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Master's Thesis of Science in Agriculture

Preparation of Yogurt with Exopolysaccharide-producing *Leuconostoc garlicum* Isolated from Kimchi

김치에서 분리한 EPS 생성 *Leuconostoc garlicum*을 이용한 요구르트 제조

February 2021

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Preparation of Yogurt with Exopolysaccharide-producing *Leuconostoc garlicum* Isolated from Kimchi

A thesis
submitted in partial fulfillment of the requirements to the faculty
of Graduate School of International Agricultural Technology
for the Degree of Master of Science in Agriculture

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Abstract

Exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) are used to modify the rheological, physical, and sensory properties of fermented milk. *Leuconostoc garlicum* KCCM 43211 isolated from Kimchi is known as highly viscous EPS producing LAB. However, the effects of EPS produced by *L. garlicum* KCCM 43211 in fermented milk and its structural characteristics have not been studied. The overall objective of this study was to determine the influence of EPS produced *L. garlicum* KCCM 43211 on rheological properties of fermented milk and investigate the physicochemical characteristics of EPS (Chapter 2). After that, we studied the effects of pre-fermentation with *L. garlicum* KCCM 43211 on yogurt properties during the storage at 4 °C for 28 days due to the potential application of *L. garlicum* KCCM 43211 for yogurt production (Chapter 3).

L. garlicum KCCM 43211 were grown in raw milk and homogenized milk for 48 h at 25 °C with 100 rpm shaking in the presence or absence of sucrose. Cell growth, pH variations, EPS production and rheological properties were measured during 48 h fermentation. The results showed that viable cell count reached to 10^8 - 10^9 CFU/mL and a decrease in pH followed by an increase in bacteria was observed during fermentation for all four fermented milks. EPS production was not observed in fermented milks without sucrose. With 10% sucrose, *L. garlicum* KCCM 43211 produced 22.91 to 27.29 g/kg medium of EPS. Viscosity was also no changes without sucrose, meanwhile, increased 1.08 to 3.22 cP and 1.29 to 2.93 cP in raw-milk fermented milk and

homogenized-milk fermented milk, respectively. EPS production and the rheological analysis showed that EPS increases the viscosity in fermented milk. To investigate the physicochemical properties of EPS, EPS was isolated from fermented milk made with raw milk 10% sucrose and purified. EPS produced by *L. garlicum* KCCM 43211 was identified as linear α-(1→6) dextran with 2.9×10^6 Da of molecular weight.

In the chapter 3, The effect of EPS on the storage stability of yogurt was investigated at raw-milk yogurt and homogenized-milk yogurt. For pre-fermentation, *L. garlicum* KCCM 43211 was inoculated on raw milk and homogenized milk containing 10% sucrose and incubated for 12 h at 25 °C with shaking. After 12 h, yogurt samples were prepared by yogurt starter culture. Viable cell changes, pH variations, total solids, color differences, water holding capacity (WHC), EPS concentration, and rheological properties are studied during 28 days of refrigerated storage for all four yogurt samples. Viable cell counts decreased and pH also decreased from 4.59 – 4.60 to 4.20 – 4.30. Total solids content showed no significant difference for all samples during storage. In addition, the presence of EPS did not affect the color. The WHC results showed that homogenization treatment could be an effective factor to develop the water-holding ability of the gel network, which prevents syneresis during storage. There was slight decrease (0.4 - 0.5 g/kg medium) of EPS concentration in both yogurts (pre+main), which indicated no degradation of EPS during long cold storage. Results from flow curve showed pseudoplastic (shear thinning) behavior for four yogurt samples. There was no significant difference of apparent

viscosity by storage time. However, yogurts containing EPS showed an upward trend and smaller hysteresis loop after 14 days storage in temperature sweep measurement.

The results show that the present EPS improve the viscosity of fermented milk in the preliminary stages of gelation (> pH 4.6), however, EPS effect could be screened in the strong gel network (< pH 4.6, homogenized-milk yogurt). Based on results obtained, EPS produced by *L. garlicum* KCCM 43211 could modulate physical and rheological properties of fermented milk with weak gel network.

Key word : *Leuconostoc garlicum*, Exopolysaccharide, Homogenization, Fermented milk, Yogurt

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Chapter 1.

Research background

1. Lactic acid bacteria

LAB can be seen in various environments, including gastrointestinal tracts, plant surface, and fermented foods (Endo et al., 2019). LAB have been used to lactic acid fermentation by the important reason which is the tolerance for acidic conditions, heat treatments, and cold-storage (Kudo and Sasaki, 2019; Hemme and Foucaud-Scheunemann, 2004). LAB fermentation improves the preservation by lowering pH of the food matrix, contributes positively to sensory properties through producing aroma and flavor compounds and increases health benefits (van Geel-Schutten, 2000; Ruas-Madiedo, 2002; Walstra et al., 2005; Papadimitriou et al., 2016). Also, some strains of LAB can enhance the texture of final products via EPS production during fermentation (Papadimitriou et al., 2016). The most common genera of LAB regarded as food related are *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc*, *Oenococcus*, *Tetragenococcus*, *Carnobacterium*, *Bifidobacterium*, and *Weissella* (Table 1.1) (Ali, 2010; Bong et al., 2013; Papadimitriou et al., 2016; Fessard and Remize, 2017). Among those genera, *Streptococcus*, *Lactobacillus*, and *Leuconostoc* are remarkable due to production of dextran, slimy EPS with a high molecular size. Especially, some *Leuconostoc* strains produce dextran applied in commercial market with novel

physico-chemical properties (Capek et al., 2011).

Table 1.1. Fermented foods and their LAB (Ali, 2010; Bong et al., 2013; Fessard and Remize, 2017).

Fermented foods	LAB
Dairy products	
Fermented milk	*Lb. casei*, *Lb. acidophilus*, *Lb. rhamnosus*, *Lb. johnsonii*, *B. lactis*, *B.bifidum*, *B. brevis*
Yogurt	*Lb. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*
Cheeses	*Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis* var. *diacetylactis*, *L. mesenteroides* subsp. *cremoris*, *Lb. delbrueckii* subsp. *lactis*, *Lb. helveticus*, *Lb. casei*, *Lb. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*
Butter and buttermilk	*Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *lactis* var. *diacetylactis*, *Lc. lactis* subsp. *cremoris, L. mesenteroides* subsp. *cremoris*
Kefir	*Lb. kefir*, *Lb. kefiranofacies*, *Lb. brevis*
Fermented vegetables	
Kimchi	*Lb. casei*, *L. mesenteroides*, *Lb. acidophilus*, *Lb. fermentum*, *Lb. plantarum*, *Lb. sakei*
Sauerkraut	*L. mesenteroides*, *Lb. plantarum*, *P. acidilactici*
Pickles	*L. mesenteroides*, *P. cerevisiae*, *Lb. brevis*, *Lb. plantarum*
Soy sauce	*T. halophilus*
Fermented meat products	
Sausages	*P. acidilactici*, *P. pentosaceus*, *Lb. sakei*, *Lb. curvatus*, *Lb. plantarum*, *Lb. pentosus*, *Lb. casei*, *Lc. lactis*
Fermented cereals	
Sourdough	*Lb. sanfransiscensis*, *Lb. farciminis*, *Lb. fermentum*, *Lb. brevis*, *Lb. plantarum*, *Lb. amylovorus*, *Lb. reuteri*, *Lb. pontis*, *Lb. panis*, *Lb. alimentarius*, *W. cibaria*
Alcoholic beverages	
Wine	*O. oeni*, *Lb. plantarum*, *Lb. hilgardii*
Rice wine	*Lb. sakei*

Lb: *Lactobacillus*, B: *Bifidobacterium*, S: *Streptococcus*, Lc: *Lactococcus*, L: *Leuconostoc*, P: *Pediococcus*, T: *Tetragenococcus*, W: *Weissella*, O: *Oenococcus*

1.1. *Leuconostoc* spp.

Leuconostoc spp. are heterofermentative LAB and play important role in the food fermentation such as kimchi, sauerkraut, meat products and fermented milks (Holland and Liu, 2011; Park et al. 2013). Among LAB, *Leuconostoc* spp. are the predominant group of EPS-producing LAB. They produce homopolysaccharides (Hops) such as dextran with mainly α-1,6-glucosidic bond from sucrose metabolism (Park et al., 2013). Table 1.2 shows EPS originated from *Leuconostoc* spp. has variety types depending on the strains. *Leuconostoc* spp. produced EPS has been used as viscosifier, stabilizer, emulsifier, sweetener, gelling agent or water-binding agent in both of food and non-food industries (Korakli and Vogel, 2006). In addition, during the milk fermentation, a cooperative relationship occurs to exist between *Lactococcus* and EPS producing *Leuconostoc* (Holland, 2011).

1.2. *Leuconostoc garlicum*

L. garlicum was first isolated from garlic surface with the characteristics such as tolerance to high garlic concentration which is an extreme environment for microorganisms and ability to survive at high temperature (> 40 ℃) (Kim et al., 2002). The characteristics of *L. garlicum* have been barely studied, nonetheless, *L. garlicum* PR and *L. garlicum* KCCM 43211 strain is known as that produces highly viscous EPS during sucrose fermentation (Capek et al., 2011; Hong et al., 2017).

Table 1.2. Source, structure, yield, and molar mass of EPS produced by *Leuconostoc* spp.

Strain	Source	EPS	Repeating units and linkages	EPS yield (g/L)	Molar mass (Da)	References
Leuconostoc garlicum						
PR	Activated sludge	Glucan	95% of α-(1→6)-linked glucopyranose units with less than 5% of α-(1→2), α-(1→3), α-(1→4)-linked D-glucopyranosyl residues	50	2×10^7	Capek et al., 2011
Leuconostoc lactis						
KC117496	*Idli*	Glucan	α-(1→6) and α-(1→3)-linked glucose with 95% α-(1→6) and 5% branching α-(1→3) linkages	4.55	4.428×10^3	Saravanan and Shetty, 2016
95A	Sourdough	Dextran	α-(1→6) glycosidic linkages with D-glucopyranosyl units	88.05	N/A	Palomba et al., 2012
Leuconostoc mesenteroides						
SN-8	*Dajiang*	Hetero EPS	Mannose, glucose, and galactose with α-(1→6) glucosidic linkages	2.42	2.0×10^5	Yang et al., 2020
BD1710	N/A	Dextran	α-(1→6)-linked D-glucopyranosyl units with 6% α-(1→3) branches	32	6.35×10^5	Han et al., 2014
Leuconostoc pseudomesenteroides						
PC	Chinese pickled cabbage	Dextran	D-glucopyranose units with α-(1→6) linkages	30.5	3.13×10^6	Wang et al., 2019
YF32	Soybean paste	Dextran	α-(1→6)-linked D-glucopyranose units	12.5	5.54×10^6	Yang et al., 2018
Leuconostoc ctireum						
NM105	Sauerkraut	Dextran	α-(1→6), α-(2→6)-linked D-glucopyranose units, and α-D-glucopyranose units at a ratio of 1:1:1 with α-(1→2) glucosidic bond	23.5	1.01×10^8	Yang et al., 2015
SK24.002	Chinese pickled vegetables	Dextran	α-(1→3), α-(1→6)-linked D-glucopyranose units with α-(1→6) linkages to α-(1→3) linkages at a ratio of 5:4	35	4.62×10^7	Miao et al., 2014

N/A: Not available

2. Exopolysaccharide (EPS)

2.1. Definition

EPS is high-molecular-mass biopolymer consisting of sugars or sugar derivatives repeating units (Wang et al., 2015). EPS is produced during the metabolism of microorganisms for instance bacteria, fungi, and blue-green algae (Gentès et al., 2013; Wang et al., 2015). Especially, some strains of LAB synthesize various EPS with generally recognized as safe (GRAS) status in fermented food such as wine, yogurt, and kimchi (van Geel-Schutten, 2000; Gentès et al., 2011; Wang et al., 2015; Kook et al., 2019). LAB-produced EPS has been used in broad spectrum of application including food products, pharmaceuticals, and chemical products. LAB-produced EPS improves the texture, mouth-feel, rheological properties, and stability of the final products such as yogurt. Due to these characteristics, EPS can be suitable candidates for natural biothickners, biostabilizers, bioemulsifiers, and gelling agents. (Duboc and Mollet, 2001; Welman and Maddox, 2003). Also, EPS has been shown to possesses physiological and biological functions, including anti-oxidation (Pan and Mei, 2010; Liu et al., 2011; Li et al., 2013; Rani et al., 2018), anti-bacterial (Jeong et al., 2017; Yu et al., 2018), immunomodulating function (Dinić et al., 2018; Ren et al., 2016; Liu et al., 2011), anti-tumor (Wang et al., 2014; Li et al., 2015), cholesterol-lowering (Korcz et al., 2018), and anti-coagulant (Hussain et al., 2017). In addition, German et al. (1999) presumed EPS in yogurt may stay in gastrointestinal tract for longer, improving

colonies by probiotics.

These various characteristics of EPS appear greatly depending on its structure such as monosaccharide composition, charge, backbone linkages, repeating units, branching, and its location; attached on the cell surface in the form of capsules or extracellularly released into the fermentation medium in the form of slime (Duboc and Mollet, 2001; Welman and Maddox, 2003; Yang et al., 2010; Mende et al., 2016).

2.2. Classification and structure

EPS from LAB can be classified into homopolysaccharides (HoPS) and heteropolysaccharides (HePS) by monosaccharides composition. HoPS is composed of single monosaccharide and consists of subgroups such as glucans, fructans, and poly-galactans (De Vuyst and Degeest, 1999; Monsan et al., 2001). Figure 1.1 (Oleksy and Klewicka, 2016) shows the various types of HoPS according to their different linkage patterns. HePS is made of various monosaccharides like D-glucose, D-galactose, and L-rhamnose in different ratios. Gellan and xanthan are common examples of HePS (Baruah et al., 2016).

HoPS consists of main backbone of single monosaccharide with different degrees of branching and linkage sites. While, HePS has repeating units of backbone of various monosaccharides with sugar or non-sugar derivatives such as N-acetylgalactosamine, or glucuronic acid (Zannini et al., 2016). These differences in structure result in

variation with molecular weight of EPS; HoPS is up to 10^5 Da, HePS ranges from 10^4 to 10^6 Da (Ruas-Madiedo et al., 2002).

Exopolysaccharide

Single-type monosaccharide

Homo-EPS

Glucans; polymer of D-glucose

Type	Linkage
Dextran	α-1,6-glucosidic bonds
Mutan	α-1,3-glucosidic bonds
Reuteran	α-1,4 and α-1,6-glucosidic bonds
Alternan	Alternating α-1,6 and α-1,3-glucosidic bonds
β-D-glucan	β-1,3-glucosidic bonds

Fructans; polymer of fructose

Type	Linkage
Levan	β-2,6-fructosyl bonds
Inulin	β-2,1-fructosyl bonds

Poly-galactans; polymer of galactose

Different-types monosaccharides

Hetero-EPS

Repeating structure of two or more monosaccharides (e.g. D-glucose, D-galactose, L-rhamnose) with carbohydrate or non-carbohydrate substituents

Fig. 1.1. Classification of EPS by monosaccharides composition (Oleksy and Klewicka, 2016).

3. Fermented milk

3.1. Definition and classification

Fermented milk has been consumed as functional foods worldwide due to its various advantages. Fermented milk includes milk products prepared by lactic acid fermentation (e.g. yogurt), a combination of LAB and yeast fermentation (e.g. Kefir), or LAB and molds fermentation (e.g. Viili) (Fig. 1.2.) (Walstra et al., 2005). During the fermentation process, LAB results in pH reduction by producing lactic acid from lactose with coagulation of milk protein (Nagaoka, 2019). According to this process, fermented milk tends to show less syneresis during storage without addition of stabilizers (Lucey, 2001). Also, fermentation provides unique tastes, flavors and textures to final products, increases shelf life and enhances digestibility of milk. Therefore, consumer's interest in fermented milk is growing (Tamime and Robinson, 2007).

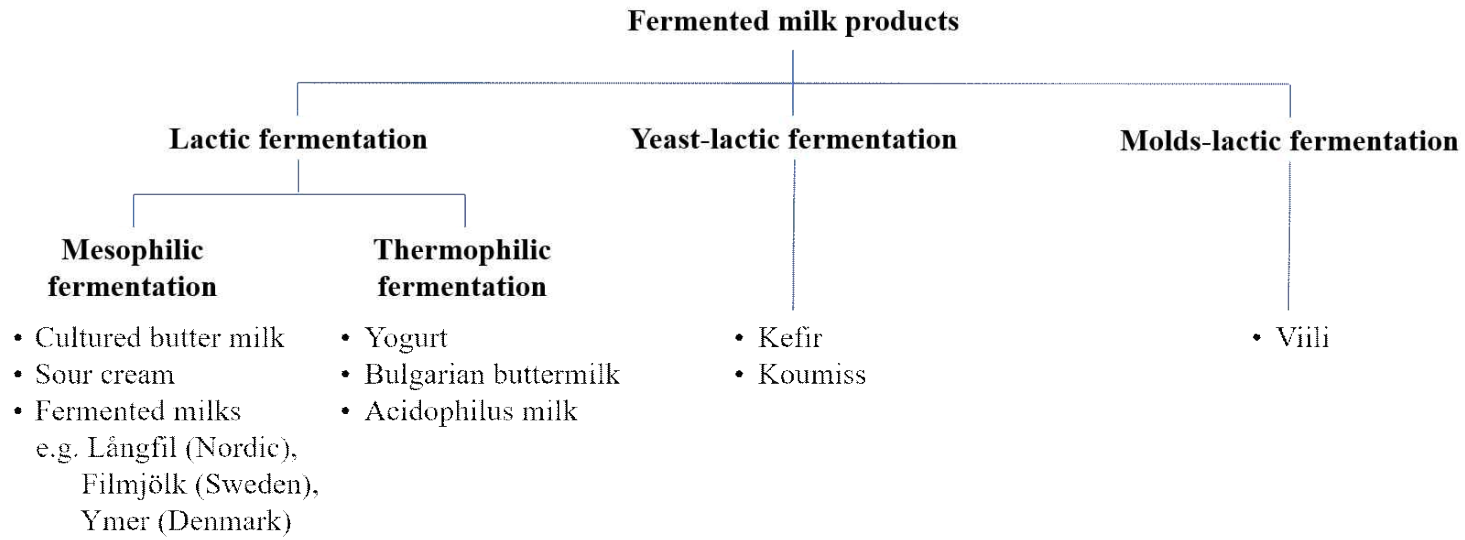


Fig. 1.2. Types of fermented milk products (Walstra et al., 2005).

3.2. Yogurt

Yogurt is best-known fermented dairy product around the world and its consumption has been driven by the health benefits and sensory properties (Mckinley, 2005). Based on the Codex standard, yogurt is produced with milk by LAB, including *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Codex, 2011). During the fermentation, these two species have symbiotic relationship. *S. thermophilus* grows rapidly at the start of fermentation producing pyruvic acid, formic acid, and traces of carbon dioxide. These metabolites stimulate the growth of *Lb. delbrueckii* subsp. *bulgaricus*, while *Lb. delbrueckii* subsp. *bulgaricus* hydrolyzes casein into polypeptides and amino acids which can utilize the growth of *S. thermophilus* (Nagaoka, 2019). Through homofermentative process, lactic acid is produced from lactose and gel structure is created by the coagulation of the milk proteins. In addition, milk components are converted into volatile flavor compounds such as acetaldehyde, diacetyl, acetone, acetoin, and 2-butanone which are important compounds organoleptically (Cheng, 2010).

3.3. Classification of yogurt

Yogurt is commonly produced in two major types according to its physical state: set and stirred yogurt (Fig. 1.3). The manufacturing process of commercial set yogurt includes standardization of milk, homogenization, pasteurization, inoculation with starter cultures,

fermentation, cooling and storage. Set type yogurt is fermented after packaging, giving a continuous gel structure in the final products. In stirred type yogurt, yogurt is prepared in large tank with breaking of coagulum by stirring after fermentation. The mechanical process causes alterations in the rheological, physical properties of stirred yogurt, following changes of original gel characteristics (Haque et al., 2001; Tamime and Robinson, 2007).

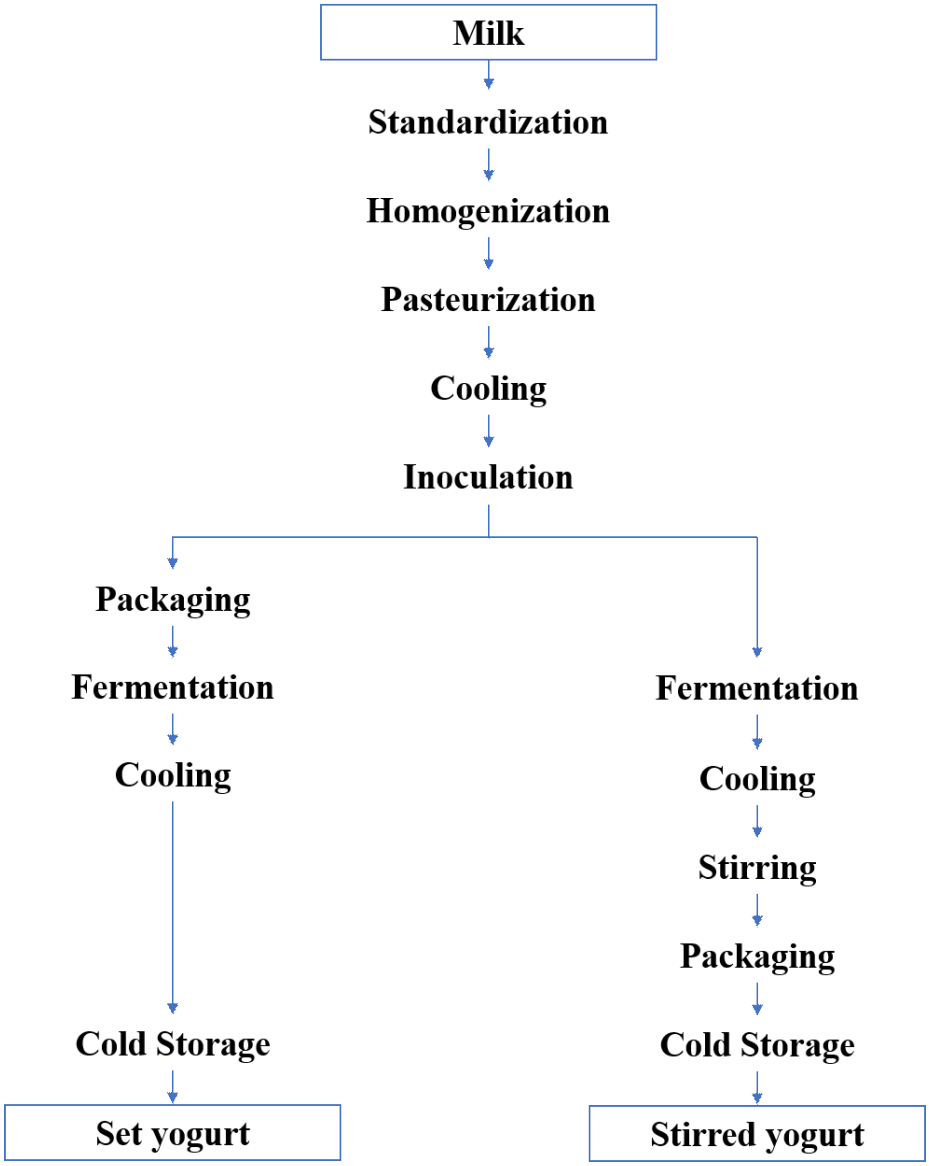


Fig. 1.3. Manufacturing process of set and stirred type yogurts (Walstra et al., 2005; Nagaoka, 2019).

3.4. Yogurt manufacture

As shown in Fig. 1.3, the manufacturing process of yogurt is mainly composed of the following four steps: (1) preparation of ingredients (2) pre-fermentation treatments such as heat treatment and homogenization (3) fermentation of the yogurt (4) post-fermentation treatments such as stirring, cooling, and packaging. All these steps affect the final quality of the yogurt.

3.4.1. Ingredient preparation

Yogurt is made of various ingredients including milk, bacterial culture, sweeteners, stabilizers, fruits, flavors, etc.

(1) Milk

Milk is the main ingredient of yogurt and milk composition is modified by dairy ingredients, such as cream, whole milk, skimmed milk and various type of milk powders. Standardization for fat and solids-not-fat (SNF) content results in fat reduction and increase in total solids such as lactose, protein, mineral, and vitamin content (Corrieu and Béal, 2016). For many commercial yogurt products, solids content of the milk is increased to 14-15% with 8.2-8.6% solid not-fat content (Ranadheera et al., 2016).

(2) Starter culture

Most of the yogurt is fermented with *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. Furthermore, optional culture including *Lb. acidophilus*, *Bifidobacterium*, and other probiotic bacteria is often

added. These strains have a slight effect on the final product characteristics, especially flavor (Chandan, 2008).

(3) Other ingredients

Sweeteners are added to improve the flavor and sensory characteristics (Weerathilake et al., 2014). Addition of stabilizers such as pectin, gelatin, xanthan gum or dextran are used to improve the texture and prevent the whey separation from the yogurt, in quantities in the order of 0.1% to 0.5%. Fruits, flavors and other additives can be added to the yogurt at various points in manufacturing process.

3.4.2. Pre-fermentation treatments

(1) Homogenization

Homogenization is an essential step in commercial yogurt processing. Milk is an oil-in-water emulsion, in which the fat globules dispersed in a continuous skim milk phase. The creaming of milk during storage is a common problem confronted in the dairy industry. High-pressure homogenization is a mechanical treatment that reduces the diameter of fat globules from 1-15 μm to 1-2 μm by passing milk under high pressure through a narrow orifice (Shah, 2003; Lee and Lucey, 2010; Weerathilake et al., 2014). For the result, it gives a counteracting effect for creaming, improves the stability toward partial coalescence, creates desirable rheological properties, and effects on sensory properties such as color, flavor, or mouthfeel (Walstra et al., 2005).

Most yogurt manufacturing uses a two-stage homogenization. The latter stage of two-stage homogenization prevents the clumping of fat globules (Fig. 1.4). Homogenization is carried out at the pressures of 10-20 MPa and 5 MPa in first and second stages, respectively and the temperature used are between 55 and 75 ℃ to prevent crystallization of the fat (Shah, 2003; Walstra et al., 2005).

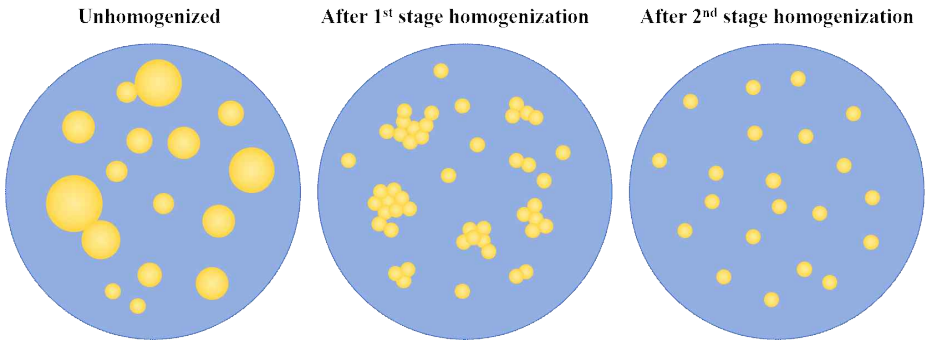


Fig. 1.4. Schematics of fat globules before and after two-stage high pressure homogenization (Bylund, 1995).

(2) Heat treatment

Heat treatment of milk is an important process. Heating is used to destroy most of unwanted microorganisms, inactivates enzymes which may cause undesirable effects, and improves the physical properties following by whey protein denaturation (Lucey, 2002).

Heat treatment is classified into several types based on its intensity; Thermalization, low pasteurization, high pasteurization, sterilization, and preheating. Thermalization is a heat treatment of lower intensity than pasteurization, usually 60-69 ℃ for 20 sec. Low temperature pasteurization requires temperature/time combinations of 63-68 ℃ for 30 min or 72-75 ℃ for 15 sec (HTST, High Temperature Short Time). High temperature pasteurization refers to heat treatment of milk at 85 ℃ for 20 min. Sterilization is the most intense heat treatment that requires temperature of 110 ℃ for 30 min, 130 ℃ for 30 sec, or 145 ℃ for 1 sec (UHT, Ultra High Temperature, short time). Preheating covers most of heating intensities between low pasteurization and sterilization (Walstra et al., 2005). Heat treatment conditions that are generally used in the yogurt industry includes 85 ℃ for 30 min or 90-95 ℃ for 5 min (Robinson, 2003).

3.4.3. Fermentation

When heat treatment is complete, the milk is cooled to incubation temperature. The starter cultures can be inoculated range from 0.5 to 5% (v/v). The optimal level is 1% each of *S. thermophilus* and *Lb.*

delbrueckii subsp. *bulgaricus* (Chandan, 2008). For optimum growth of the thermophilic starter cultures, the inoculated milk is incubated at 40-45 ℃. During the fermentation, bacterial starter cultures converts lactose into lactic acid, which decreases the pH of milk. The endpoint of fermentation is pH 4.5-4.6. After fermentation, yogurt is immediately cooled to prevent unintentional acidification (Lee and Lucey, 2010).

3.5. Formation of gel network during yogurt fermentation

Acidification of milk by bacterial fermentation results in the reduction of net negative charge on casein micelle near its isoelectric point (pH 4.6). The loss of electrostatic repulsion among the casein micelles allows them to aggregate and build the three-dimensional gel network (Tuinier and De Kruif, 2002; Lee and Lucey, 2010)

Fermentation of milk has three stages which occur the major physical changes of gelation (Fig. 1.5 and Fig. 1.6). The first stage, when milk pH is between 6.7 to 6.0, electrostatic repulsion among the casein micelles decreases following the reduction of the net negative charge on the casein surface. Still, the internal structure of the casein micelles is unchanged, because a very small amount of colloidal calcium phosphate (CCP) is solubilized at pH > 6.0 (Lee and Lucey, 2010). Between pH 6.0 to 5.0, casein micelles have major physical changes of the surface and internal structure. The net negative charge of casein micelles considerably decreases, and the

peptide chains of κ-casein shrink. Also, the CCP solubility increases, which causes weakening the micelle internal structure and increases electrostatic repulsion inside the micelle by exposed phosphoserine residues (Lee and Lucey, 2010; Peng et al., 2010). When the pH gets close to pH 4.6 which is the isoelectric point of casein, the surface charge of the casein tends to disappear. The hydrophobic electrostatic interactions among the casein micelles increases, which results in the electrostatic repulsion decreases and attractive force increases. Finally, the milk acidification process results in the three-dimensional gel network with clusters and chains of caseins (Lee and Lucey, 2010).

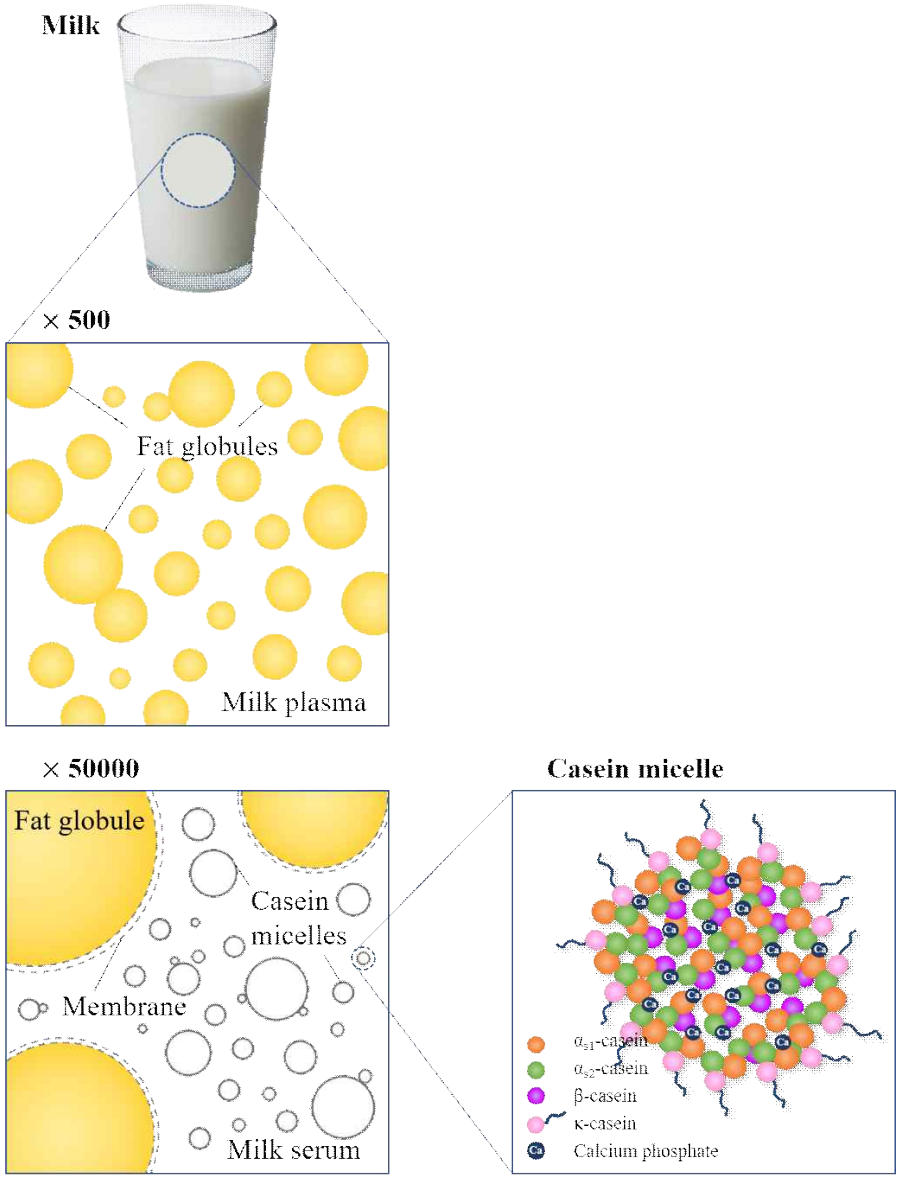


Fig. 1.5. Distribution of fat globules and casein micelles in milk (Walstra et al., 2005).

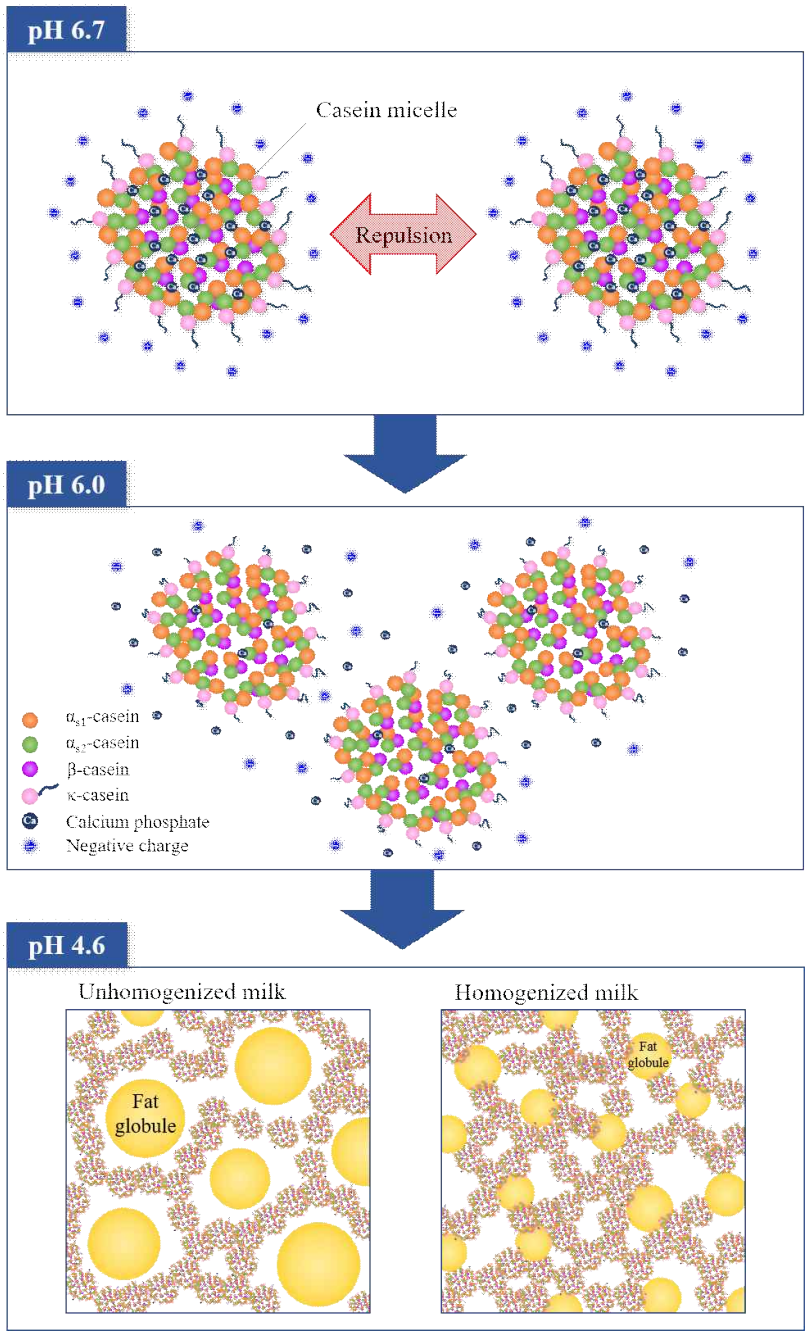


Fig. 1.6. Formation of casein particulate gel network during yogurt fermentation.

3.6. Factors influencing physical properties of yogurt

3.6.1. Total solids

During the milk preparation, supplementation such as skim milk powder (SMP), sodium caseinate, calcium caseinate, whey protein concentrate (WPC), whey protein isolate (WPI), or milk protein concentrate (MPC) is commonly added to increase the total solids content (Shah, 2003). The main purpose of supplementation is to produce firmer texture yogurt and improve the stability of yogurt by reducing whey separation. Also, supplementation increases the firmness and viscosity of set and stirred type yogurt (Jaros et al., 2002; Krasarkoopt et al., 2004). Damin et al. (2009) reported that increasing sodium caseinate resulted in an increase in yield stress, storage modulus, and firmness of nonfat stirred yogurt. As shown in the microstructure of yogurt, the gel structure is mainly composed of protein network that traps other constituents like fat globules (Hassan et al., 2003). Increasing total protein to change in the structure of protein matrix may be the reason for the increase in gel strength or the reduction in syneresis of yogurt (Jaros et al., 2002; Krasarkoopt et al., 2004).

3.6.2. Heat treatment

Heat treatment contributes to improving the rheological and sensory properties of yogurt. Penna et al. (2006) reported that increasing the heat treatment temperature results in a significant increase in consistency index and a decrease in flow behavior index.

In addition, Célia et al. (2017) compared the viscosity and syneresis of yogurt with various heat process (non-heat treatment, pasteurization, and UHT) and reported that intense heat treatment is positive for viscosity and syneresis of final products.

Several studies find out that heat treatment can result in irreversible changes in milk protein structure. When milk is heated above 65 ℃, whey proteins unfold and expose hydrophobic groups including thiol (-SH) groups. Following unfolding, denatured α -lactalbumin and β-lactoglobulin form disulfide linkage with other proteins. Especially, β-lactoglobulin attached to κ-casein in the casein micelles that results in heat-induced protein aggregates. These changes at molecular level suggest that denatured whey proteins contributed to gel characteristics of yogurt (Cano-Ruiz and Richter, 1997; Walstra et al., 2005; Raikos, 2010).

3.6.3. Homogenization

High-pressure homogenization is the non-thermal processing which is applied to improve the rheological properties and syneresis resistance of yogurt, by breaking up the fat globules and denaturation of proteins (Massoud et al., 2016). Serra et al. (2008) studied that high-pressure homogenization improves the density of the gel, aggregation rate, and water retention, which may result in the production of more desirable yogurt. Also, high-pressure homogenization treatment seems to favor the growth of yogurt starter culture, as a result increasing viscosity index and gel firmness of final products (Shah, 2003; Lanciotti et al., 2004).

High-pressure homogenization has three factors that contribute to enhancing the stability of milk. First, high-pressure homogenization decreases the mean diameter of the fat globules, which results in high stability toward partial coalescence according to Stokes' law. For another factor, it also narrows the size distribution down, therefore the speed of creaming of fat globules becomes similar and prevents tendency to cluster together. The last factor is the growth in density of the fat globules because of the absorption of plasma protein following the increase in the surface area of fat globules (Walstra et al., 2005; Massoud et al., 2016).

3.6.4. EPS production

The EPS produced by LAB have potential ability to replace stabilizer, emulsifier, thickening, and gelling agent (Lucey, 2004). EPS-producing starter cultures are preferred for fermented milk products manufacture to improve rheological and sensory properties (Yilmaz et al., 2015). EPS-producing starter cultures have been studied that increases apparent viscosity, storage modulus (G′), and mouth thickness in stirred yogurt or decreases syneresis than using non-EPS-producing starter cultures (Duboc and Mollet, 2001; Folkenberg et al., 2006). However, Hassan et al. (2003) reported the structural breakdown was increased in stirred yogurt made with EPS-producing starter cultures, but immediate syneresis is not shown compared with yogurt without EPS.

The EPS-producing starter culture affects the physical properties of yogurt by engaging their gel network. In set type yogurt, the protein

network is not broken therefore EPS may interfere with gel structure, which leads to a decrease in gel strength or firmness. Meanwhile, in stirred yogurt, disruption of the protein network is highly possible to reduce the effect of the gel network, but increase gel characteristics by EPS (Lucey, 2002).

4. Interactions between milk protein and EPS

4.1. Protein-polysaccharide interactions

Proteins and polysaccharides are present together as two major biopolymers in various food colloids. These macromolecules contribute to the physicochemical properties such as structure, texture, and stability of food through thickening, stabilizing, emulsifying, and gelling behavior (Goh et al., 2020). When proteins and polysaccharides are dispersed in an aqueous phase, one of the four phenomena can occur complex coacervation, co-solubility, thermodynamic incompatibility, and depletion interaction (Goh et al., 2020). The formation, deformation, and solubility of polysaccharide-protein complexes depend on several factors like nature of biopolymers, charge, concentration, pH, ionic strength, or temperature (Ghosh and Bandyopadhyay, 2012).

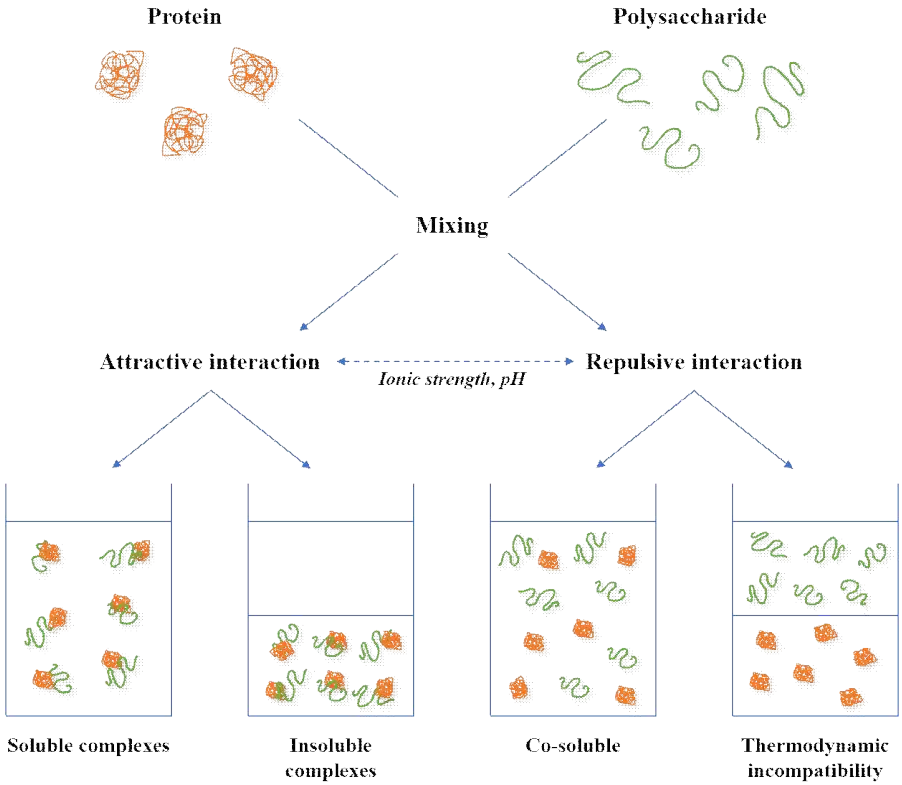


Fig. 1.7. Schematic illustration of the proteins-polysaccharides interaction.

4.1.1. Attractive interactions

Attractive interaction includes complex coacervation which are the result from electrostatic interactions between two oppositely charged biopolymers (Timilsena et al., 2019; Goh et al., 2020). Complex coacervation usually occurs between the isoelectric point of the protein and the pK_a of the polysaccharide in food hydrocolloids (Ye, 2008). At pH values below the isoelectric points, protein molecules have a net positive charge and act like polycation. Then, polysaccharide with anionic carboxyl group behaves as polyanions. As a result, electrostatic interbiopolymer complexes are formed at pH between 2 to 5 (Tolstoguzov, 2002).

There are two types of attractive interaction according to the charge density between the protein and polysaccharide molecules. For soluble complexes, the coacervated polymers may exist as stable suspensions (single phase) in the aqueous medium. Instead, insoluble complexes contain two phases which are a complex matrix with both biopolymers and a depleted biopolymer phase (Goh et al., 2020).

4.1.2. Repulsive interactions

Repulsive interaction includes co-solubility and thermodynamic incompatibility. Co-soluble state is a stable homogeneous single phased solution that two biopolymers do not interact (Goh et al., 2020). When small biopolymers are mixed, like as monosaccharides and hydrophilic amino acids, a co-soluble system is formed. Though, when the mixture exceeds the co-solubility threshold with increasing

molecular weight or concentration of the polymers, the system turns into a thermodynamic incompatibility (Ghosh and Bandyopadhyay, 2012). Thermodynamic incompatibility is occurred by the repulsive interactions between protein and polysaccharide molecules and results in two different phases, a protein-rich and a polysaccharide-rich phase (Tolstoguzov, 2002). Also, depletion flocculation can occur due to incompatibility especially with spherical particles of biopolymers with phase separation (Goh et al., 2020).

4.2. Milk protein-EPS interactions

The stability of solutions mixed with the protein and EPS depends on biopolymer ratio, total concentration, molecular weight, charge, and environmental factors, like solvent, ionic strength, pH, and temperature (van de Velde et al., 2015). At very low concentration of the protein and EPS, two biopolymers exist in a co-soluble state. At higher concentration, the stability of the solution is determined by the interaction between the protein and EPS. When the two biopolymers are compatible with each other, coacervate complexes are formed. On the other hand, if they have the same surface charge, which results in phase separation. Despite the two polymers have repulsive force with similar charges, protein and polysaccharide remain stable in the solution (de Kruif and Tuinier, 2001; Tolstoguzov, 2003).

Most of interaction between polysaccharides and food proteins known as thermodynamic incompatibility (Corredig et al., 2011). When neutral EPS and casein micelles (colloidal spherical particle)

are mixed in a solution, they do not compatible and commonly result in phase separation (de Kruif and Tuinier, 2001). Also, Tuinier et al. (2000) and Weinbreck et al. (2003) reported, in heated milk system, whey protein aggregates act as the spherical colloidal particles, then the non-absorbing EPS lead depletion flocculation to both of casein micelles and whey protein aggregates.

Attractive interactions usually occur between charged EPS and milk protein due to electrostatic attraction. Because the surface charge of milk protein varies with pH, electrostatic interaction between the proteins and EPS also depends on pH (Corredig et al., 2011). Weinbreck et al. (2003) observed electrostatic interaction between whey protein and negatively charged EPS at near isoelectric point of β-lactoglobulin, pH 5.2. The interaction between milk protein and charged EPS may not always be a stable system, because of partial bridging flocculation, especially with gelling polysaccharides. However, the polysaccharide concentration as enough as cover all of the protein molecules, results in stable mixture (Maroziene and De Kruif, 2000).

5. Overall objectives

The aim of this study was to investigate the influence of exopolysaccharide (EPS) produced by *Leuconostoc garlicum* (KCCM 43211) on rheological properties of fermented milk and co-cultured yogurt.

Taking the above objectives, in Chapter 2, the physicochemical properties including molar mass, monosaccharide composition, linkage, and type of EPS during raw milk fermentation are characterized. Also, the effects of EPS on the cell growth and rheological properties of fermented milk with or without homogenization were discussed.

Then, in Chapter 3, raw and homogenized yogurt cultured with *Leuconostoc garlicum*, *Streptococcus thermophilus*, and *Lactobacillus delbrueckii* subsp. *bulgaricus* are stored for 28 days to study the effects of EPS on cell growth, physical properties, and rheological properties. The schematic of the research strategy is in Fig. 1.8.

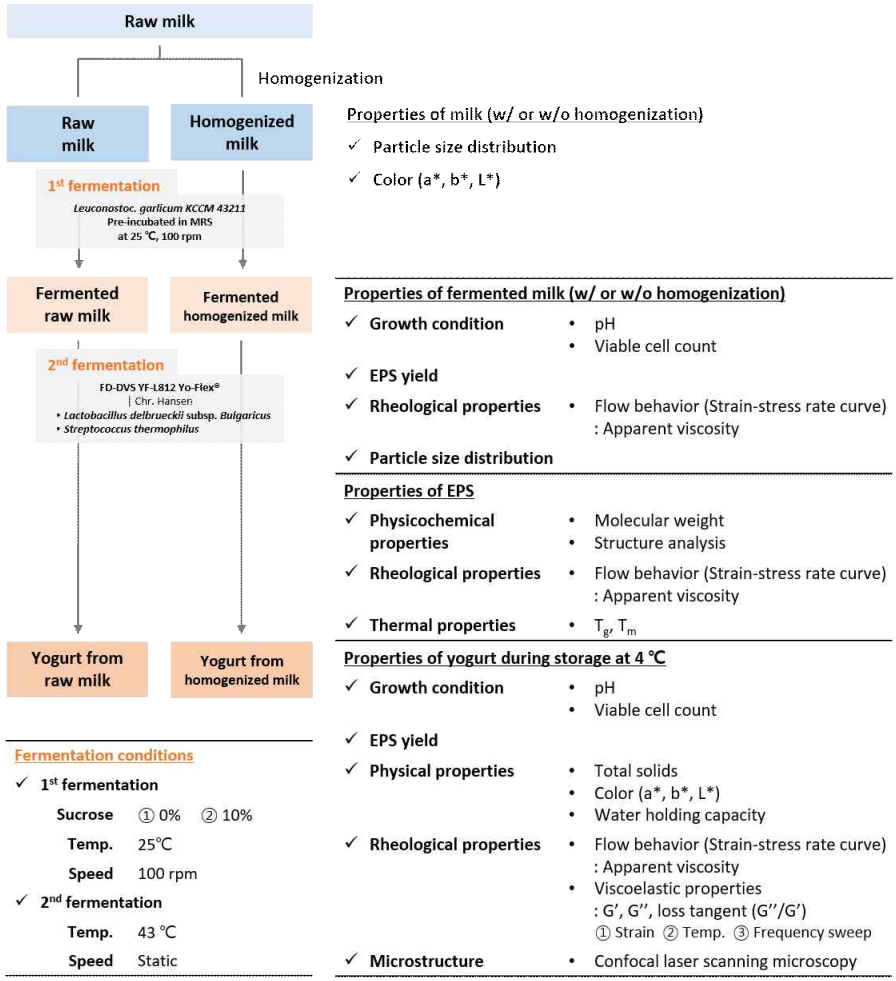


Fig. 1.8. Research strategy to prepare yogurt with *L. garlicum* KCCM 43211.

Chapter 2.

Characterization of milk fermentation by exopolysaccharide-producing *Leuconostoc garlicum* KCCM 43211 isolated from Kimchi

1. Introduction

Some strains of LAB can produce a wide range of EPS during fermentation (Ruas-Madiedo et al., 2002). Interest in EPS produce by LAB is growing because these biopolymers are able to modify the texture, rheological properties, and stability of fermented milk (Duboc and Mollet, 2001). EPS from LAB can be divided into HoPS and HePS by monosaccharides composition. HoPs is composed of only one type of monosaccharide, and HePS is containing various monosaccharides such as glucose, galactose, and rhamnose (De Vuyst and Degeest, 1999; Monsan et al., 2001). HoPS have high molecular weights, up to 10^5 Da, while HePS ranges from 10^4 to 10^6 Da (Ruas-Madiedo et al., 2002). The EPS derived from different LAB shows wide variation in composition, charge, rigidity and ability to interact with proteins (Duboc and Mollet, 2001).

Among the LAB, *Streptococcus*, *Lactobacillus*, and *Leuconostoc* are remarkable due to production of EPS, especially, dextran which is composed of glucose with linear or branched structure (Capek et al., 2011). Dextrans are high molecular size polymers and mostly

consisted of α-(1→6) glycosidic linkages. The structural properties such as type of glycosidic linkage, degree of branch, type of branch, molecular weight distribution, and conformation of chain depend on EPS produced LAB species (Capek et al., 2011). Most of EPS produced by *Leuconostoc* ssp. was glucan, a HoPS composed of repeating units of glucose. The EPS produced by *L. garlicum* PR was a dextran consisting of glucose with α-(1→6) glucopyranose repeating units linked glycosidic links (Capek et al., 2011). Palomba et al. (2012) found that *L. lactis* 95A strain produced dextran consisting of D-glucopyranosyl units with α-(1→6) glycosidic links. Leuconostoc spp. also produces HePS. For example, L .mesenteroides SN-8 was reported to produce a HePS consisting of mannose, glucose, and galactose, which were linked by α-(1→6) glucosidic bonds (Li et al., 2020).

L. garlicum was isolated from garlic surface with the characteristics such as tolerance to high garlic concentration and ability to survive at high temperature by Kim et al. (2002). Among the strains, *L. garlicum* KCCM 43211 which isolated from Kkakdugi (cubed radish Kimchi) is known as that produces highly viscous EPS during sucrose fermentation (Hong et al., 2017).

In this study, raw and homogenized milks were fermented with *L. garlicum* KCCM 43211, an EPS-producing LAB, in the presence of 10% sucrose in order to explore the potential applications of *L. garlicum* KCCM 43211 for yogurt production. The effect of homogenization and sucrose on the milk fermentation, including the

changes in pH, viable cells, flow behavior, and EPS production were investigated. In addition, the EPS produced by *L. garlicum* KCCM 43211 was characterized for monosaccharide composition, molar mass, and chemical structure.

2. Materials and methods

2.1. Materials

L. garlicum KCCM 43211 (Hong et al., 2017) was obtained from Korean Culture Center of Microorganisms (Seoul, Korea). The stock cultures were preserved at -80 ℃ in 15% (v/v) sterile glycerol. The microorganisms were activated in MRS (de Man-Rogosa-Sharpe) medium (Oxoid, Hampshire, UK) at 25 ℃ for 12 h with shaking at 100 rpm. Fresh raw milk was obtained from animal farm of Seoul National University (Pyeongchang, Korea).

2.2. Preparation of fermented milks

Four types of fermented milk were prepared; raw milk fermented with or without sucrose and homogenized milk fermented with or without sucrose (Fig. 2.1).

For the homogenization, milk was heated to 60 ℃ and two-stage homogenized using a high-pressure homogenizer (MN250A, Micronox, Seongnam, Korea). During the first stage, a pressure of 15 MPa was applied, followed using 6.5 MPa during the second

stage. Sucrose was added to raw or the homogenized milk at 10% (w/w). 4 L of each milk sample pasteurized at 68 ℃ for 30 min, cooled to 25 ℃, inoculated with 1 % (v/w) *L. garlicum* KCCM 43211, and divided to 300 mL volumes. The milk samples were incubated at 25 ℃ for 48 h with 100 rpm shaking.

During the fermentation, the milk samples were sampled at 0, 6, 12, 24, 36, and 48 h and analyzed as described below.

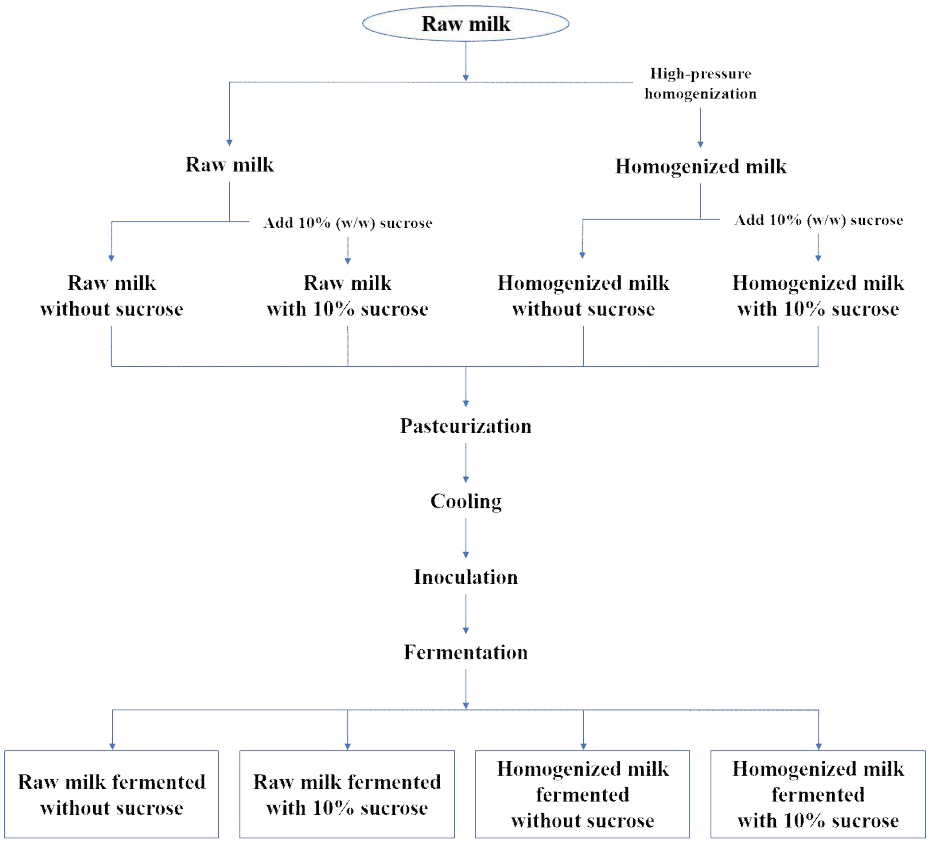


Fig. 2.1. Preparation of fermented milk samples.

2.3. Particle size analysis

The volume-weighted mean diameter ($d_{4,3}$) and particle size distribution of raw and homogenized milks were determined by laser diffraction particle size analyzer model (1190, Cilas, Buffon, France) based on Fraunhofer theory. The two milks were diluted in distilled water until an appropriated obscuration (10-15%) was obtained in the diffractometer cell.

2.4. Color and pH measurements

The color of milk samples was assessed using CIE-L*a*b* system. The measurements were done using a CR-400 chromameter (Konica Minolta, Osaka, Japan). The L* value represents the lightness from 0 (black) to 100 (white), the a* value represents the degree of redness (+a*) or greenness (-a*) and the b* value represents the degree of yellowness (+b*) or blueness (-b*). Calibration was carried out with a white calibration plate. The pH of milk samples was measured using a pH meter (Starter 3100, Ohaus Instrument Co., Ltd., Parsipanny, NJ, USA).

2.5. Determination of viable cell count

A volume of 1 mL of each milk sample was suspended in 9 mL of sterile 0.85% (w/w) NaCl solution and mixed uniformly, followed by serial dilution by a factor of 10 to 10^6. Each dilution (100 μL) was plated on MRS agar and incubated at 25 ℃ for 72 h. The viable cell were counted and expressed as colony forming units

(CFU) per mL of sample.

2.6. Determination of flow behavior and apparent viscosity

The flow curves (shear stress versus shear rate) were obtained for the milk samples using a hybrid rheometer (DHR-3, TA Instruments Inc., New Castle, DE, USA), equipped with a cone and plate geometry (60 mm diameter and 2.0˚ cone angle). Each milk sample was gently stirred and placed on the bottom plate. The top plate was slowly lowered until the gap was 1,000 μm, and excess sample was removed. The temperature was maintained at 25 ℃ using a circulating water bath. A flow sweep was conducted in a shear rate range from 0.1 to 250 s^{-1}. The apparent viscosity (η_{app}, mPa · s) was calculated at a shear rate 100 s^{-1}.

2.7. Isolation and purification of EPS

EPS was isolated from the fermented milk samples according to the method described by Prasanna et al. (2012) with some modifications. The solution of 60% (w/w) trichloroacetic acid (TCA) was added to 100 g of each milk sample to give a final TCA concentration of 12% (w/w) for protein precipitation. After 30 min of gentle stirring, protein precipitates were removed by centrifugation at 9,588 × g for 30 min at 4 ℃. The supernatants were collected and added to a three-fold volume of 99.9% cold ethanol and kept overnight at 4 ℃ to precipitate EPS. After centrifugation at 9,588 × g for 30 min at 4 ℃, the pellets were collected. The crude EPS

was obtained by repeating the previous steps, followed by freeze drying. The yield of EPS was expressed as g crude EPS/kg milk sample.

For the purification of EPS, the pellets of crude EPS from the raw milk fermented with 10% sucrose were dissolved and dialysed against deionized water at 4 ℃ for 2 days with two-time water change per day, using a dialysis membrane tube of molar mass cut-off value of 1,000 Da (Spectra/Por® 6, Repligen Corp., Waltham, MA, USA). The purified EPS pellets were precipitated using cold ethanol and freeze-dried for further characterization.

2.8. Monosaccharide composition of EPS

Monosaccharide composition was determined by hydrolyzing EPS sample in trifluoroacetic acid. Analysis was carried out using a High-performance anion-exchange chromatography (HPAEC) system (Dionex Corp., Sunnyvale, CA, USA) equipped with pulsed amperometric detection (PAD) and CarboPac^{TM} PA1 column. The monosaccharide was eluted by linear gradient of NaOH solution (18 mM and 200 mM) and the flow rate was 1mL/min. The reference monosaccharides (fucose, rhamnose, arabinose, galactose, glucose, xylose, and fructose) were chosen as standards.

2.9. Molar mass determination

The molar mass of the EPS were measured by gel-permeation chromatography (GPC) in Ultimate 3000 Dionex HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) with refractive index

(RI) detector using Waters Ultrahydrogel column (Waters Corp., Milford, MA, USA). Lyophilized EPS was dissolved by deionized water and 20 μL of sample were injected. Operation temperature was 40 ℃ and flow rate was 1 mL/min. The EPS molar mass (Da) were determined using linear regression equations constructed using pullulan standards of known molecular mass, 342 to 803,000 Da.

2.10. Nuclear magnetic resonance (NMR) spectra

Lyophilized sample was dissolved in D_2O, and then freeze dried. High-resolution ^{1}H NMR and ^{13}C NMR spectra were performed at 600 and 150 MHz, respectively, on a NMR spectrometer (Avance 600, Bruker, Germany) equipped with 5 mm broad band probe. Homonuclear 1H/1H correlation spectroscopy (COSY) and heteronuclear 1H/13C correlation experiments (HMBC and HSQC) were run using the standard Bruker pulse sequence.

2.11. Statistical analysis

All experiments were performed in triplicate, and the statistical process of the experimental results was analyzed using SPSS 26.0 program (SPSS Inc., Chicago, IL, USA). The results treatment were analyzed with Kruskal-Wallis test using SPSS 26.0, and the significance of the average values ($p \leq 0.05$) was determined using Mann-Whitney test.

3. Results and discussion

3.1. Effect of homogenization on milk properties

Figure 2.2 shows the effect of homogenization on the particle size of milk. In raw milk, the peak started at 0.2 μm, likely refering to the casein micelles, and the major peak was found at 4.0 μm, corresponding to milk fat globules (Amador-Espejo et al., 2014). After the homogenization, definite changes were observed in particle size distribution. First, the fat globule mean diameter ($d_{4,3}$) was reduced from 3.17 μm to 0.98 μm, which also confirmed by considerable increasing in the peak of around 1.0 μm in Fig. 2.2. Also, span value which indicates the width of the size distribution was decreased from 0.968 to 0.804 with significant difference ($p \leq 0.05$). The results support other studies that high-pressure homogenization reduces the fat globules diameter from 1-15 μm to 1-2 μm (Shah, 2003; Lee and Lucey, 2010; Weerathilake et al., 2014).

Figure 2.3 shows the effect of homogenization on milk color. Homogenization increased the lightness (L*) of milk compared to raw milk from 88.54 to 92.48. The redness (a*) significantly increase by homogenization ($p \leq 0.05$), while yellowness (b*) values showed no significant difference ($p > 0.05$). Walstra et al. (2005) reported that homogenization makes milk whiter by increasing the number of scattering particles. The result that homogenization increases the redness of milk have not been reported. However,

Chudy et al. (2020) reported that milk fat contained β-carotene which has various color from yellow to deep red-orange. The increase in redness might be occurred by exceeding of β-carotene from fat globule membrane by homogenization.

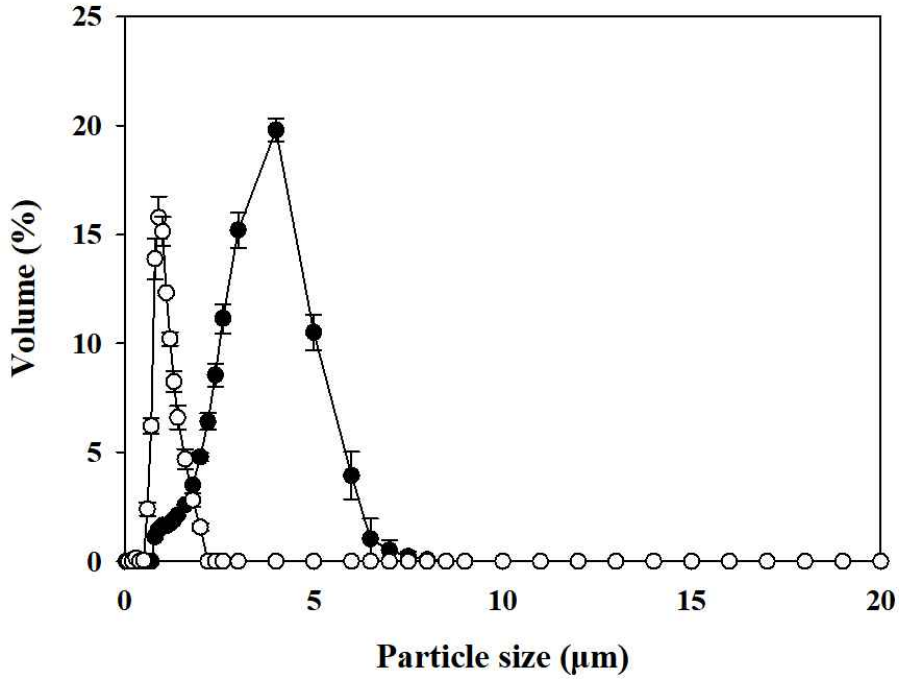


Fig. 2.2. Particle size distribution of raw milk (●) and homogenized milk (○). High pressure homogenization was performed in two stage at 15 MPa and 6.5 MPa.

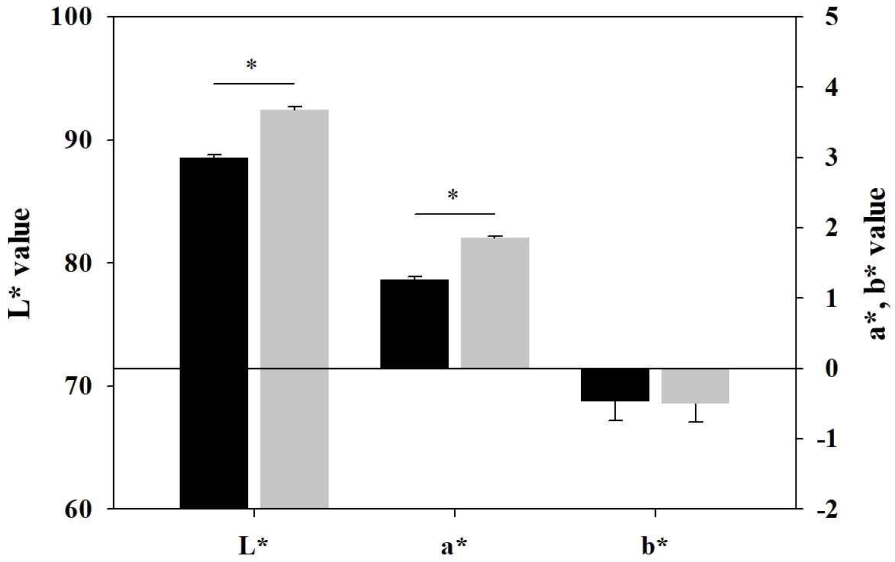


Fig. 2.3. Color attributes (CIE L*, a*, and b*) of raw milk (■) and homogenized milk (■). High pressure homogenization was performed in two stage at 15 MPa and 6.5 MPa. Asterisks indicate significant differences between the values ($p \leq 0.05$).

3.2. Effects of homogenization and sucrose on fermentation

Figure 2.4a shows the changes in pH during the fermentation of raw and homogenized milks in the presence or absence of sucrose. For the all four types of sample, the pH value decreased from 6.47-6.70 to 5.40-5.67. The decrease of pH may be attributed to the production of lactic acid by L. garlicum KCCM 43211 (Hong et al., 2017). After 48 h fermentation, the homogenized milk showed significantly lower pH values than the raw milk regardless of sucrose presence ($p \leq 0.05$). This is probably because homogenization treatment induces fat autoxidation by changes in the surface layer of fat globules such as increased surface area which contacts with residual oxygen (Walstra et al., 2005; Chudy et al., 2015).

Figure 2.4b shows the growth of *L. garlicum* KCCM 43211 in raw and homogenized milk in the absence or presence of sucrose. The bacterial growth was fast during the first 6 h and then entered stationary phase. After 12 h fermentation, mean viable cell counts in raw and homogenized milks fermented without sucrose reached 1.67×10^8 and 1.36×10^8 CFU/mL, respectively. Meanwhile, much higher cell counts were obtained when the milks were fermented in the presence of sucrose (1.15×10^9 CFU/mL for raw milk and 9.50×10^8 CFU/mL for homogenized milk). The results showed that the bacterial growth strongly depended on the presence of sucrose rather than the homogenization.

3.3. Effects of homogenization and sucrose on apparent viscosity and EPS production

Figure 2.5 shows the flow curves of the fermented milks during 48 h fermentation with *L. garlicum* KCCM 43211 by shear stress versus shear rate. Raw milk fermented without sucrose and fermented milks regardless of sucrose presence showed linear characteristic indicates Newtonian behavior as the apparent viscosity kept constant values with the increase in shear rate (Bylund, 1995) for all fermentation time. Raw milk fermented with 10% sucrose showed Newtonian behavior for 0, 6, and 24 h fermentation, however, it changed to slight logarithm characteristic which indicates pseudoplastic (shear-thinning) behavior for 12, 36, and 48 h fermentation time.

Figure 2.4c shows the apparent viscosity during the fermentation. Raw milk fermented and homogenized milk fermented without sucrose had no significant changes in apparent viscosity during 48 h fermentation. The initial apparent viscosity of raw milk fermented and homogenized milk fermented without sucrose was 0.82 mPa · s and 0.97 mPa · s, respectively, the final value was 0.87 mPa · s and 0.98 mPa · s, which is nearly not changed as a Newtonian fluid. The initial apparent viscosity of raw fermented milk and homogenized fermented milk with 10% sucrose was 1.08 mPa · s and 1.29 mPa · s. It presumed that the slight difference followed by sucrose concentration is due to the viscosity of sucrose itself in fermented milks. The maximum apparent viscosity under presence of

sucrose was 7.29 mPa · s in raw milk fermented and 2.93 mPa · s in homogenized milk fermented at 48 h. Contrary to this result, homogenization is known as increase the apparent viscosity in the fermented milk (Lee and Sherbon, 2002; Walstra et al., 2005; Li et al., 2018). In our study, final pH was only 5.64 in raw milk fermented and 5.40 in homogenized milk fermented in presence of sucrose. On the other hand, most of fermented milk pH is below 4.6, the point which is casein micelles forming gel network. Homogenized fat globules is becoming the part of gel network to make dense and stable gel network, which producing higher apparent viscosity (Walstra et al., 2005).

Figure 2.4d shows the EPS production properties of *L. garlicum* KCCM 43211 grown in fermented milks at 25 ℃. *L. garlicum* KCCM 43211 barely produce EPS in both of raw milk fermented and homogenized milk fermented without sucrose, the maximum amount of EPS is 0.47 g/kg milk sample and 0.30 g/kg milk sample, repectively. The presence of polysaccharide was found in both fermented milks with 10% sucrose, as indicated by ethanol precipitation. As this result, EPS production by *L. garlicum* KCCM 43211 was clearly affected by the sucrose concentration (Hong et al., 2017). The rate of EPS production increased with the increase of apparent viscosity. The amount of EPS increased throughout the 48 h fermentation time, and reached to 27.29 and 22.91 g/kg milk sample in raw fermented milk and homogenized fermented milk with 10% sucrose, respectively. Differnce in the apparent viscosities were reflected in the difference in EPS yields. The EPS production of raw

milk fermented was only 1.2-fold higher than homogenized milk fermented, however, the apparent viscosity values had 2.5-fold differences. Accordingly, the presence of EPS surely improve the viscosity, but it is assumed that the large fat globule also act as resistance to the flow.

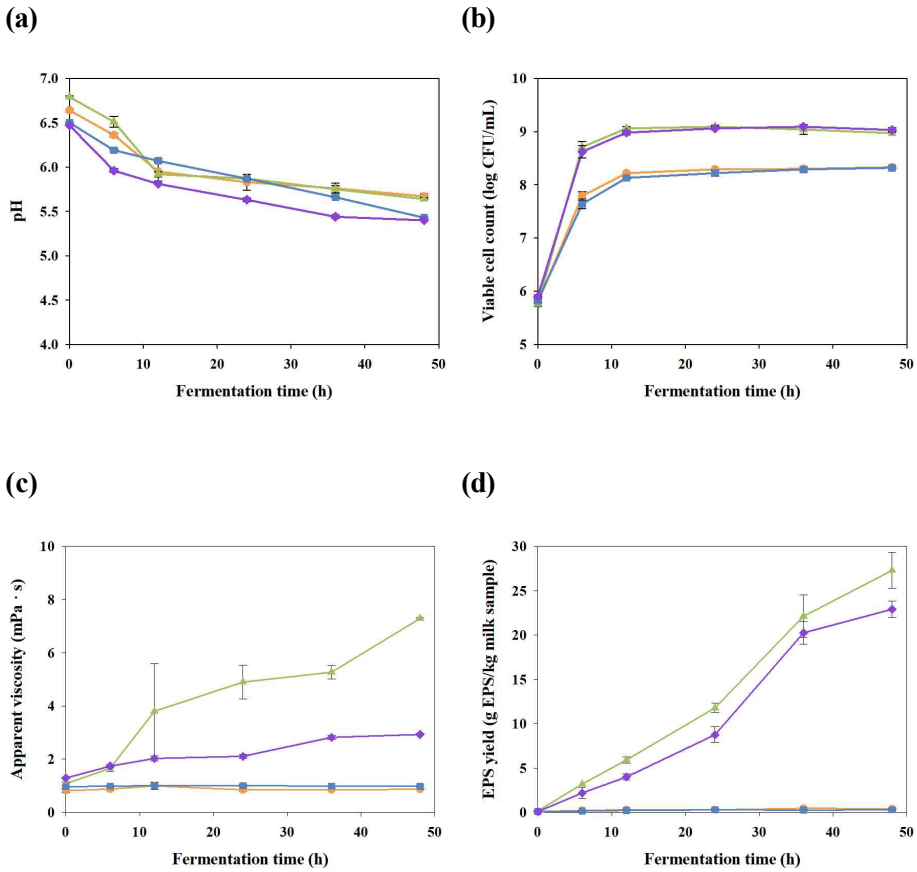


Fig. 2.4. Changes in (a) pH, (b) viable cell count, (c) apparent viscosity, and (d) EPS yield during the fermentation with *L. garlicum* KCCM 43211 of raw milk without sucrose (●), raw milk with 10% sucrose (▲), homogenized milk without sucrose (■), and homogenized milk with 10% sucrose (◆).

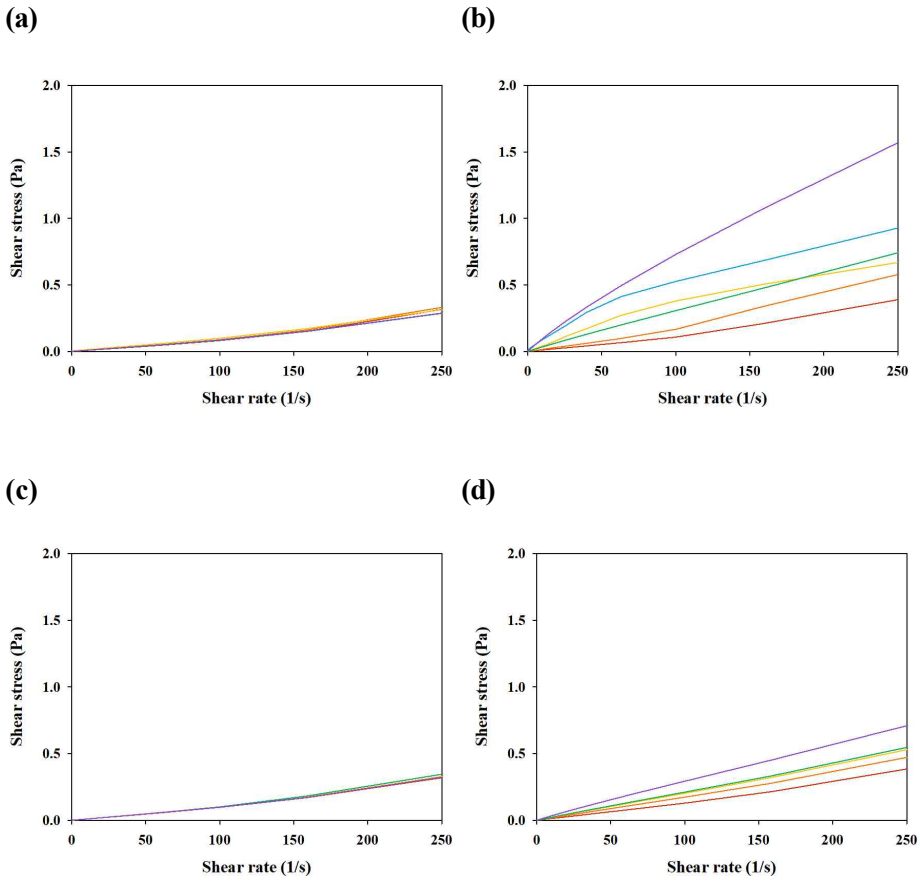


Fig. 2.5. Flow curve (shear stress versus shear rate) of (a) raw milk fermented without sucrose, (b) raw milk fermented with 10% sucrose, (c) homogenized milk fermented without sucrose, and (d) homogenized milk fermented with 10% sucrose at 0 h (red line), 6 h (orange line), 12 h (yellow line), 24 h (green line), 36 h (blue line), and 48 h (purple line) fermentation time.

Fig. 2.6. EPS produced by *L. garlicum* KCCM 43211 on Elliker agar plate with viscous translucent status.

3.4. Characteristics of EPS produced by *L. garlicum* KCCM 43211

3.4.1. Monosaccharide composition

Figure 2.7 shows monosaccharide composition analysis that the EPS only contained glucose, and other monosaccharides such as fucose, rhamnose, arabinose, galactose, xylose, and fructose were not detected. This results showed that EPS was a homoexopolysaccharide (HoPS) composed of glucose as expected by Hong et al. (2017). Most of EPS produced by *Leuconostoc* spp. is known as glucan or dextran which is composed of glucose, but some EPS exhibits various monosaccharide composition including mannose, glucose, and galactose (Yang et al., 2020) depending on the strains and the medium environments.

3.4.2. Molar mass of EPS

Figure 2.8 shows the chromatogram of EPS that indicated a symmetrical narrow peak, confirming the homogeneity of the EPS sample. The molecular weight of EPS was calculated to be 2.9×10^6 Da. EPS exhibited a narrow-dispersed peak as shown in the elution profile of GPC with the polydispersity index (M_w/M_n) of 1.98. The molecular characteristics of EPS derived from GPC, including molecular weight, listed in Table 2.1.

3.4.3. Chemical structure of EPS

Figure 2.9 shows the ^{1}H-NMR spectrum of EPS. ^{1}H signals were

observed at 4.97, 3.57, 3.71, 3.51, 3.90, and 3.74/3.98 ppm which were ascribed as H-1, H-2, H-3, H-4, H-5, and H-6a/b, repectively. The ^{1}H spectrum showed a typical dextran α-(1→6) chain-extending anomeric signal centered at 4.97 ppm (Bounaix et al., 2009). Two small anomeric protons were also assigned to α-(1→3) and α-(1→2) glucosidic residues.

The ^{13}C spectrum included anomeric carbon (95-110 ppm) and ring carbons regions (50-85 ppm) (Fang et al., 2018). In the ^{13}C-NMR spectrum (Fig. 2.10), the carbon was assigned at 97.77, 71.46, 73.46, 69.91, 70.24, and 65.64 ppm for C-1, C-2, C-3, C-4, C-5, and C-6, repectively. The major resonance in the anomeric regions occur at about 90 ppm indicated that the C-1 is linked. Also, The signal at 60 ppm indicated that most of the C-6 is linked (Majumder et al, 2009). From HSQC spectrum, single-bond correlations between the protons and the corresponding carbons obtained, H1/C1, H2/C2, H3/C3, H4/C4, H5/C5, H6a/C6, and H6b/C6 (Fig. 2.11) (Agrawal, 1992).

On the basis of the above spectrum data, the EPS from *L. garlicum* KCCM 43211 was linear dextran with α-(1→6) linkages and with a few α-(1→2) and α-(1→3) branching glucosidic linked residues.

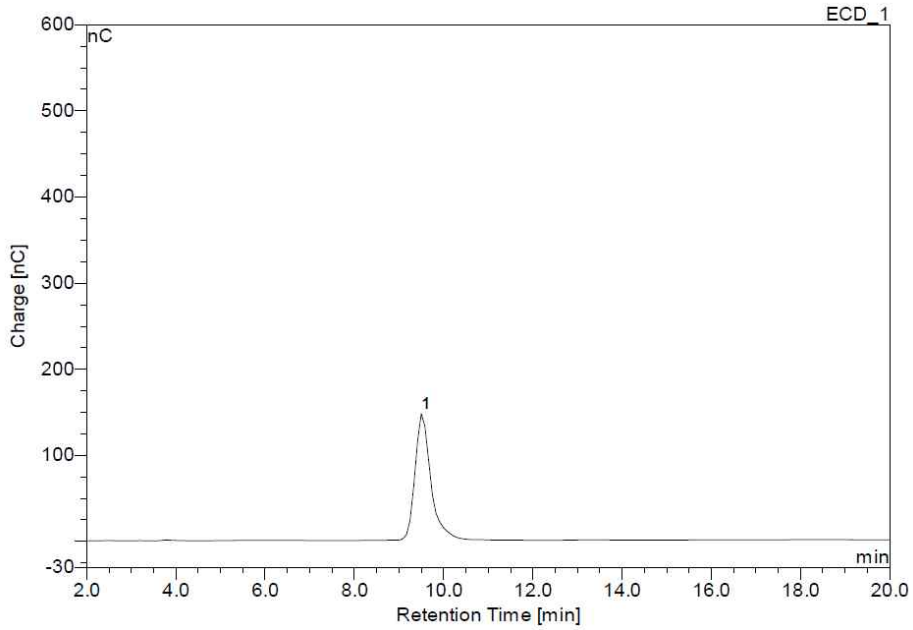


Fig. 2.7. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) chromatogram of the EPS produced during the milk fermentation with *L. garlicum* KCCM 43211.

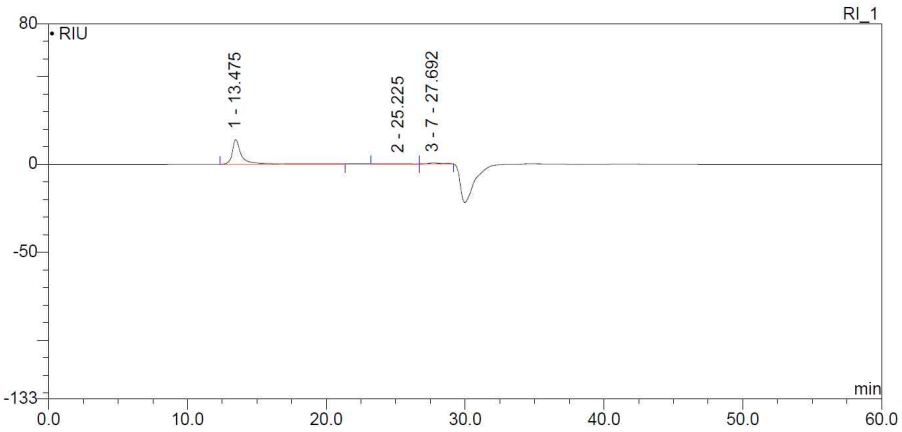


Fig. 2.8. Gel permeation chromatography (GPC) chromatogram of EPS produced during the milk fermentation with *L. garlicum* KCCM 43211. Retention time is 13.475, 25.225, and 27.692 min for peak 1, 2, and 3, respectively.

Table 2.1. Weight average molar mass (M_w) of EPS procuced from *L. garlicum* KCCM 43211.

Parameter	M_w (Da)		
	Peak 1	Peak 2	Peak 3
M_w	2943715	3242	357
% Total peak area	91.69	1.42	6.89

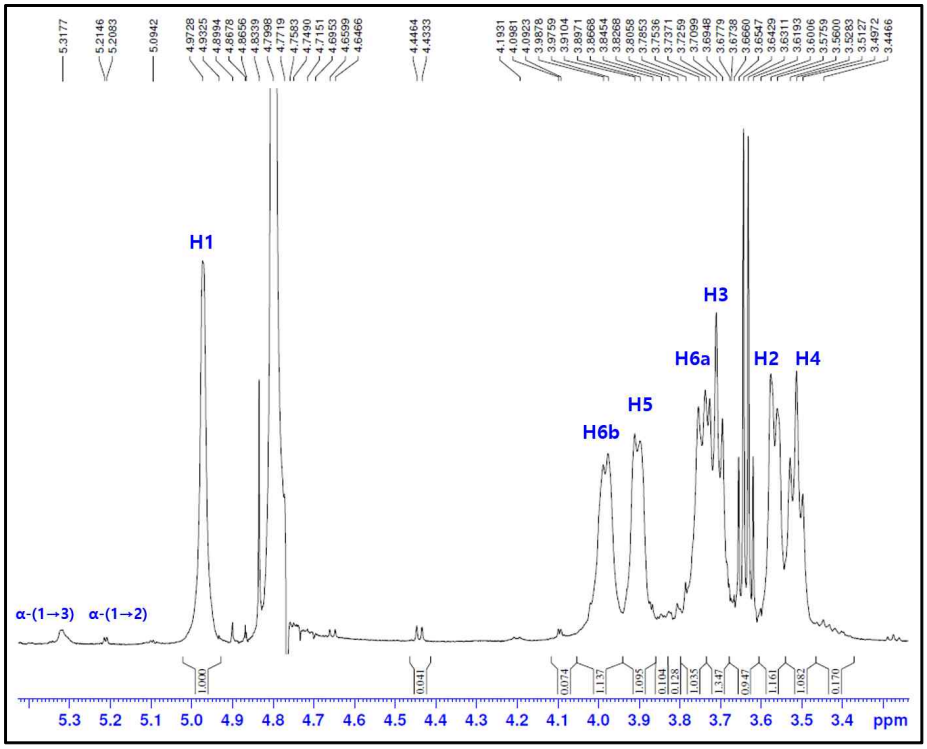


Fig. 2.9. The 1D ^{1}H nuclear magnetic resonance (NMR) spectrum of EPS produced by *L. garlicum* KCCM 43211, recorded at 600 MHz in D_2O. The peaks are referenced to internal ethanol.

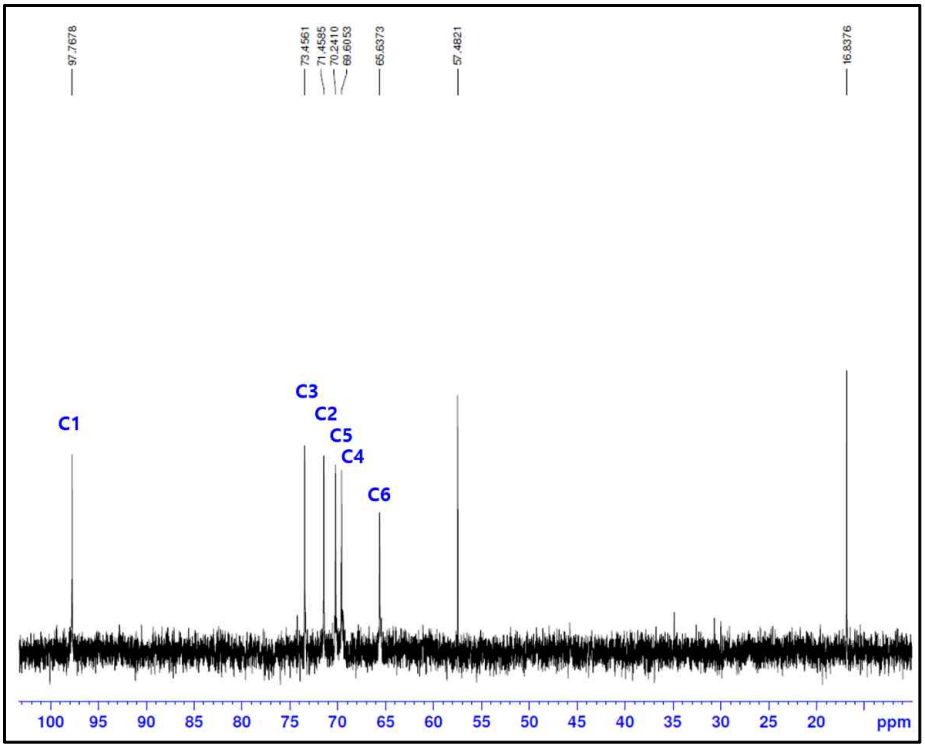


Fig. 2.10. The 1D ^{13}C nuclear magnetic resonance (NMR) spectrum of EPS produced by *L. garlicum* KCCM 43211, recorded at 150 MHz in D_2O.

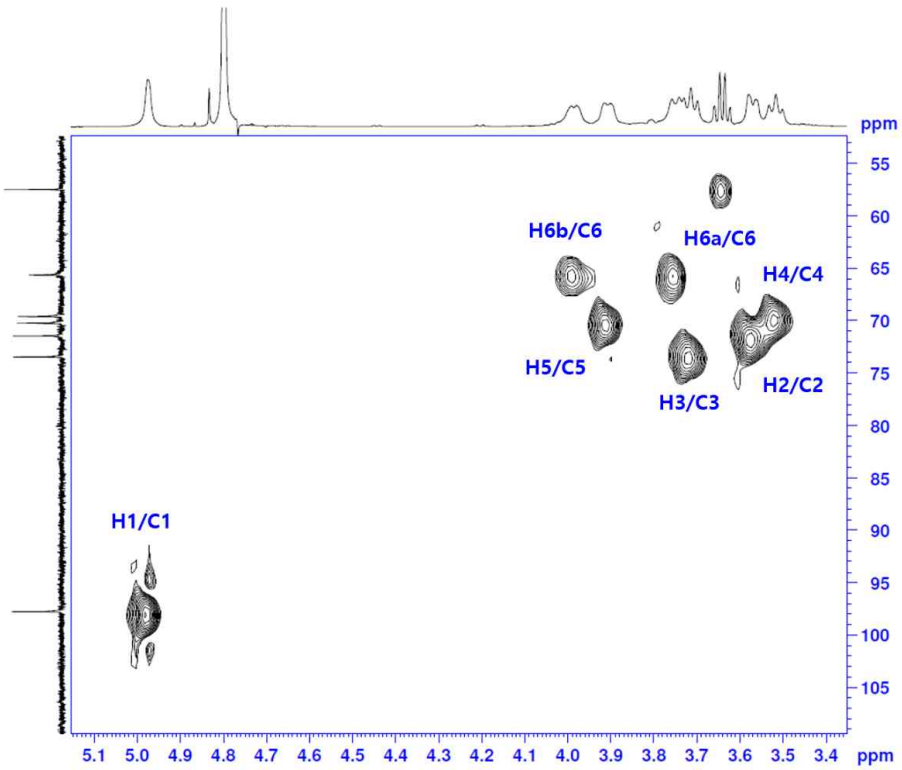


Fig. 2.11. Heteronuclear single quantum coherence spectroscopy (HSQC) spectrum of EPS produced by *L. garlicum* KCCM 43211 recorded at 600 MHz in D_2O.

4. Conclusions

L. garlicum KCCM 43211 shown the remarkable growth in both fermented milks regardless of the presence of sucrose. In the raw milk fermented and homogenized milk fermented, the maximum growth was 10^8 CFU/mL without sucrose, and 10^9 CFU/mL with 10% sucrose. Also, pH reduction in all fermented milks was observed followed by lactic acid production throughout 48 h fermentation. As shown in Fig. 2.4d, without sucrose, EPS could not be produced from *L. garlicum* KCCM 43211 in raw milk fermented and homogenized milk fermented. In contrast, with the presence of 10% sucrose, the yield of EPS increased during the fermentation in both fermented milks. EPS production by *L. garlicum* KCCM 43211 was maximal with raw milk fermented with 10% sucrose as 27.29 g/kg medium at 48 h fermentation. As indicated in Fig. 2.4c and 2.4d, production of EPS by *L. garlicum* KCCM 43211 improved on the rheological properties of fermented milk with 10% sucrose. Apparent viscosity of raw milk fermented and homogenized milk fermented with 10% sucrose increased proportionally with the EPS production. Higher EPS contents resulted in higher apparent viscosity.

EPS samples isolated from the raw milk fermented with 10% sucrose and purified to chemical analysis. The monosaccharide composition analysis revealed *L. garlicum* KCCM 43211 produced EPS is HoPS composed of glucose. ^{1}H and ^{13}C NMR showed that EPS produced by *L. garlicum* KCCM 43211 is linear and neutral dexran linked by α-(1→6) glycosidic bonds with a few α-(1→2) and

α-(1→3) linked residues. In addition, the molecular mass was confirmed in 2.9 × 10^6 Da. The rheological behavior of fermented milk was determined by both the dispersed and continuous phase. The continuous phase was responsible for the viscoelastic properties, whearas the dispersed phase accounted for viscosity (Rao, 2010). The linear high molecular EPS was determined affect on apparent viscosity of fermented milk. This result can be explained EPS could be solved in dispersed phase as viscosifier.

Chapter 3.

Effects of pre-fermentation with exopolysaccharide -producing *Leuconostoc garlicum* KCCM 43211 on yogurt properties during cold storage

1. Introduction

Yogurt is one of the most popular fermented dairy products (Mckinley, 2005). Yogurt is manufactured by LAB including *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*. During the fermentation, these two species have symbiotic relationship, as a result, LAB is synthesized from lactose, causing a decrease in pH and producing gel network (Nagaoka, 2019). There are factors related to yogurt formation such as heat treatment, homogenization, starter culture, fermentation temperature, and presence of supplementation which are known as important to determine yogurt texture (Lee and Lucey, 2010). The syneresis of yogurt may occur during storage, caused by the rearrangement of casein gel network in yogurt (Macit and Bakirci, 2017). In order to prevent structure deformation, stabilizers are added to interact with the casein network (Nobuhara et al., 2014).

EPS is high-molecular-mass biopolymer produced during microorganism metabolism (Gentès et al., 2013; Wang et al., 2015). EPS is widely used to improve the rheological quality of stirred

yogurt (Walstra et al., 2005). Also, EPS-producing strain improves the final texture of the fermented dairy product. Since EPS influenced to a polymer-like behavior of dispersed phase, which could prevent syneresis and increase viscosity of yogurt (Gentès et al., 2013).

The objective of this study was to investigate the effects of pre-fermentation with *L. garlicum* KCCM 43211, an EPS producing LAB, on yogurt properties during the storage at 4 ℃ for 28 days, in order to explore the potential application of *L. garlicum* for yogurt production.

2. Materials and methods

2.1. Materials

Two starter cultures are used for the experiment. *L. garlicum* KCCM 43211 is used to obtain the EPS and YoFlex® YF-L812 (Christian Hansen Lab., Hørsholm, Denmark) which is included *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* is used to produce the yogurt. *L. garlicum* KCCM 43211 are pre-incubated in MRS broth for 12 h at 25 ℃ with shaking of 100 rpm. YF-L812 is direct vat set (DVS) starter which can be directly added to the milk.

2.2. Preparation of yogurt

Four types of yogurt were prepared (Fig. 3.1); yogurts prepared by the pre-fermentation of raw and homogenized milks with *L. garlicum* followed by the main fermentation with a yogurt starter culture and two control yogurts prepared by the fermentation of raw and homogenized milks with a yogurt starter culture without the pre-fermentation (Raw (only main) and Homo (only main)).

To produce Raw (pre+main) and Homo (pre+main), raw milk and homogenized milk with 10% sucrose are prepared followed by Chapter 2, Section 2.2. After inoculating 1% of *L. garlicum* KCCM 43211, milk samples are incubated in 25 ℃ for 12 h with 100 rpm shaking to obtain EPS in fermented milks. After 12 h, pre-fermented milk samples and pasteurized raw milk and homogenized milk with 10% sucrose are ready. YF-L812 was inoculated in all milk samples

with the producers recommended concentration, 0.0472 g per L of milk. After gently stirring 5 min, samples are divided into 300 mL volumes. The fermentation process was carried out in an incubator at 43 ℃. When pH reached to 4.6, samples were stirred manually for 1 min to obtain stirred yogurts. The stirred yogurt samples were poured into 100 mL sterile polyethylene terephthalate (PET) bottle and closed with the lids provided with the bottle. Afterward, samples were stored in refrigerator at 4 ℃ and samples were taken for analysis after 1, 7, 14, 21, and 28 days.

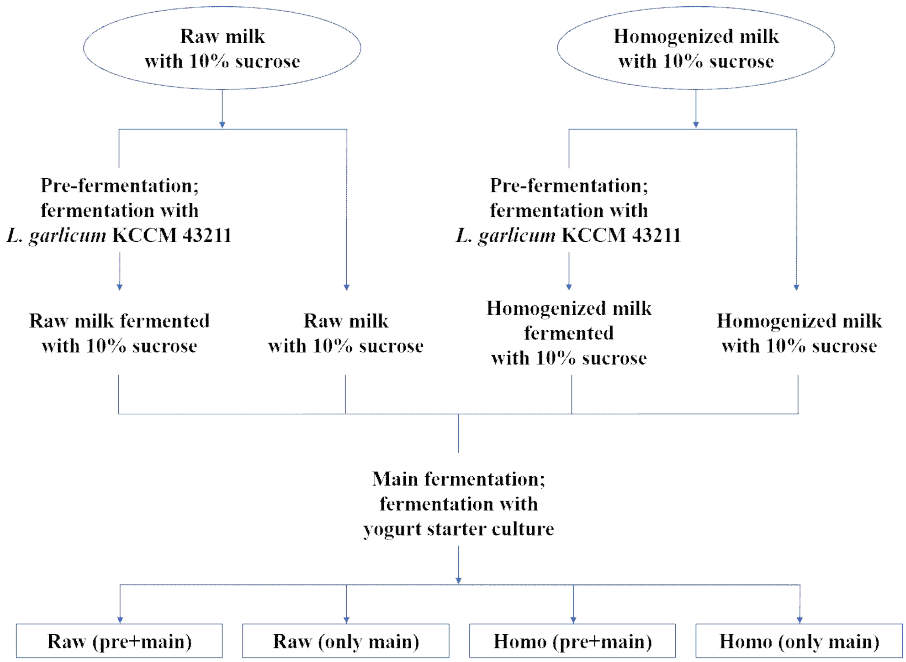


Fig. 3.1. Preparation of four types of yogurt samples.

2.3. Determination of viable cell count

The counts of *L. garlicum*, *S. thermophilus*, and *Lb. delbrueckii* ssp. *bulgaricus* was carried out by plating serial dilution of yogurt at 1, 7, 14, 21, and 28 days during storage. 1 mL of yogurt sample was decimally diluted in sterile 0.85% (w/w) NaCl solution. After uniform mixing, subsequent serial decimal dilutions were prepared in 900 μL of sterile NaCl solution, followed by inoculation of 100 μL of each dilution in two Petri dishes. The enumeration of *L. garlicum*, *S. thermophilus*, and *Lb. bulgaricus* was performed using Elliker agar (Kisan Bio, Seoul, Korea). The plates was incubated at 37 ℃ for 72 h. The results were expressed as colony forming units (CFU) per mL of yogurt sample.

2.4. Total solid measurement

Total solids is caculated from total moisture content, which is described in Korea Food Code. Approximately 2 g of yogurt samples were weighed in crucible and subsequently placed into atmospheric oven at 105 ℃ for 5 h. Then, the crucibles were gently moved to dessicator to cool down. After 30 min, the crucibles were weighed until get constant weight.

$$\text{Total solids}\ (\%, \text{w/w}) = 100 - \left(\frac{\text{Weight after drying} - \text{Weight of empty crucible}}{\text{Weight before drying} - \text{Weight of empty crucible}} \times 100\right) \quad (1)$$

2.5. Color and pH measurements

The color of yogurt samples was analyzed using CIE L*a*b* system. The measurement method was carried out as described in Chapter 2,

Section 2.4. The pH was measured using a pH meter (Starter 3100, Ohaus Instrument Co., Ltd., Parsipanny, NJ, USA).

2.6. Determination of water holding capacity (WHC)

The water holding capacity of yogurt was determined by a centrifuge method. 30 g of yogurt samples at 222 × g for 10 min at 4 ℃ using 1236R centrifuge (Labogene, Daejeon, Korea). Whey expelled during each centrifugation was removed and the pellet was weighed.

$$\text{WHC}\ (\%, \text{w/w}) = \frac{\text{Weight of tube with pellet} - \text{Weight of empty tube}}{\text{Weight of tube with sample} - \text{Weight of empty tube}} \times 100 \quad (2)$$

2.7. Isolation of EPS

EPS isolation was followed ethanol precipitation method, which was described in Chapter 2, Section 2.8

2.8. Rheological analysis

Rheological analysis of the yogurt samples was done by using a hybrid rheometer (DHR-3, TA Instruments Inc., New Castle, DE, USA), equipped with a cone and plate geometry (60 mm diameter and 2.0˚ cone angle). Each yogurt sample was gently stirred before testing the rheological properties and placed on the bottom plate. The top plate was slowly lowered until the gap was 1000 μm. Excess sample was removed. The temperature of the plates was maintained at 4 ℃ by a circulating water bath. Rheological measurements were performed at 1, 7, 14, 21, and 28 days after yogurt production and the sample is replaced to a fresh sample for each analysis.

2.8.1. Strain sweep test

Amplitude sweep was first carried out in a controlled strain mode with applied strain at a fixed range 0.01 to 300.0 strain % to determine the linear viscoelastic range (LVR) of the yogurt samples at constant frequency of 1.0 Hz.

2.8.2. Determination of flow behavior and apparent viscosity

The flow curve was produced by measuring the shear stress as a function of shear rates from 0.1 to 250 s^{-1}. Apparent viscosity (η_{app}) was caculated at 100 s^{-1} shear rate.

2.8.3. Frequency sweep test

To determine the viscoelasic properties of yogurt, frequency sweep tests were conducted at a constant strain of 0.1%, and frequency of oscillation ranged from 0.01 to 100 Hz. Dynamic moduli (G′; storage modulus, G″; loss modulus) and tan δ were caculated using the TRIOS software (TA Instruments Inc., New Castle, DE, USA).

2.8.4. Temperature sweep test

Dynamic temperature ramp test were performed at stress and frequency of 0.1 strain % and 1.0 Hz, repectively, in a temperature range of 4 to 50 ℃ (heating) and 50 to 4 ℃ (cooling), at a rate of 5 ℃/min. Storage modulus (G′) and loss modulus (G″) were recorded as a function of temperature.

2.9. Statistical analysis

All experiments were performed in triplicate, and the statistical process of the experimental results was analyzed using SPSS 26.0 program (SPSS Inc., Chicago, IL, USA). The results treatment were analyzed with Kruskal-Wallis test using SPSS 26.0, and the significance of the average values ($p \leq 0.05$) was determined using Mann-Whitney test.

3. Results and discussion

3.1. Microbial growth in yogurts during storage

Raw (pre+main) and Homo (pre+main) samples were contained *L. garlicum*, *S. thermophilus*, and *Lb. delbrueckii* subsp. *bulgaricus*, on the other hand, Raw (only main) and Homo (only main) did not have *L. garlicum*. Fig. 3.2a shows that the viable cells decreased during storage significantly ($p \leq 0.05$) in all samples. There was 2.7 log decrease in both EPS+ yogurts after 28 days storage. Meanwile, EPS- yogurts had only 1.6 log decrease. This result could not be explained that the exact amount number of difference was caused by the presence of *L. garlicum*, because undefined interaction between bacteria could exist.

3.2. pH of yogurts during storage

Figure 3.2b shows that the initial pH for all samples were 4.59 ± 0.01. The pH of all yogurt samples containing EPS had a normal decrease during storage as well as the control samples (only main). After 28 days storage, the final pH were 4.24 ± 0.02, 4.30 ± 0.01, 4.20 ± 0.01 and 4.25 ± 0.01 for Raw (only main), Raw (pre+main), Homo (only main), and Homo (pre+main), respectively. There was only significant difference ($p \leq 0.05$) between Raw (pre+main) and Homo (only main). During the fermentation, LAB produce lactic acid from lactose, resulting in lowering the pH (Nagaoka, 2019). In this study, pH decreases in yogurt samples was due to lactic acid

production by lactic fermentation.

3.3. Total solids of yogurts during storage

Table 3.2 shows that total solids did not change throughout the storage period. Results of the investigation on total solids contents of yogurt samples showed that there was no significant difference ($p > 0.05$) between samples containing EPS and the control samples.

3.4. Color of yogurts during storage

The color of yogurt represents reflection of physico-chemical changes in the product. Table 3.2 shows that color variation of yogurt samples for 28 days storage. L*, a*, and b* represents lightness, redness, yellowness, respectively. There was no significant difference ($p > 0.05$) between samples with EPS and the control samples. At day 1, there were significant differences ($p \leq 0.05$) on L*, a*, and b* between raw-milk yogurts and homogenized-milk yogurts. It seems to originate from the effects of homogenization.

3.5. Water holding capacity of yogurts during storage

Figure 3.2c presents data on the water holding capacity of yogurts during storage. Homogenized-milk made yogurt with EPS exhibited the highest water holding capacity, and yogurt made with raw-milk without EPS had the lowest. These results confirm the ability of homogenization to bind sufficient water to significantly affet yogurt texture. However, the presence of EPS showed significant differnces ($p \leq 0.05$) in raw-milk made yogurt after 7 days storage.

3.6. EPS production during storage

Figure 3.2d shows that yogurt starter culture YF-L812 did not produce the EPS. EPS yield after 12 h *L. garlicum* fermentation was 7.32 ± 0.45 and 6.38 ± 0.44 g/kg milk sample for raw milk fermented and homogenized milk fermented, respectively. During 28 days, EPS yield slightly decreased to 6.87 ± 0.58 g/kg medium and 5.96 ± 0.30 g/kg medium but there was significant difference (p > 0.05) in both (pre+main) yogurts. Some studies revealed that EPS degradation is observed during long-term storage at 4 ℃ (Gorret et al., 2001; Feldmane et al., 2014). In this study, EPS produced by *L. garlicum* KCCM 43211 was not degraded itself during 28 days storage at 4 ℃.

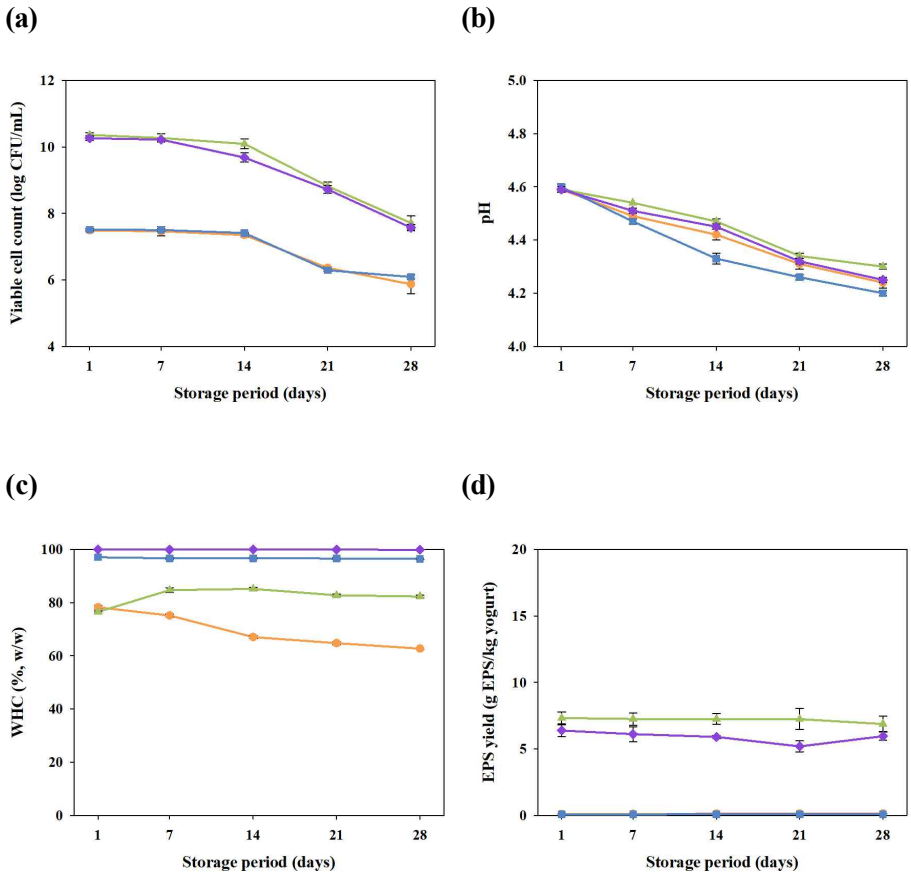


Fig. 3.2. Changes in (a) viable cell counts, (b) pH, (c) water holding capacity (WHC), and (d) EPS yield during the storage at 4 °C for 28 days of Raw (only main, ●), Raw (pre+main, ▲), Homo (only main, ■), and Homo (pre+main, ◆).

Table 3.1. Total solids content of yogurt stored at 4 ℃ for 28 days. Asterisks indicate significant differences between the values ($p \leq 0.05$).

Storage period (days)	Total solids (%, w/w)			
	Raw (only main)[1]	Raw (pre+main)[2]	Homo (only main)[3]	Homo (pre+main)[4]
1	19.59 ± 0.15*	20.49 ± 0.05	19.83 ± 0.54	19.92 ± 0.20
7	19.99 ± 0.15	20.54 ± 0.11	20.06 ± 0.74	20.06 ± 0.26
14	20.14 ± 0.18	20.26 ± 0.22	20.15 ± 0.80	20.01 ± 0.25
21	20.66 ± 0.36	20.55 ± 0.23	20.22 ± 0.74	20.00 ± 0.18
28	22.20 ± 0.50*	20.60 ± 0.05	19.98 ± 0.54	20.25 ± 0.45

[1]Raw (only main) represents the yogurt made with raw milk by yogurt starter culture only.

[2]Raw (pre+main) represents the yogurt made with raw milk by *L. garlicum* KCCM 43211 for pre-fermentation and yogurt starter culture for main fermentation.

[3]Homo (only main) represents the yogurt made with homogenized milk by yogurt starter culture only.

[4]Homo (pre+main) represents the yogurt made with homogenized milk by *L. garlicum* KCCM 43211 for pre-fermentation and yogurt starter culture for main fermentation.

Table 3.2. Color attributed (CIE L*, a*, and b*) of yogurts stored at 4 ℃ for 28 days. Asterisks indicate significant differences between the values ($p \leq 0.05$).

Storage period (days)	Raw (only main)[1]			Raw (pre+main)[2]			Homo (only main)[3]			Homo (pre+main)[4]		
	Color			Color			Color			Color		
	L*	a*	b*	L*	a*	b*	L*	a*	b*	L*	a*	b*
1	88.77 ± 0.18	1.62 ± 0.02	0.12 ± 0.03	89.05 ± 0.08*	1.66 ± 0.03*	1.71 ± 0.11*	90.46 ± 0.24*	2.11 ± 0.02	-0.55 ± 0.05	90.80 ± 0.14	1.89 ± 0.01*	2.04 ± 0.01*
7	88.57 ± 0.33	1.62 ± 0.03	0.10 ± 0.05	88.91 ± 0.07	1.78 ± 0.01	1.61 ± 0.10	90.65 ± 0.23	2.05 ± 0.05	-0.38 ± 0.26	90.74 ± 0.23	1.94 ± 0.03	1.76 ± 0.41
14	88.69 ± 0.12	1.65 ± 0.04	0.33 ± 0.16*	88.48 ± 0.22	1.89 ± 0.02	1.11 ± 0.25	91.40 ± 0.15	2.01 ± 0.05	0.88 ± 0.45*	90.94 ± 0.02	2.00 ± 0.04	1.60 ± 0.31
21	88.46 ± 0.30	1.64 ± 0.04	0.38 ± 0.34	87.64 ± 0.16*	1.85 ± 0.03	0.92 ± 0.17	91.10 ± 0.10	1.93 ± 0.03*	0.36 ± 0.14	90.78 ± 0.23	2.11 ± 0.02	0.97 ± 0.17
28	88.60 ± 0.64	2.75 ± 0.37	-2.36 ± 0.93*	88.70 ± 0.13	2.58 ± 0.18*	0.44 ± 0.39*	92.64 ± 0.05*	2.82 ± 0.19*	-0.86 ± 0.60*	91.51 ± 0.17	2.92 ± 0.05*	-0.70 ± 0.21*

[1]Raw (only main) represents the yogurt made with raw milk by yogurt starter culture only.

[2]Raw (pre+main) represents the yogurt made with raw milk by *L. garlicum* KCCM 43211 for pre-fermentation and yogurt starter culture for main fermentation.

[3]Homo (only main) represents the yogurt made with homogenized milk by yogurt starter culture only.

[4]Homo (pre+main) represents the yogurt made with homogenized milk by *L. garlicum* KCCM 43211 for pre-fermentation and yogurt starter culture for main fermentation.

3.7. Rheological properties of yogurts during storage

3.7.1. Flow behavior and apparent viscosity

Figure 3.3 shows the flow curves of the yogurt samples on 1, 7, 14, 21, and 28 days by shear rate versus shear stress. The logarithm characteristic indicates a deviation from pseudoplastic (shear-thinning) behavior.

Apparent viscosities (η_{app}) caculated at 100 s^{-1} shear rate. Figure 3.3. shows that the initial apparent viscosities were 119.512 ± 3.21, 90.85 ± 6.97, 122.46 ± 4.10 and 92.16 ± 7.50 for Raw (only main), Raw (pre+main), Homo (only main), and Homo (pre+main), repectively. During 28 days of storage, there was no significant difference ($p > 0.05$) of apparent viscosity on each days in same samples. Meanwhile, the presence of EPS was significant factor for apparent viscosity of RY and HY.

3.7.2. Frequency sweep behavior

The dynamic modulus (storage modulus and loss modulus) representing elastic properties and viscous properties, respectively. Figure 3.5 shows that the change in the storage modulus (G′) and loss modulus (G′) for yogurts during storage at 4 ℃. The linear increase was observed in all yogurt samples. This result could be indicate that yogurt samples had a concentrated solution behavior. Also, G′ was greater than G″ at all frequency values. This indicates

that all yogurt samples exhibited solid-like behavios and weak elastic gel structures (Steffe, 1996).

3.7.3. Temperature sweep behavior

Figure 3.7 shows the storage modulus (G′) and loss modulus (G″) versus temperature for yogurts during storage at 4 ℃. The G′ value of all yogurt samples was greater than the G″ value over the whole temperature range. In addition, there was no crossover occurred between the modulus. It was confirmed by the results of the frequency sweep measurements (Fig. 3.4). The G′ and G″ values of all yogurt samples were decreased with the temperature increased from 4 to 50 ℃. The decrease could be explained the weakening of the gel network structure as the temperature rises (Fu et al., 2018). The G′ and G″ values of all yogurt samples were increased with decreasing the temperature from 50 to 4 ℃. There was no significant effect of EPS on G′ and G″, but, smaller hysteresis loop was observed with EPS existence in RY samples on day 21 and day 28. According to the results, temperature changes may affect the properties of yogurts and EPS could affect on the stability of yogurts in weak gel.

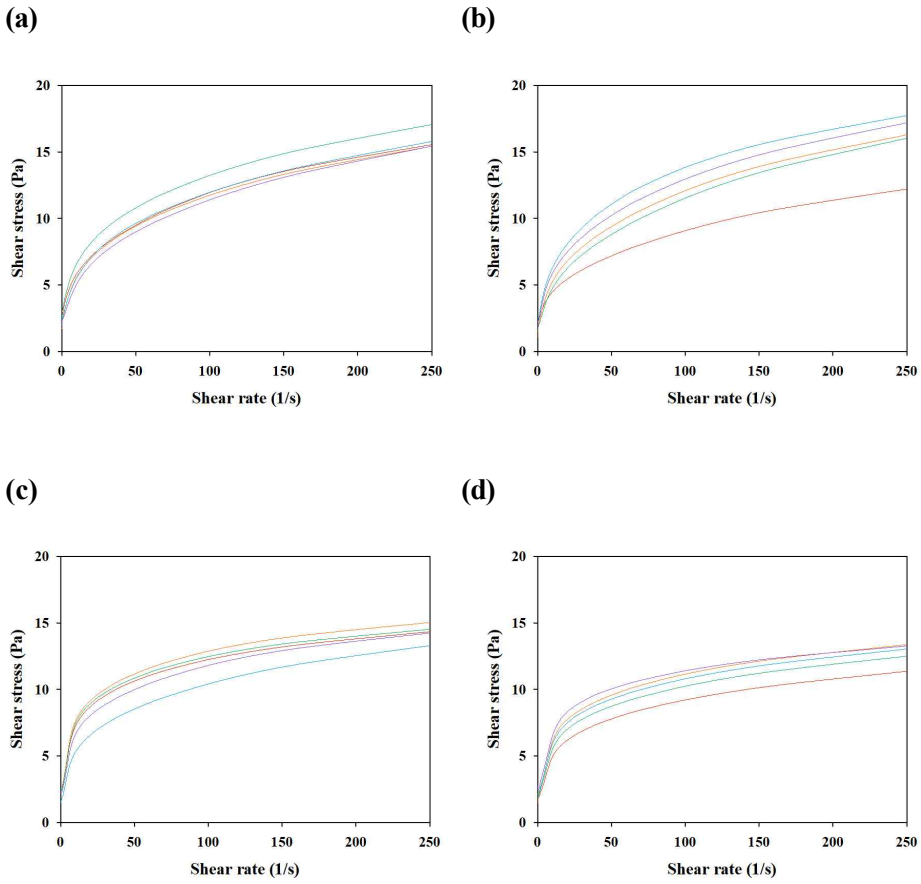


Fig. 3.3. Flow curve (shear stress versus shear rate) of (a) Raw (only main), (b) Raw (pre+main), (c) Homo (only main), and (d) Homo (pre+main) on day 1 (red line), day 7 (orange line), day 14 (green line), day 21 (blue line), and day 28 (purple line) storage time at 4 ℃.

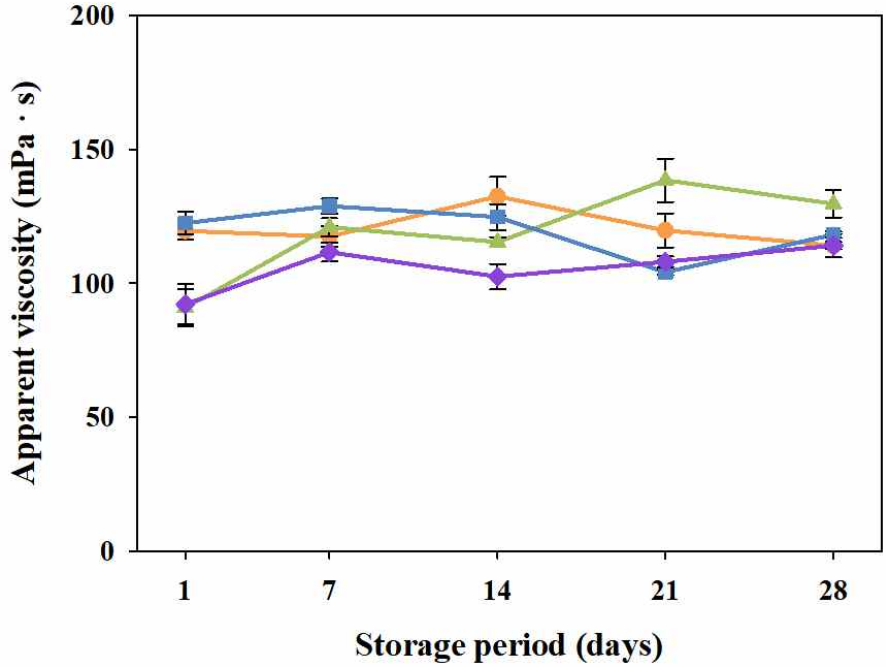


Fig. 3.4. Apparent viscosity (measured at 100 s^{-1}) of Raw (only main; ●), Raw (pre+main; ▲), Homo (only main; ■), and Homo (pre+main; ◆) during storage at 4 ℃ for 28 days.

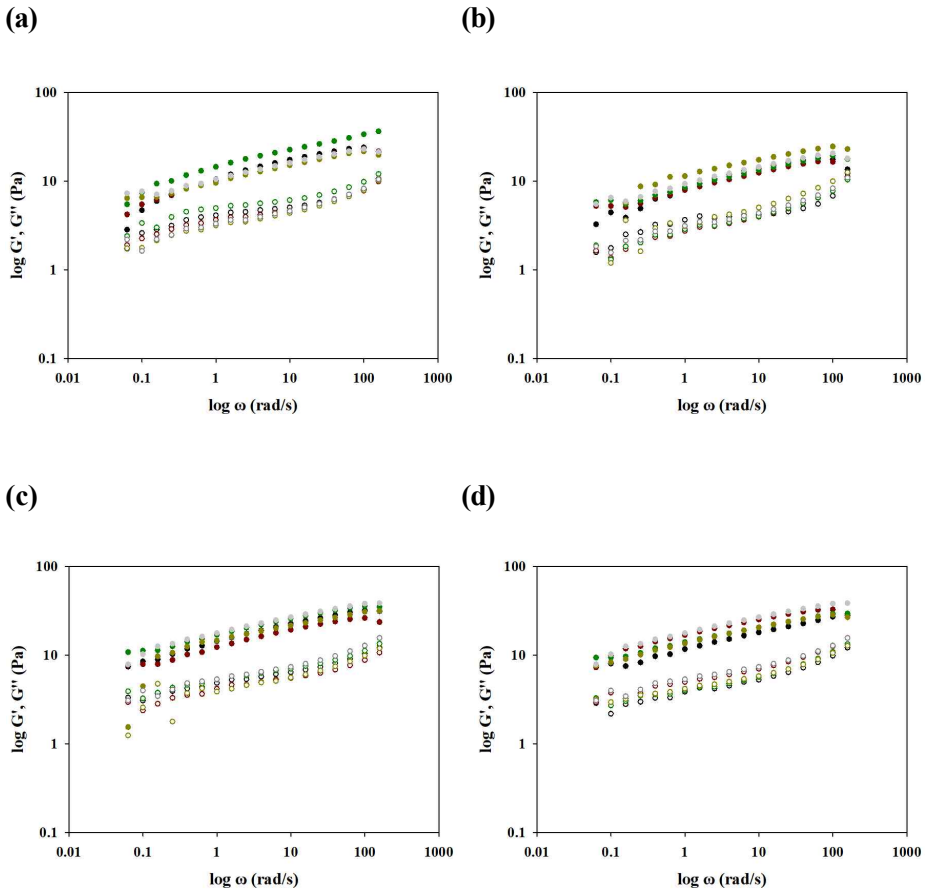
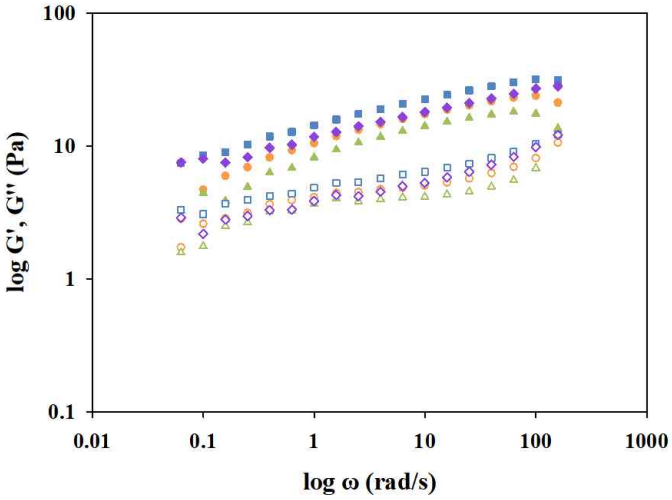


Fig. 3.5. Storage modulus (G′, solid symbol) and loss modulus (G″, open symbol) with respect to frequency (ω) (a) Raw (only main), (b) Raw (pre+main), (c) Homo (only main), and (d) Homo (pre+main) for Day 1 (●, ○), Day 7 (●, ○), Day 14 (●, ○), Day 21 (●, ○), and Day 28 (●, ○) storage time at 4 ℃.

(a)



(b)

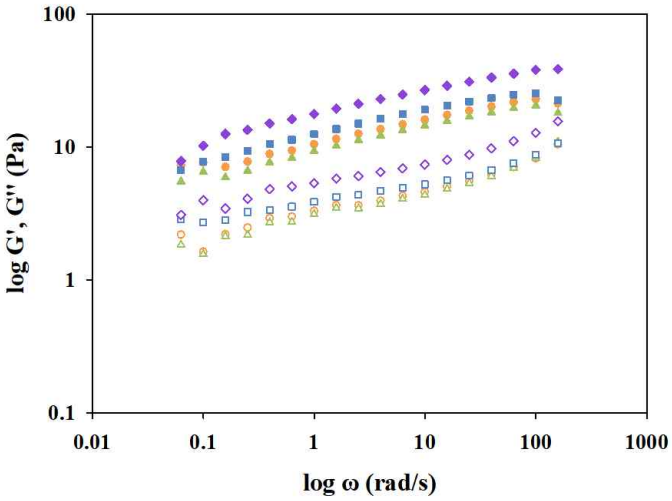
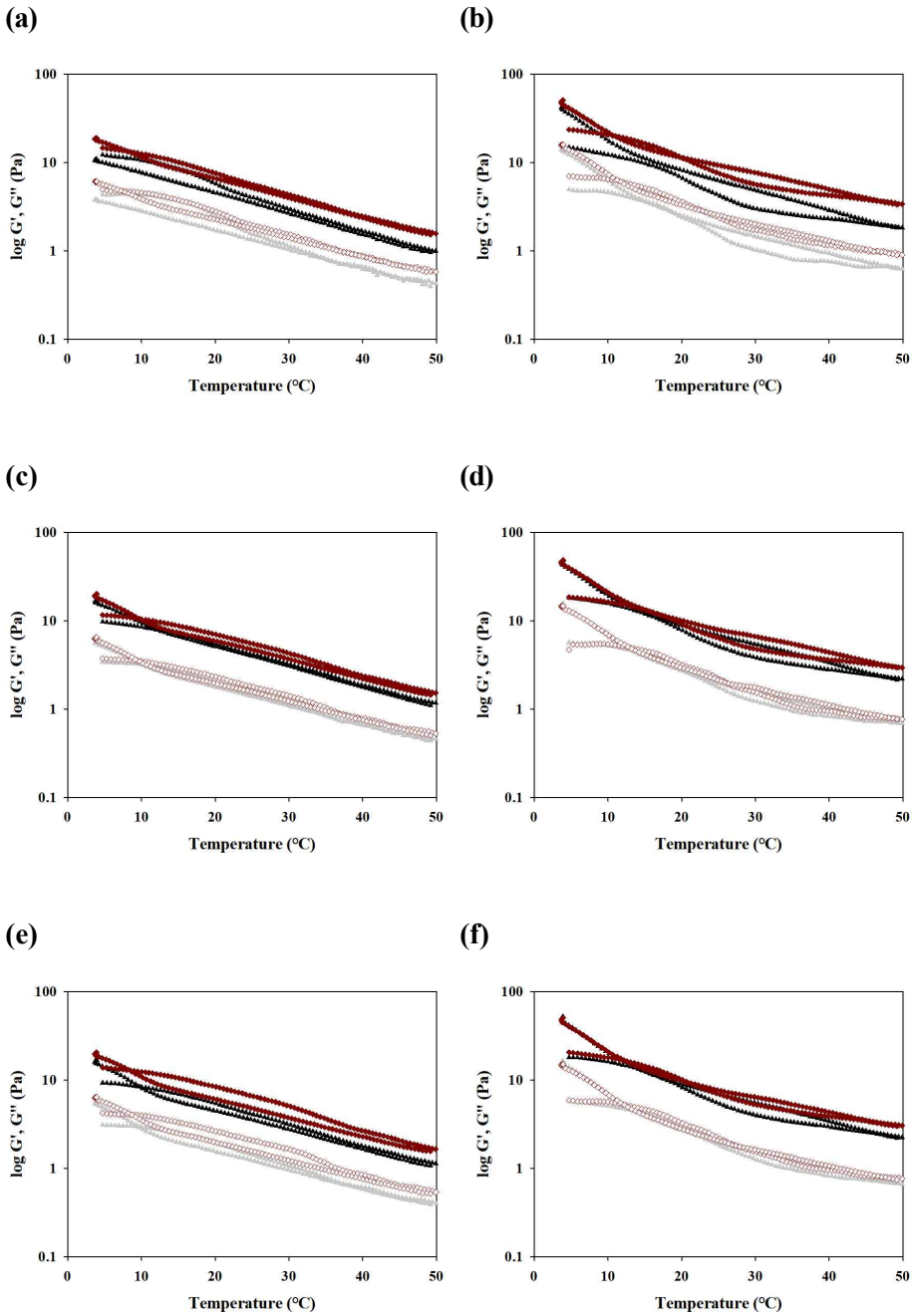


Fig. 3.6. Storage modulus (G′, solid symbol) and loss modulus (G″, open symbol) with respect to frequency (ω) of Raw (only main; ●, ○), Raw (pre+main; ▲, △), Homo (only main; ■, □), and Homo (pre+main; ◆, ◇) at (a) day 1 and (b) day 28 storage time at 4 ℃.


(a)
(b)
(c)
(d)
(e)
(f)
log G', G'' (Pa)
Temperature (°C)
100
10
1
0.1
0
10
20
30
40
50

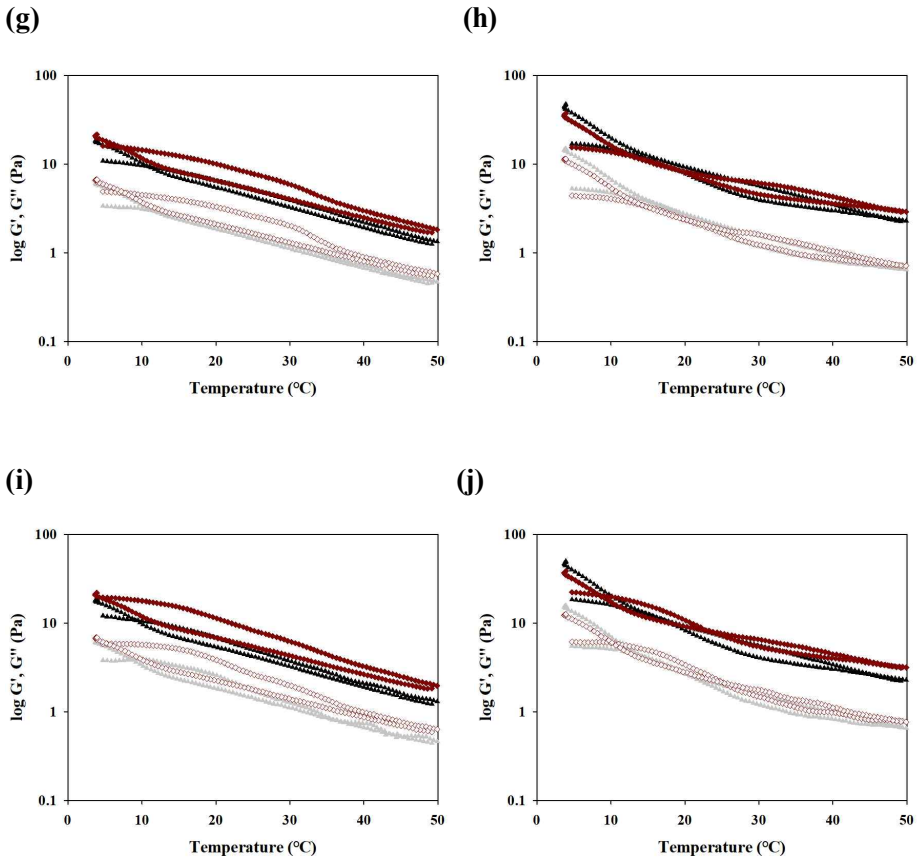


Fig. 3.7. Temperature dependence of storage modulus (G′; ◆ (only main), ▲ (pre+main)) and loss modulus (G″; ◇ (only main), ▲ (pre+main)) during initial heating from 4 to 50 °C and subsequent cooling from 50 to 4 °C at a rate of 5 °C/min: (a) Raw on day 1, (b) Homo on day 1, (c) Raw on day 7, (d) Homo on day 7, (e) Raw on day 14, (f) Homo on day 14, (g) Raw on day 21, (h) Homo on day 21, (i) Raw on day 28, and (j) Homo on day 28.

4. Conclusions

The microbial, physical, and rheological properties of yogurt made with raw and homogenized milk with or without EPS production after 28 days of storage at 4 ℃ were evaluated in this study. Growth of *L. garlicum*, *S. thermophilus*, and *Lb. delbrueckii* subsp. *bulgaricus* in all yogurt samples was significantly decreased during 28 days storage. In addition, pH decreased from 4.59 to 4.20 - 4.30 during storage by lactic acid formation. The normal pH of commercial yogurt products is known as ranged from pH 4.0 to 4.6 (Chandan, 2008). Total solids had similar concentration in all yogurt samples during storage. Color differences showed only between raw yogurt and homogenized yogurt. Water holding capacity results indicated that presence of EPS had a positive effect on yogurt characteristics. Homogenization was remarkably effective on the ability to hold in yogurt gel network. However, the presence of EPS is significant factor in raw-milk yogurt. There are some studies that long-cold storage can occur degradation of EPS. But, in this study, EPS yield was constant during storage. The result of strain sweep test showed that all yogurt samples had pseudoplastic behavior and frequency sweep measurements indicated the yogurts made up with weak elastic gel structure. Temperature sweep test showed that EPS could be act as stabilizer in non-homogenized milk products with long (> 21 days) cold storage.

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Abstract in Korean

유산균이 생성하는 세포외다당류(EPS)는 발효유의 유변학적, 물리적, 관능적 특성을 조절하는데 사용된다. 김치에서 분리한 *L. garlicum* KCCM 43211은 높은 점도를 가지는 EPS를 생성한다고 알려져 있다. 하지만, 발효유 내에서 EPS의 효과와 EPS의 구조적 특징은 아직 연구된 바가 없다. 따라서, 본 연구의 목적은 *L. garlicum* 43211이 생성하는 EPS가 발효유의 유변학적 특징에 미치는 영향을 밝히고, 생성된 EPS의 물리화학적 특징을 연구하는 것이다(Chapter 2). 그다음, 요구르트 생성에 있어서 *L. garlicum* KCCM 43211의 잠재적 적용을 확인하기 위해 본 균주를 이용하여 전 발효를 거친 요구르트를 4 °C의 조건에서 28일 동안 저장하며 그 특징을 연구하였다(Chapter 3).

발효유를 제조하기 위해 자당의 유무에 따른 원유와 균질 우유에 *L. garlicum* KCCM 43211을 접종하여 25 °C, 100 rpm 진탕 조건에서 48시간 동안 발효시켰다. 48시간 발효 중 균의 성장, pH의 변화, EPS 생성 및 발효유의 유변학적 특성을 실험하였다. 생균수 측정 결과 모든 발효유에서 10^8에서 10^9 CFU/mL의 성장을 보였다. pH는 모든 발효유에서 발효 기간 동안 균주의 성장과 함께 감소한 것을 확인할 수 있었다. 자당을 함유하지 않은 발효유에서는 EPS의 생성을 관찰할 수 없었다. 하지만, 10% 자당을 함유한 발효유에서는 22.91에서 27.29 g/kg medium의 EPS를 생성한 것을 확인할 수 있었다. 자당을 함유하지 않은 발효유에서는 점도의 변화 또한 관찰되지 않았으며, 자당을 함유한 원유 생성 발효유는 1.08 cP에서 3.22 cP, 균질 우유 생성 발효유는 1.29 cP에서 2.93 cP로 증가하였다. EPS의 생성과 점도 변화의 결과는 발효유 내에서 EPS가 점도 증가에 영향

을 주는 것을 보였다. EPS의 물리화학적 특성을 확인하기 위하여, 10% 자당을 함유한 원유를 이용하여 만든 발효유에서 EPS를 추출 및 정제하였다. 분석을 통해 *L. garlicum* KCCM 43211이 생성하는 EPS는 선형의 α-(1→6) 결합을 가진 분자량이 2.9×10^6 Da인 덱스트란인 것을 확인하였다.

Chapter 3에서는 원유와 균질 우유로 생성한 요구르트의 저장안정성에 미치는 효과를 연구하였다. 10% 자당을 함유한 원유 및 균질 우유에 요구르트 균주를 접종하여 생성된 요구르트를 대조군으로 사용하였다. EPS를 함유한 요구르트를 만들기 위해, *L. garlicum* KCCM 43211을 이용하여 12시간 동안 25 ℃, 100 rpm 진탕 조건에서 발효하였으며, 그다음 요구르트 균주를 접종하여 요구르트를 생성하였다. 네 종류의 요구르트를 28일 동안 냉장 보관하며 생균수 변화, pH 변화, 총 고형분 함량, 색 변화, 보수력, EPS 함량 및 유변학적 특성을 관찰하였다. 28일 저장 기간 중 모든 요구르트 샘플에서 생균수는 감소하였으며, 젖산의 생성으로 pH도 초기 4.59 – 4.60에서 4.20 – 4.30 수준으로 감소하였다. 총 고형분 함량은 저장 기간 동안 모든 샘플에서 유의적인 차이를 보이지 않았다. EPS의 존재는 색에도 영향을 미치지 않았다. 보수율의 결과는 균질화 과정이 젤 네트워크 내에서 저장 기간 동안 이수(syneresis)를 방지하며 보수 능력을 증진하는데 효과적인 요인이 될 수 있음을 보여주었다. 또한, 두 종류의 EPS 함유 요구르트에서 EPS가 약간 감소함을 보였고, 이는 긴 시간 저온 저장 중 EPS의 분해가 없음을 나타내었다. 흐름곡선의 결과는 네 종류의 요구르트 모두 유사가소성 유체임을 보여주었다. 저장 기간 동안 겉보기 점도의 유의미한 변화는 보이지 않았지만, 온도 변화 시험(temperature sweep test)를 통해 EPS의 존재는 14일 저장 기간 이후 겉보기 점도 상승 및 더 작은 이력 루프

(hysteresis loop)를 보여주며 요구르트의 유변학적 특성에 긍정적인 영향을 주었음을 확인할 수 있었다.

결과적으로, EPS는 젤 생성의 초기 단계(> pH 4.6)에서 발효유의 점도를 향상시키지만, 강한 젤 네트워크(< pH 4.6, 균질 우유 생성 요구르트)에는 그 효과가 가려질 수 있다는 것을 확인하였다. 이러한 결과를 바탕으로 *L. garlicum* KCCM 43211이 생성하는 EPS는 약한 젤 네트워크를 가진 발효유 내에서 물리적, 유변학적 특징을 조절하는 데 도움을 줄 수 있을 것이라 생각된다.