

Structural Elucidation and Antioxidant Activities of Exopolysaccharide from *L. helveticus* SMN2-1

Lijuan Bai^{a, b}, Long Wang^a, Shujuan Ji^{*a}

^a Department of Food Science, Shenyang Agricultural University, Shenyang 110866, P.R.China;

^b College of Food Science and Engineering, Jinzhou Medical University, Jinzhou 121001, P.R.China.

jsjsyau@sina.com

Koumiss is a traditional fermented dairy product and known for its unique physiological actions. An attempt was made to purify exopolysaccharide produced by LAB in Koumiss and antioxidant activity of the exopolysaccharide. In total, 55 EPS-producing strains were isolated from 12 koumiss samples and 3 strains with ability of higher EPS-producing were identified by 16S rRNA gene sequence as *Lactobacillus Helveticus*, *Enterococcus duran* and *Leuconostoc lactis* respectively. The structural analysis of purified EPS-I produced by *Lactobacillus Helveticus* SMN2-1 was performed by FTIR analysis. The EPS as important extracellular bioactive molecules had a molecular mass of 8.5×10^5 Da and good antioxidant activity with high hydroxyl and superoxide radicals scavenging abilities. The diversity of lactic acid bacteria in koumiss and structure and antioxidant activity of exopolysaccharide from *L. helveticus* SMN2-1 were verified that might be contributed to koumiss for its physiological actions.

1 Introduction

Koumiss, a traditional fermented dairy product is developed and known for the diversity of lactic acid bacteria (LAB) and its unique physiological actions, such as beneficial health effects on cholesterol levels, immune response, and treatment of some diseases (Sari and Donmez, 2014). In recent years, lactic acid bacteria (LAB) have received increased attention because of their ability to secrete extracellular polysaccharides and exopolysaccharides (EPS) that are naturally produced during the fermentation process (Blanco-Rodriguez et al., 2016). EPS is extracellularly synthesized by cell wall-anchored enzymes and can be secreted into the external environment. Furthermore, the quantity and structure of produced EPS varies by bacterial strain. EPS produced by LAB are of great technological interest because of their role in modulating the rheological and physical properties to improve texture, stability, and mouth-feel of fermented milk products. Furthermore, some EPS have shown beneficial bioactivities on human health, including prebiotics, antitumor activity (Wang et al., 2015) and antioxidant activity (Abdhu et al., 2014).

There are some reports on EPS-producing LAB from traditional milk products. However, few reports exist on EPS from the koumiss. Isolation and identification of the EPS-producing LAB and biological activity of exopolysaccharide in koumiss will yield valuable knowledge, such as to improve quality (Behare et al, 2013) and reveal the physiological function of koumiss (Donmez et al., 2014). We evaluated EPS-producing LAB genera from koumiss and explored the EPS-producing capacity of these isolates. The EPS was separated, purified and its structure and antioxidant activity were analysed.

2. Materials and methods

2.1 Sample collection and enumeration of microorganisms

12 samples of koumiss were collected from different households in Eastern Inner Mongolia and different colonies with distinct morphologies (such as size, shape, and colour) were selected. Gram-positive, catalase-negative isolates stored at -80°C were purified and identified by physiological and biochemical properties (Grosu-Tudor et al., 2013).

2.2 16S rRNA sequence identification

The bacterial isolates were identified by 16S rRNA sequencing analysis. Genomic DNA was extracted from adding each isolate into Takara Lysis buffer for Microorganism to Direct PCR (code no.9164) 85°C, 15 min. The 16S rRNA gene was amplified using the Takara 16s rDNA Bacterial Identification PCR Kit: 16S-FA: 5'-GAGCGGATAACAATTTTCACACAGG-3'; 16S-RE: 5'-CGCCAGGGTTTTCCCAGTCACGAC-3' Takara Mini BEST Agarose Gel DNA Extraction Kit ver. 4.0 (code NO.9762) was used by isolating the PCR product from the agarose gel. The purified PCR fragments were analysed by Takara Biotechnology (Dalian, China). The 16S rRNA gene nucleotide sequences of the isolates were identified and analysed using the BLAST program on the NCBI website (<http://www.ncbi.nlm.nih.gov/>). Sequences were aligned and phylogenetic trees were constructed using MEGA 5.0.

2.3 EPS separation and purification

After cultivation, the cells were removed and the EPS was gained by repetitive ethanol precipitation, dialysed 24 h with distilled water at 4°C, and lyophilized (Behare et al, 2013). DEAE-52-Cellulose ion exchange chromatography column was used for fractionating the crude EPS and further purification of the EPS was performed by a SepharoseCL-6B column. Phenol-sulfuric acid method was used for analysis the carbohydrate content (Ma et al., 2015).

2.4 Determination of molecular weight (Mw)

The Mw of the EPS was measured by gel-filtration chromatography using SephadexG-200 column (16 mm×100 cm), and it was calibrated by standard dextran (10, 40, 70, 100, and 500 KDa) at a concentration of 5 mg/ml. The Mw of purified EPS was determined by graphic plot of the log Mw of the dextran against elution volume (Ernstsen J et al., 2016).

2.5 Monosaccharide composition analysis of EPS

5 mg of purified EPS were hydrolysed with 2 ml trifluoroacetic acid (TFA) at 120 °C for 2 h. The hydrolysed EPS was freeze-dried, redissolved in deionized water, and measured by high performance anion exchange chromatography (HPAEC) system (Dionex, Sunnyvale, CA, USA), equipped with a quaternary GP50 gradient pump (Dionex), and ED40 conductivity detector (Dionex). The parameter was used according to the conditions described by Zhang (2013). Standard arabinose, mannose, rhamnose, fucose, glucose, galactose and xylose were prepared for comparison.

2.6 Functional group analysis

The major structural groups of the purified EPS were detected using Fourier transform infrared (FT-IR) spectroscopy, and the spectrum of the EPS was obtained using a KBr method. The polysaccharide samples were pressed into KBr pellets at a sample: KBr ratio of 1:100. The FT-IR spectra were recorded on a Bruker Tensor 27 instrument in the region of 4, 000 – 400 cm⁻¹ (Fontana et al., 2015).

2.7 Antioxidant activity tests in vitro

The DPPH radical-scavenging capacity of EPS was measured according to the method described by Zhang (2013). The scavenging ability was Eq (1):

$$\text{DPPH scavenging ability (\%)} = \left[1 - \frac{(A_{\text{sample}} - A_{\text{blank}})}{A_{\text{control}}} \right] \times 100 \quad (1)$$

The hydroxyl radical scavenging effect of EPS was determined with the Fenton reaction described as Eq (2). A_s is the absorbance in the presence of the sample, A_0 is the absorbance of the control in the absence of the sample, and A is the absorbance without the sample and Fenton reaction system.

$$\text{Hydroxyl radical scavenging activity (\%)} = \left[\frac{(A_s - A_0)}{(A - A_0)} \right] \times 100 \quad (2)$$

The superoxide anion radical scavenging activity was determined according to the method described by Wang with slight modifications (Wang et al., 2015). ΔA_1 is the difference of the absorbance values per 10 s for samples of different concentrations and ΔA_0 is the difference of the absorbance values per 10 s without samples.

$$\text{Superoxide anion radical scavenging activity (\%)} = \left[\frac{(\Delta A_0 - \Delta A_1)}{\Delta A_0} \right] \times 100 \quad (3)$$

3. Results and discussion

3.1 Identification of the EPS-producing strains

72 LAB were separated and identified including 55 EPS producing strains. The average EPS ranged from 10.35 mg/L to 110.39 mg/L based on fermentation, isolation, and purification conditions, as determined by phenol-sulphuric acid analysis method. Three strains of the average high EPS were *Lactobacillus helveticus*,

Enterococcus and *Leuconostoc lactis* which yields of EPS were 110.39 mg/L, 86.79 mg/L and 60.32 mg/L respectively.

3.2 16S rRNA sequence identification and phylogenetic analysis

It was reported that conventional physiological and biochemical properties and saccharide fermentation are limited, complicated, and lack certainty because of the increasing number of species that have varied characters. 16S rRNA sequence analysis produces more accurate and reliable subspecies identification than conventional methods.

The obtained sequences (approximately 1.5 kbp) of higher EPS-producing strains in GenBank were assigned the accession nos. KP408149–KP408151. They were SMN1-2 (KP408149), SMN2-1 (KP408151) and SMN2-1-2 (KP408150) respectively and phylogenetic analysed by 16S rRNA sequence.

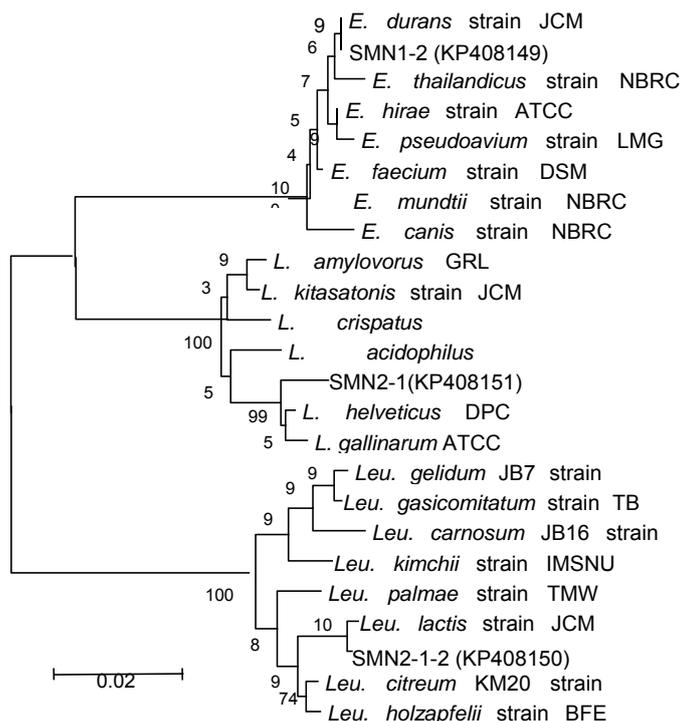


Figure 1: 16S rRNA phylogenetic tree of representative EPS-producing isolates from koumiss

Phylogenetic analysis (Figure 1) revealed that SMN1-2 (KP408149) and SMN2-1 (KP408151) were more homogenous to each other than SMN2-1-2 (KP408150). The results revealed that the more same gene segments in the two strains. Combining the result of biochemical properties and saccharide fermentation with the analysis of 16S rRNA sequence, we notarized that SMN1-2 (KP408149) belonged to *Enterococcus durans*, SMN2-1-2 (KP408150) was *Leu. Lactis* and SMN2-1 (KP408151) was *L. helveticus*, because they exhibited a high degree of sequence similarity with each other (Jebava et al., 2014). Similar results were found by Saravanan et al (2016) who demonstrated that they have the ability to biosynthesise EPS (Saravanan and Shetty, 2016). *L. helveticus* SMN2-1 had the highest yield of EPS, we had analysed its structure and antioxidant activity of EPS deeply.

3.3 Isolation and purification of EPS

After *L. helveticus* SMN2-1 was statically cultured in MRS broth for 24 h at 36°C, the crude EPS was gained by repetitive ethanol precipitation and fractionated on a DEAE-52-Cellulose column, resulting in a major peak of EPS eluted with deionized water and 0.1–0.4 mol/L NaCl solutions. Figure 2 (A) showed that two fractions were collected, and yield of EPS-I was far more than EPS-II. Further purification of the collected fractions were performed by a SepharoseCL-6B column and eluted with 0.85% (w/v) NaCl. As result, one sub-fraction EPS-I was gained (Figure 2 (B)). The polysaccharide fractions were detected, pooled, dialyzed and lyophilized for further investigation.

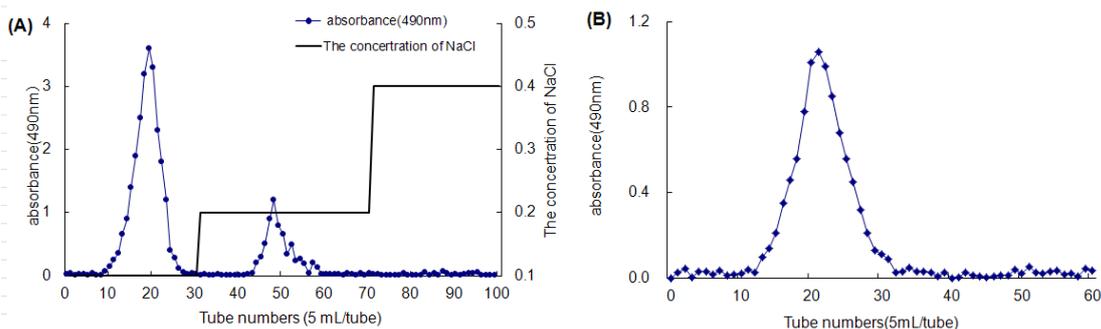


Figure 2: Anion exchange chromatography of the crude EPS on a DEAE-cellulose-52 (A) and gel permeation chromatography on Sepharose CL-6B (B).

3.4 Mw and monosaccharide composition analysis of EPS

The Mw of the EPS was measured by gel-filtration chromatography using SephadexG-200 column and calibrated by standard dextran. Based on a calibration curve ($\text{Log Mw} = -20.4V_e/V_0 + 11.45$, $R^2 = 0.9927$), obtained from standard dextran with various Mw cut-offs. The Mw of EPS-I was 8.5×10^5 Da and EPS-II was 5.2×10^3 Da that calculated by the calibration curve. The Mw of EPS-I was right in the average Mw of EPS from *L. helveticus* between 1×10^5 and 1×10^6 (Fontana et al., 2015) and EPS-II was not involved.

The monosaccharide composition of EPS-I was analysed by HPAEC by comparing with the reference standards. Result showed that EPS-I from *L. helveticus* SMN2-1 was mainly composed of glucose, galactose, and mannose in a molar ratio of 4:3:1. It just was identical to the result of Li W et al (2013) that EPS from *L. helveticus* was mainly composed of galactose, glucose, and mannose (Li et al., 2015), but it was different from a molar ratio.

3.5 Functional group analysis

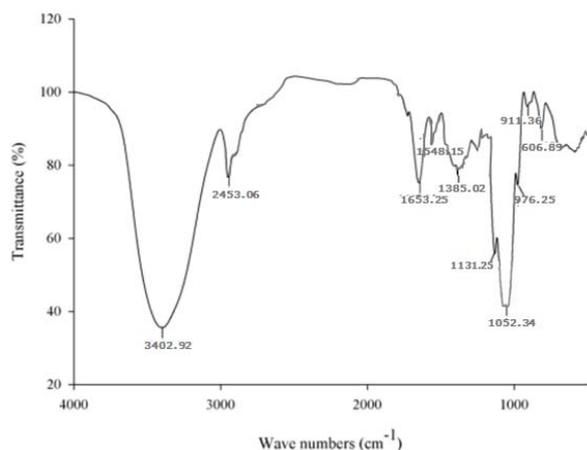


Figure 3: FT-IR spectrum of purified EPS-I polysaccharides from *L. helveticus* SMN2-1

Figure 3 showed the infrared spectrum of the major functional groups of EPS-I from *L. helveticus* SMN2-1. The EPS showed that peaks are ranging from 3,402.92 to 606.89 cm^{-1} and no peaks in the range of 260-290 cm^{-1} , it clearly indicated the sample did not have any proteins and nucleic acids. A broad stretching characteristic peak at 3,402.92 cm^{-1} was characteristic for O-H existed in hydrogen bond of polymer and a weak peak at 2,453.06 cm^{-1} for an asymmetrical C-H stretching band. The bands in the region of 1,653.25 cm^{-1} were assigned to the stretching vibration of the carboxyl group (C=O) and due to the associated water which indicates the presence of organic substances such as the ring stretching of mannose or galactose (Wang et al., 2015). The relatively weak absorption peak at 1,548.15 cm^{-1} might be ascribed to the N-H (Zhang et al., 2013). The absorption peak at 1385.02 cm^{-1} was possibly due to the symmetric stretching of the COO- group. In addition, the band within the 1,200-1,000 cm^{-1} region was assigned to the vibrations of the

C=O and C-O-C glycoside bands. The strong absorption peak at $1,052.34\text{ cm}^{-1}$ indicated the presence of carbohydrates (Abdhul et al., 2014).

3.6 Antioxidant activity tests in vitro

The superoxide and hydroxyl radicals are considered to be a highly potent oxidant and can react with all biomolecules. DPPH as a stable free radical can accept hydrogen radical or an electron to become a stable diamagnetic molecule. Therefore, antioxidants played important roles against various diseases. In this study, the antioxidant activity in vitro of the EPS-I from *L. helveticus* SMN2-1 was investigated by DPPH, hydroxyl and super oxide radical scavenging activity and compared with those of ascorbic acid (Vc) (Ma et al., 2015).

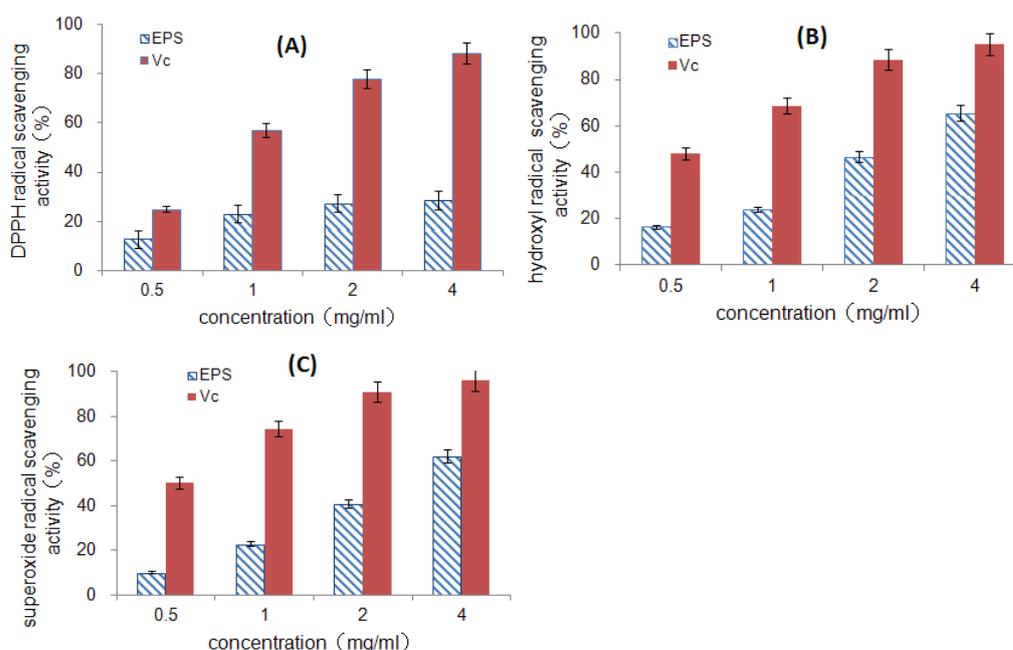


Figure 4: Scavenging activities on the DPPH radical (A), hydroxyl radical (B) and superoxide radical (C) of the purified EPS-I from *L. helveticus* SMN2-1 and ascorbic acid (Vc). Data are presented as the mean \pm SD of triplicates.

Figure 4 showed that both EPS-I from *L. helveticus* SMN2-1 and ascorbic acid exhibited concentration-dependent scavenging activities against DPPH, hydroxyl and super oxide radical. EPS-I could scavenge DPPH (Figure 4 (A)), hydroxyl (Figure 4 (B)) and super oxide radical (Figure 4 (C)) at concentrations between 0.5 and 4.0 mg/ml, with lower scavenging activity than the reference ascorbic acid at every concentration point. At the high dose of 4.0 mg/ml, EPS-I exhibited relatively stronger radical scavenging activity with DPPH radical (28.40%), hydroxyl radical (65.30%) and superoxide radical (61.97%). Our results showed EPS-I from *L. helveticus* SMN2-1 with higher scavenging activity of hydroxyl and super oxide radical than DPPH radical. This suggested that EPS-I from *L. helveticus* SMN2-1 might be an effective scavenger for hydroxyl and super oxide radical, which could react with the bio-macromolecules in living cells resulting in severe damage to the adjacent macromolecules. However, the scavenging activity of the EPS-I was differing from different isolates (Abdhul et al, 2014). Similarly to the reported earlier, the antioxidant activity of EPS might be affected by different factors such as the extraction and isolation methods used as well as monosaccharide composition, Mw et al (Wang, 2016).

4. Conclusion

55 EPS-producing strains were isolated and identified from 12 koumiss samples. *L. helveticus* SMN2-1 was notarized as the highest EPS-producing LAB. The EPS as important extracellular bioactive molecules produced by *L. helveticus* SMN2-1 showed a molecular mass of 0.85×10^5 Da and good antioxidant activity with high hydroxyl and superoxide radicals scavenging abilities in vitro. This study suggests that the diversity of LAB in koumiss and antioxidant activity of EPS from LAB may lead to intensive study of koumiss as a characteristic fermented product as well as for its unique physiological actions.

References

- Abd hul K., Ganesh M., Shanmughapriya S., Kanagavel M., Anbarasu K., Natarajaseenivasan K., 2014, Antioxidant activity of exopolysaccharide from probiotic strain *Enterococcus faecium* (BDU7) from Ngari, *Int J Biol Macromol*, 70, 450-454, DOI: 10.1016/j.ijbiomac.2014.07.026
- Behare P.V., Singh R., Nagpal R., Rao K.H., 2013, Exopolysaccharides producing *Lactobacillus fermentum* strain for enhancing rheological and sensory attributes of low-fat dahi, *J Food Sci Technol*, 50, 1228-1232, DOI: 10.1007/s13197-013-0999-6
- Blanco-Rodriguez A., Campo F., Morales O., Valiente R., Lambert B., Becherán L., Garcia-Ramirez A., De Melo Lisboa H., 2016, Development an Electronic Nose to Identify and Classify Odours from Beverages, *Chemical Engineering Transactions*, 54, 337-342, DOI: 10.3303/CET1654057
- Donmez N., Kisadere I., Balaban C.Kadiralieva N., 2014, Effects of traditional homemade koumiss on some hematological and biochemical characteristics in sedentary men exposed to exercise, *Biotech Histochem*, 89, 558-563, DOI: 10.3109/10520295.2014.915428
- Ernstsen J., Nazir S., Roed B.K., Manca D., 2016, Systemising Performance Indicators in the sessment of Complex Sociotechnical Systems, *Chemical Engineering Transactions*. 53, 187-192, DOI: 10.3303/CET1653032
- Fontana C., Li S., Yang Z., Widmalm G., 2015, Structural studies of the exopolysaccharide from *Lactobacillus plantarum* C88 using NMR spectroscopy and the program CASPER, *Carbohydr Res*, 402, 87-94, DOI: 10.1016/j.carres.2014.09.003
- Grosu-Tudor S.S., Zamfir M., Van Der Meulen R., De Vuyst L., 2013, Isolation of novel homopolysaccharide-producing lactic acid bacteria from Romanian raw milk and fermented dairy products, *European Food Research and Technology*, 237, 609-615, DOI: 10.1007/s00217-013-2038-2
- Jebava I., Chuat V., Lortal S., Valence F., 2014, Peptidoglycan hydrolases as species-specific markers to differentiate *Lactobacillus helveticus* from *Lactobacillus gallinarum* and other closely related homofermentative lactobacilli, *Curr Microbiol*, 68, 551-557, DOI: 10.1007/s00284-013-0512-5
- Li W., Ji J., Chen X., Jiang M., Rui X., Dong M., 2014, Structural elucidation and antioxidant activities of exopolysaccharides from *Lactobacillus helveticus* MB2-1, *Carbohydr Polym*, 102, 351-359, DOI: 10.1016/j.carbpol.2013.11.053
- Li W., Xia X., Tang W., Ji J., Rui X., Chen X., Jiang M., Zhou J., Zhang Q., Dong M., 2015, Structural characterization and anticancer activity of cell-bound exopolysaccharide from *Lactobacillus helveticus* MB2-1, *J Agric Food Chem*, 63, 3454-3463, DOI: 10.1021/acs.jafc.5b01086
- Ma Z., Cui F., Gao X., Zhang J., Zheng L., Jia L., 2015, Purification, characterization, antioxidant activity and anti-aging of exopolysaccharides by *Flammulina velutipes* SF-06, *Antonie Van Leeuwenhoek*, 107, 73-82, DOI: 10.1007/s10482-014-0305-2
- Polak-Berecka M., Wasko A., Szwajgier D.Chomaz A., 2013, Bifidogenic and antioxidant activity of exopolysaccharides produced by *Lactobacillus rhamnosus* E/N cultivated on different carbon sources, *Pol J Microbiol*, 62, 181-188.
- Saravanan C., Shetty P.K., 2016, Isolation and characterization of exopolysaccharide from *Leuconostoc lactis* KC117496 isolated from idli batter, *Int J Biol Macromol*, 90, 100-106, DOI: 10.1016/j.ijbiomac.2015.02.007
- Sari E.K., Bakir B., Aydin B.D., Sozmen M., 2014, The effects of kefir, koumiss, yogurt and commercial probiotic formulations on PPARalpha and PPAR-beta/delta expressions in mouse kidney, *Biotech Histochem*, 89, 287-295, DOI: 10.3109/10520295.2013.844274
- Torino M.I., Mozzi F., Font De-Valdez G., 2005, Exopolysaccharide biosynthesis by *Lactobacillus helveticus* ATCC 15807, *Appl Microbiol Biotechnol*, 68, 259-265, DOI: 10.1007/s00253-004-1865-2
- Wang J., Zhao X., Yang Y., Zhao A., Yang Z., 2015, Characterization and bioactivities of an exopolysaccharide produced by *Lactobacillus plantarum* YW32, *Int J Biol Macromol*, 74, 119-126, DOI: 10.1016/j.ijbiomac.2014.12.006
- Wang Y., Yan H., Yin Z., 2016, A quality traceability system for seafood based on bill of lots, *Revista de la Facultad de Ingeniería*.31, 49-58.
- Zhang L., Liu C., Li D., Zhao Y., Zhang X., Zeng X., Yang Z., Li S., 2013, Antioxidant activity of an exopolysaccharide isolated from *Lactobacillus plantarum* C88, *Int J Biol Macromol*, 54, 270-275, DOI: 10.1016/j.ijbiomac.2012.12.037