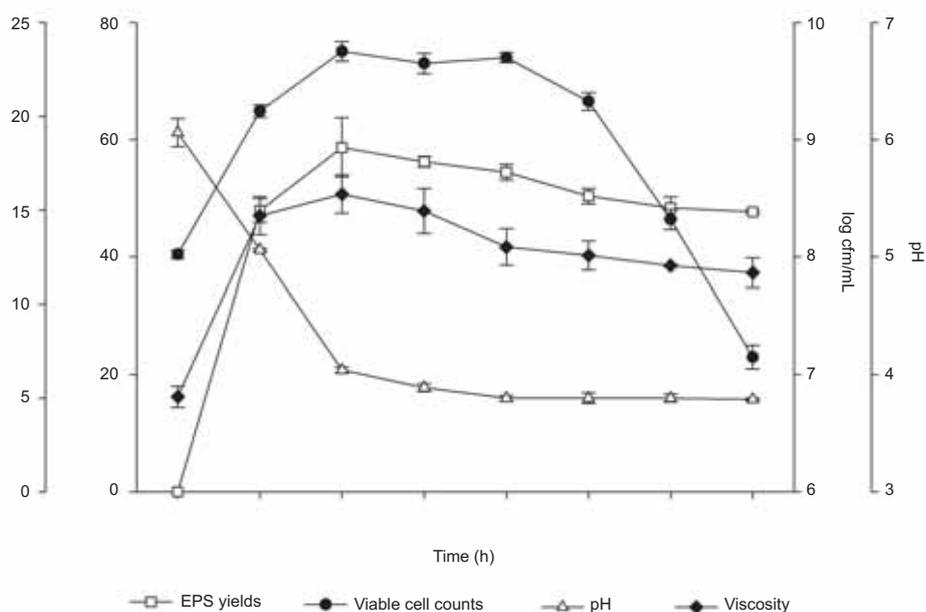


FIGURE 1. EPS yield, pH, viable counts and viscosity by *L. plantarum* SKT109 in SDM batch cultures at 37°C over time from 0 to 56 h. Data are presented as means  $\pm$  SD of triplicates.

TABLE 1. Volatile compounds of the fermented milk made with EPS-producing *L. plantarum* SKT109 at 1 d of storage as compared to those of the control fermented milk.

No.	Compounds	Retention time (min)	RI <sup>1</sup>	Area <sup>2</sup> (%)		ANOVA
				SKT109 fermented milk	Control fermented milk	
1	Acetone	4.239	829	9.53±0.06 <sup>a</sup>	8.83±0.63 <sup>a</sup>	NS <sup>4</sup>
2	Ethyl acetate	5.304	882	15.53±0.51 <sup>a</sup>	13.78±0.44 <sup>b</sup>	**
3	Acetic acid ethenyl ester	7.237	998	18.65±0.94 <sup>a</sup>	17.02±0.20 <sup>b</sup>	*
4	2,3-Pentanedione	9.411	1056	8.66±0.84 <sup>a</sup>	15.17±0.83 <sup>b</sup>	***
5	Dimethyl disulfide	9.824	1092	3.72±0.48 <sup>a</sup>	7.47±0.11 <sup>b</sup>	***
6	Hexanal	10.088	1024	ND <sup>3</sup>	0.62±0.08	
7	2-Heptanone	13.035	1180	1.70±0.63 <sup>a</sup>	1.25±0.03 <sup>a</sup>	NS
8	Dimethyl sulfone	13.473	1216	ND	0.62±0.03	
9	3-methyl-1-Butanol	13.965	1206	2.69±0.85 <sup>a</sup>	2.97±0.08 <sup>a</sup>	NS
10	Heptanal	15.148	1183	ND	0.72±0.01	
11	1-Pentanol	15.173	1256	1.13±0.23	ND	
12	3-hydroxy-2-Butanone	16.238	1286	13.04±0.84 <sup>a</sup>	9.34±0.22 <sup>b</sup>	***
13	1-Hexanol	18.047	1345	0.25±0.40 <sup>a</sup>	0.57±0.01 <sup>b</sup>	NS
14	Dimethyl trisulfide	18.905	1410	ND	0.62±0.04	
15	Nonanal	19.107	1392	0.19±0.30 <sup>a</sup>	0.12±0.07 <sup>a</sup>	NS
16	2-Nonanone	19.122	1386	0.27±0.02	ND	
17	Acetic acid	20.839	1435	5.98±0.48 <sup>a</sup>	6.12±0.62 <sup>a</sup>	NS
18	2-ethyl-1-Hexanol	21.668	1494	1.03±0.01	ND	
19	Benzaldehyde	22.664	1515	0.60±0.01 <sup>a</sup>	0.61±0.03 <sup>a</sup>	NS
20	Butanoic acid	25.469	1630	11.18±0.95 <sup>a</sup>	5.41±0.65 <sup>b</sup>	***
21	Hexanoic acid	30.579	1833	5.51±0.92 <sup>a</sup>	8.10±0.36 <sup>b</sup>	**
22	Phenylethyl alcohol	31.612	1903	0.33±0.02 <sup>a</sup>	0.23±0.02 <sup>b</sup>	**
23	Octanoic acid	35.221	2075	ND	0.41±0.08	

<sup>1</sup> Retention index; <sup>2</sup> Means and standard deviations of the identified compounds expressed as percentage of peak areas from triplicates; <sup>3</sup> ND, not detected; <sup>4</sup> Means in the same row followed by the same letter are not significantly different ( $P>0.05$ ); <sup>5</sup> NS, not significant; \*  $P<0.05$ ; \*\*  $P<0.01$ ; \*\*\*  $P<0.001$ .

The UV-vis analysis of the EPS shows no absorption at 260 nm or 280 nm, indicating no protein and nucleic acids present in the purified EPS sample.

### EPS Characterization

The molecular weight of the EPS produced by strain SKT109 was estimated to be  $2.1 \times 10^6$  Da. The polydispersity index of the EPS was 1.205, which indicated a rather narrow molecular weight distribution of the EPS, and thus a homogeneous EPS material in the sample. Determination of molecular weight of EPS was considered very important for understanding potential structure-function relationship of EPS in fermented milk [Vaningelgem et al., 2004b]. High molecular weight of EPS contributed to limiting the susceptibility to syneresis and improving the texture of fermented milk by cross-linking of the EPS into a network structure with the proteins during

fermentation [Hassan et al., 2003]. Although different molecular weights of EPSs produced by different *L. plantarum* strains have been reported, they are generally between  $10^5$  and  $10^6$  Da. For example, the molecular weights of the EPSs from *L. plantarum* 70810 [Wang et al., 2014b], *L. plantarum* EP56 [Tallon et al., 2003], and *L. plantarum* C88 [Zhang et al., 2013b] were  $1.70 \times 10^5$  Da,  $8.5 \times 10^5$  Da, and  $1.15 \times 10^6$  Da, respectively. In comparison, the EPSs produced by *S. thermophilus* ST 111 ( $> 5000$  kDa) and *L. delbrueckii* subsp. *bulgaricus* NCFB2074 ( $> 1800$  kDa) had higher molecular weights [Vaningelgem et al., 2004a; Harding et al., 2005]. The difference in molecular weights of the EPSs could be attributed to the different producing strains, the culture media, and the growth conditions used [Salazar et al., 2009].

Monosaccharide analysis indicated that the EPS from strain SKT109 consisted of fructose and glucose in an ap-

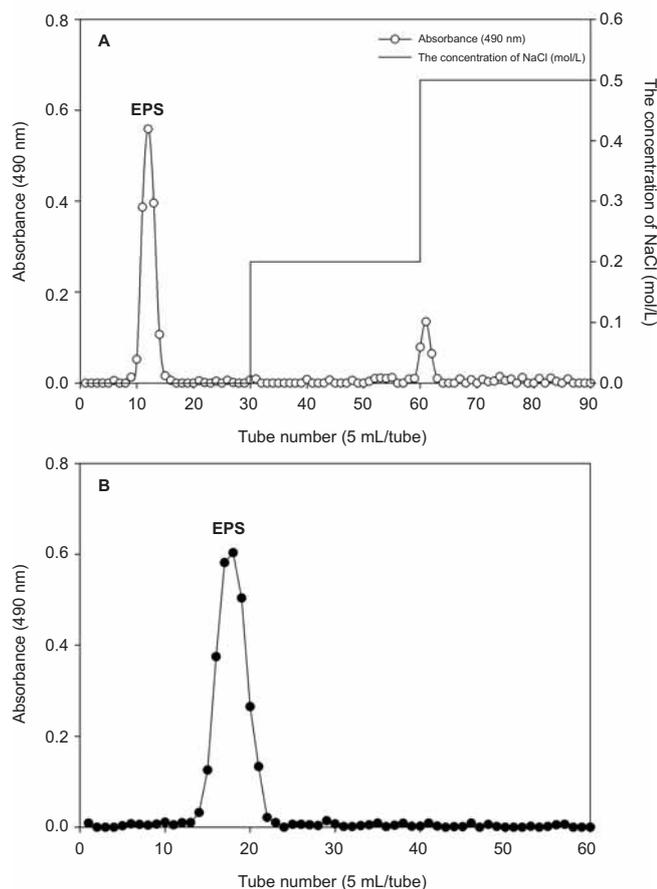


FIGURE 2. Anion-exchange chromatography of crude EPS from *L. plantarum* SKT109 (A) on DEAE-cellulose column chromatography and further gel permeation chromatography (B) on Sepharose CL-6B.

proximate molar ratio of 3:1. This was different from those reported earlier specially fructose was seldom found in the EPSs produced by *L. plantarum* strains. For example, *L. plantarum* EP56 produced an EPS consisting of glucose, galactose and N-acetylgalactosamine [Tallon *et al.*, 2003]; the EPS from *L. plantarum* C88 was composed of galactose and glucose [Zhang *et al.*, 2013b]; *L. plantarum* 70810 produced two EPSs, both containing glucose, mannose and galactose with different molar ratios [Li *et al.*, 2014c]; three monomers such as mannose, glucose and galactose were found in the EPS produced by *L. plantarum* KF5 [Wang *et al.*, 2010]. The commonly used yogurt strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were found to produce EPSs mainly composed of glucose and galactose, and sometimes rhamnose [Qin *et al.*, 2011; Mende *et al.*, 2014; Faber *et al.*, 2001; Lamothe *et al.*, 2002; ]. *L. delbrueckii* subsp. *bulgaricus* rr produced an EPS containing a branched heptasaccharide repeating unit of galactose, glucose and rhamnose in a molar ratio of 5:1:1 [Gruter *et al.*, 1993].

Rheological studies (Figure 3) of the purified EPS and xanthan gum aqueous solutions (1%, w/v) showed that both the EPS and xanthan gum solutions exhibited a shear thinning behavior, a rapid decrease in viscosity with increasing shear rates from 0 to 50 s<sup>-1</sup>, and subsequently a gentle decrease in viscosity with further increase in shear rates up to 300 s<sup>-1</sup>. The shear thinning behavior of EPS, which was

caused mainly by breakdown of structural units in the EPS by hydrodynamic shear forces, was considered to be important for yielding desired sensory properties such as mouth-feel and flavor release properties, as well as some processing operations such as stirring, pouring, pumping, spray drying [Zhou *et al.*, 2014]. In addition, the xanthan gum solution showed higher viscosity and the prominent shear thinning property than the EPS solution over the whole shear rate range, which may be due to the higher molecular weight for xanthan gum (Mw ~2 × 10<sup>6</sup> to 5 × 10<sup>7</sup> Da, Sigma) [Papagianni *et al.*, 2001] compared to the EPS from strain SKT109 (Mw ~2.1 × 10<sup>6</sup> Da).

SEM was considered to be a powerful tool to study the morphological features of polysaccharides and could be used to elucidate their physical properties [Wang *et al.*, 2010]. Observation by SEM of the EPS from strain SKT109 (Figure 4A) revealed a compact structure composed of similar size of cubes. At a higher magnitude (Figure 4B, 5000 ×), additional details of the microstructure of the EPS were visible. The EPS from strain SKT109 had a smooth cube surface, which was different from previous observations of other EPSs produced by LAB with the sheet-like or porous web-like structures [Wang *et al.*, 2010; Prasanna *et al.*, 2012]. Therefore, the EPS from strain SKT109 may be potentially used for making plasticized films due to its highly compact structure, an important property of the material used to make such kind of films [Wang *et al.*, 2010]. Additionally, the SEM scan showed that the EPS from strain SKT109 was made of homogeneous matrix, indicating the structural integrity especially important in film making [Ahmed *et al.*, 2013].

AFM could be employed as a powerful tool to characterize the force-induced conformational transitions, the dynamics, and super molecular structures of polysaccharides at the molecular level [Ahmed *et al.*, 2013]. Although this technique was less used in studies of LAB EPSs, the present study demonstrated the topographical AFM images of the EPS from strain SKT109 as shown in Figure 4C and 4D. There was presence of many spherical lumps, ranging from 10 nm

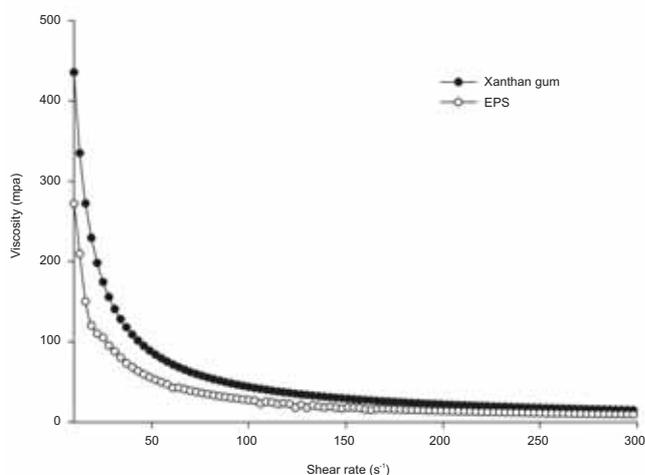


FIGURE 3. Viscosity as a function of shear rate (from 10 to 300 s<sup>-1</sup>) of aqueous solutions of the EPS from *L. plantarum* SKT109 and xanthan gum, at concentrations of 1% (w/v).

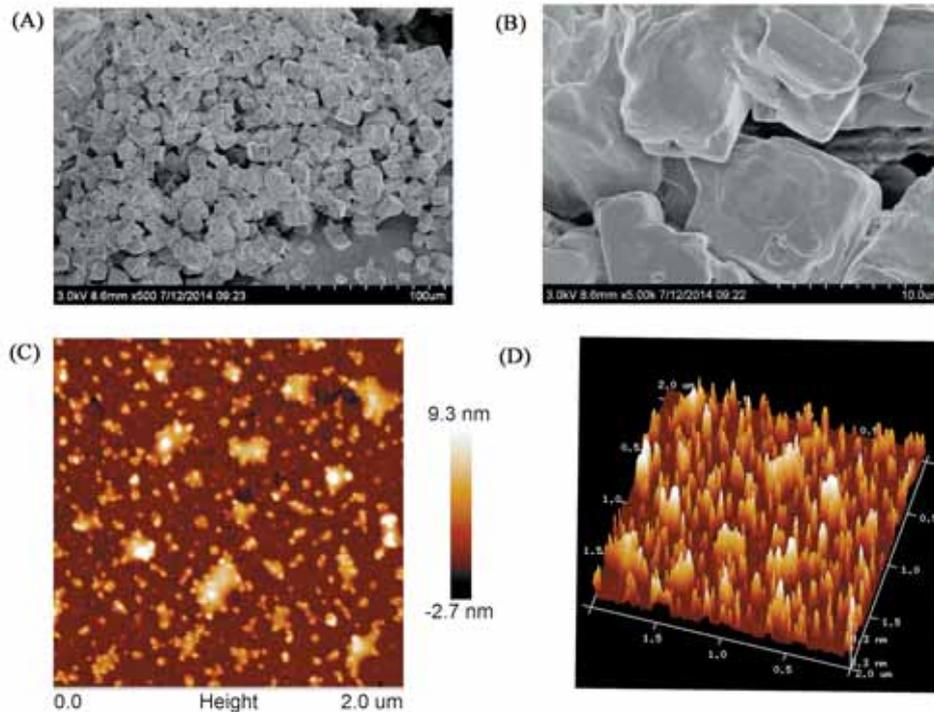


FIGURE 4. SEM images (A: 500  $\times$ ; B: 5000  $\times$ ) and AFM images (C: planar; D: cubic) of the purified EPS.

to 50 nm of diameter, and the height of the lumps ranged from 1 nm to 9.3 nm. The heights of the spherical structures of the EPS were much higher than that of a single polysaccharide chain (about 0.1–1 nm), suggesting that inter- and/or intra-molecular aggregation might be involved. Similar structural property was also observed earlier with another EPS from *Bifidobacterium animalis* RH that had spherical lumps and a ring-like network structure [Shang *et al.*, 2013]. This aggregation property of the polysaccharide molecules may be beneficial to improving the viscous behavior of the EPS from strain SKT109 when used in fermented milk.

#### Microbiological and physicochemical analyses of fermented milk

*L. plantarum* SKT109 was found to produce EPS in fermented milk with yield of about 40 mg/L, and it showed good survival and maintained at relatively stable counts ( $\sim 8.0$  log cfu/g) during the storage at 4°C for 21 days (Figure 5A). Previously, EPS-producing *L. plantarum* strains were also found to survive better than the EPS-nonproducing strains during refrigerated storage of fermented milk [Brinques & Ayub, 2011]. The good viability of strain SKT109 in fermented milk might benefit from its production of EPS that was generally of protective nature.

Figure 5A also shows that the use of strain SKT109 resulted in only slightly decreased (about 0.02) pH of the fermented milk as compared to the control, indicating that strain SKT109 did not cause post-acidification of fermented milk during the storage period. Similarly, no significant changes in pH of fermented milk containing *L. plantarum* RL19 and RL29 strains were observed during 21 days of storage at 4°C [Georgieva *et al.*, 2009]. *L. casei* AST18 exerted no significant influence on the pH of fermented milk,

and the post-acidification effect of *L. casei* AST18 was low [Li *et al.*, 2013]. However, use of several other LAB strains in fermented milk caused significantly decreased pH during cold storage of the products [Maragkoudakis *et al.*, 2006; Casarotti *et al.*, 2014; Mirlohi *et al.*, 2014]. These differences could be attributed to the different types and combination of starters used for fermented milk making by these researchers. As an important physicochemical parameter for fermented milk, WHC can directly influence the texture of final product. Figure 5A indicates that the fermented milk made with strain SKT109 had significantly higher ( $P < 0.05$ ) WHC than the control fermented milk during the whole period of storage, probably due to the formation of three-dimensional (3D) network structure resulting from EPS and protein interactions that increased moisture retention and decreased the syneresis of fermented milk [Prasanna *et al.*, 2013].

Frequency sweep (from 0.1 to 10 Hz) experiments of the control and the fermented milk sample containing the strain SKT109 (Figure 5B) at 21 d of storage gave almost straight lines with increasing frequencies, with the values of the elastic modulus ( $G'$ ) being higher than those of the viscous modulus ( $G''$ ).  $G'$  of the fermented milk sample containing the strain SKT109 was higher than the control, indicating more solid-like behavior of the fermented milk sample of the former. Similarly high  $G'$  values leading to more solid-like (elastic) properties of fermented milk were also reported when using other EPS-producing strains [Purohit *et al.*, 2009].

#### Volatile analysis of fermented milk

Table 1 shows the volatile compounds detected in the fermented milk sample made with EPS-producing strain SKT109 as compared to the control sample, revealing a large

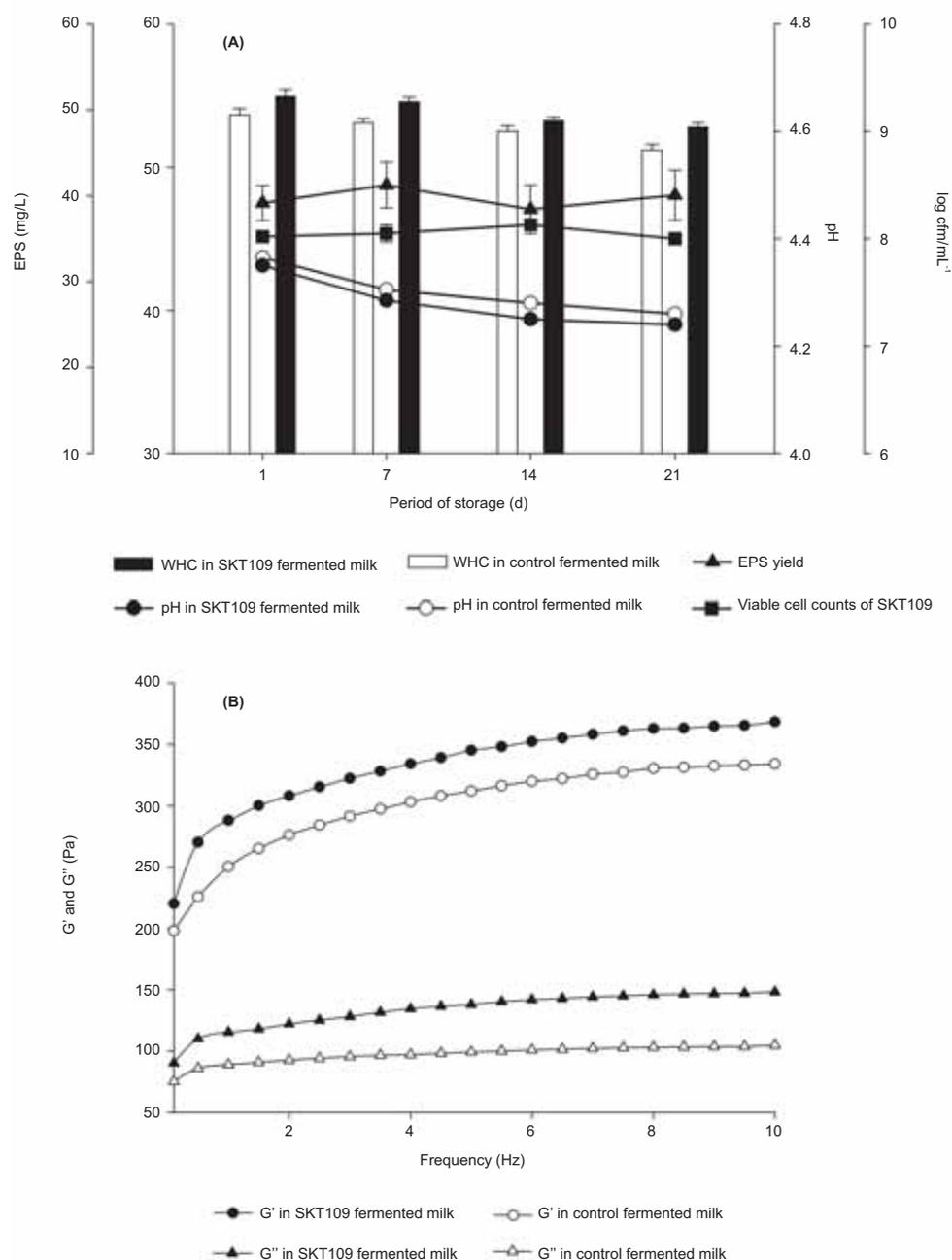


FIGURE 5. Changes of pH, viable cell counts, WHC and EPS yield in fermented milk during storage at 4 °C for 21 days (A). Elastic modulus ( $G'$ ) and storage modulus ( $G''$ ) of fermented milk at 21 d of storage (B). Data are presented as means  $\pm$  SD of triplicates.

difference between the two samples. There were total 18 volatile compounds identified in the fermented milk made with strain SKT109 (Figure 6A), including 2 esters (34.18%), 5 ketones (33.20%), 3 acids (22.67%), 5 alcohols (5.43%), 1 sulfur compound (3.72%) and 2 aldehydes (0.79%), while 20 compounds were identified in the control fermented milk (Figure 6B), including 4 ketones (34.59%), 2 esters (30.80%), 4 acids (20.04%), 3 sulfur compound (8.71%), 3 alcohols (3.77%) and 4 aldehydes (2.07%). Several volatile compounds such as ethyl acetate, acetic acid ethenyl ester and butanoic acid with fruity, sweet and cheesy flavors had higher concentrations in the fermented milk sample with strain SKT109 than in the control fermented milk. One characteristic flavor compound in fermented milk, 3-hydroxy-butanone with good

smell of fragrance, also had a higher concentration (13.04%) in the fermented milk made with EPS-producing strain SKT109 than that (9.34%) in the control. Moreover, some compounds such as 1-pentanol, 2-nonanone and 2-ethyl-1-hexanol were present in the fermented milk made with strain SKT109, but not in the control. Other compounds with disgusting flavors such as dimethyl sulfone, dimethyl trisulfide and octanoic acid had low concentrations in the control fermented milk, but they were not detected in the fermented milk made with strain SKT109. These results suggested that the addition of EPS-producing *L. plantarum* SKT109 strain could improve fermented milk flavor. Li *et al.* [2014c] also reported that soymilk fermented with EPS-producing *L. plantarum* 70810 had more characteristic flavor compounds

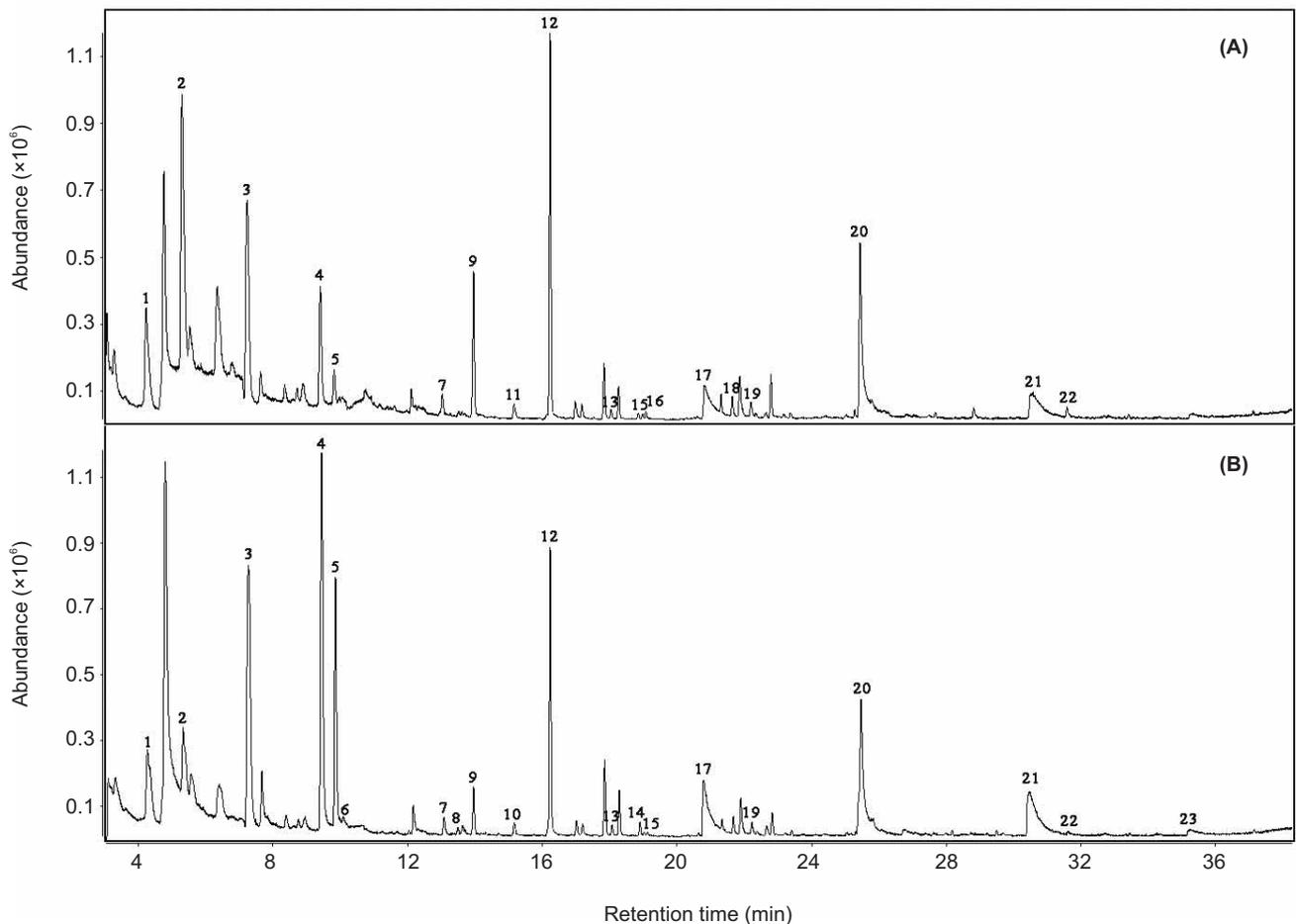


FIGURE 6. Total ion chromatogram of volatile compounds in SKT109 fermented milk (A) and control fermented milk (B). Peak numbers correspond to Table 1.

and lower beany flavor compounds than those in the original soymilk base. The EPS-producing *Lactobacillus* strains were not found to produce excessive amount of disgusting flavor compounds when used in fermented milks [Salazar *et al.*, 2009]. Additionally, fermented milk fermented with EPS-producing *S. thermophilus* 05-34 exhibited significantly higher scores on sensory property than the fermented milk containing non-EPS-producing *S. thermophilus* 05-32 [Qin *et al.*, 2011]. Taken together, EPS-producing LAB strains, including those from *L. plantarum* species, may be used to improve the flavor of fermented products.

## CONCLUSION

*L. plantarum* SKT109 was isolated and identified from Tibet Kefir. It produced an EPS consisting of fructose and glucose in an approximate ratio of 3:1, with a molecular mass of  $2.1 \times 10^6$  Da. Microstructural characterization of the purified EPS indicated that the EPS had a highly compact structure with smooth cube surface, facilitating formation of films; the EPS was composed of many spherical lumps with different diameters and heights, suggesting good ability of molecular aggregation. Furthermore, *L. plantarum* SKT109 survived well in fermented milk, improved the rheological properties of the product, but did not cause pot-acidification during

storage. Particularly, the use of the EPS-producing strain improved the fermented milk flavor by increasing the concentration of characteristic flavor compounds and eliminating those with disgusting flavors. Future studies will be focused on further characterization of the EPS structure, its biological functions and possible roles in determining the potential probiotic properties of the strain in question.

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