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Synbiotic Synthesis of Oligosaccharides During Milk Fermentation by Addition of Leuconostoc Starter and Sugars

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Abstract Synthesis of oligosaccharides during milk fermentation was attempted by inoculating *Leuconostoc citreum* with *Lactobacillus casei*, *Lb. delbrueckii* subsp. *bulgaricus*, and *Streptococcus thermophilus* as starters. Dextransucrase of *Ln. citreum* worked as a catalyst for the transglycosylation reaction of sugars; sucrose was added as the glucose donor, and lactose or maltose acted as the acceptor compound for the reaction. When 4% sucrose was added in milk, glucosyl-lactose was synthesized (about 1%, w/v) after 1–2 days of fermentation at 15 or 25°C. Alternatively, when sucrose and maltose (2% each, w/v) were added, panose (about 1%, w/v) and other isomaltooligosaccharides were made in a day at 15–35°C. Growth patterns of lactobacilli and streptococci starters were not affected by the coculture of leuconostoc starter, but the rate of acid synthesis was slightly slowed at every temperature. Addition of sugars in milk did not give any adverse effect on the lactate fermentation. Accordingly, the use of leuconostoc starter and addition of sugars in milk allowed the production of oligosaccharides-containing fermented milk, and application of this method will facilitate the extensive development of synbiotic lactate foods.

Keywords: Dextransucrase, fermented milk, glucosyl-lactose, isomaltooligosaccharides, *Leuconostoc citreum*, panose, synbiotics, yogurt

Fermented milk is made from the milk of cows, goats, and horses by adding lactic acid bacteria to convert lactose to lactic acid. As probiotics and their fermented foods products are beneficial for health, the consumption is increasing every year [1, 19, 21, 30]. In general, the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Bifidobacterium* are used for yogurt [12], and *Leuconostoc* and yeasts are used for kefir as additional starter strains. Prebiotics are defined as food ingredients that beneficially affect the host by selectively stimulating the growth and activity of benefical bacteria that can improve host health in the colon [8]. The prebiotic components are nondigestible disaccharides or oligosaccharides, and they may be derived from plants or produced by technological means, *e.g.*, transglycosylation with enzymes [14, 31].

Many studies suggest that intake of synbiotic products containing both probiotics and prebiotics [8] has greater beneficial effects on the human health than probiotic or prebiotic products alone [9, 24, 28, 29]. Indeed, the presence of probiotics and prebiotics in a single food improved survival of probiotic bacteria during the storage of the product and during the passage along the intestinal tract. Moreover, the synbiotic product may allow an efficient implantation of probiotic bacteria in the colonic microbiota, because prebiotics have a stimulating effect on the growth and/or activities of the exogenous and endogenous bacteria [23]. Addition of probiotics along with prebiotics results in increasing production costs, which can slow down the development of new synbiotic products. Hence, the use of probiotic bacteria that are able to synthesize prebiotics during the milk fermentation can provide an effective process for the manufacture of synbiotic products [15].

Leuconostoc spp. are heterofermentative lactic acid bacteria and are the major bacterial population in *kimchi* or sauerkraut from the initial to the middle stages of fermentation [7, 16]. During these stages, these bacteria produce various constituents, such as lactic acid, acetic acid, CO_2, and mannitol, all of which contribute to the flavor of fermented foods. The number of these bacteria is highest during the optimum ripening period. Dextransucrase (E.C. 2.4.1.5) excreted by *Leuconostoc* spp. transfers the glucose moiety of sucrose to form dextran and also catalyzes the transfer of glucose from sucrose (donor) to other carbohydrates (acceptors) by mainly linking an α-(1→6)-glucosyl bond [25]. When the acceptor is a monosaccharide or disaccharide, a series of oligosaccharide acceptor-products is usually produced, where maltose and lactose are recognized as the best and moderate acceptor molecules, respectively, based

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on an experiment of *Ln. mesenteroides* NRRL B-512F [27]. The usefulness of this enzyme was proved in synthesis of isomaltooligosaccharides comprising mainly panose (6^2-α-D-glucopyranosylmaltose) after addition of sucrose and maltose during *kimchi* fermentation [10].

In this study, we performed a novel synbiotic fermentation process of cow's milk by coculture of *Ln. citreum* with typical yogurt starters and by using the transglycosylation reaction of its dextransucrase, thereby to synthesize various oligosaccharides along with lactate fermentation.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Lactobacillus casei ATCC 7469, *Lb. delbrueckii* subsp. *bulgaricus* KCTC 3188, *Streptococcus thermophilus* KCTC 2185, and *Leuconostoc citreum* KACC 91035 were used as starters for the fermentation of milk. *Ln. citreum* KACC 91035 is a psychrotrophic strain secreting highly active dextransucrase at broad temperature ranges [6]. The glycosyltransfer pattern of dextransucrase of this strain was determined as being the same as that of *Ln. mesenteroides* NRRL B-512F, polymerizing dextran with 95% of α-(1→6)-glucosyl linkage and 5% of α-(1→3)-linkage. The starter strains were cultivated in 100 ml of MRS broth (Difco, Detroit, MI, U.S.A.) under aerobic condition for 12 h at 28°C.

Preparation of Fermented Milk

Cow's milk was purchased from a local grocery store. Precultured starter strains of *Lb. casei, Lb. delbrueckii* subsp. *bulgaricus*, and *St. thermophilus* (10 ml each) were inoculated in 1,000 ml of autoclaved milk. This culture was referred to as the yogurt control. For the glycosyltransfer reaction, an additional starter, *Ln. citreum*, was cocultured in the control sample (designated as sample I). Another fermented milk, sample II, was prepared by adding 4% (w/v) sucrose in sample I. The third fermented milk, sample III, was prepared by adding 2% (w/v) sucrose and 2% maltose (w/v) to the sample I. The compositions of starters and sugars added in each fermented milk are summarized in Table 1. The above milk products were fermented at the temperatures of 15, 25, or 35°C, respectively.

Table 1. Composition of fermented milks used in this study.

Composition	Types of fermented milk			
	Control	I	II	III
Milk (containing 4% lactose)	○	○	○	○
Lb. casei	○	○	○	○
Lb. delbrueckii subsp. *bulgaricus*	○	○	○	○
St. thermophilus	○	○	○	○
Ln. citreum		○	○	○
Sucrose			○[a]	○[b]
Maltose				○[b]

[a]4% of sugar was added.
[b]2% of sugar was added.

pH and Total Acidity in Fermented Milk

The pH of fermented milk was measured using a pH meter (IQ 240, I.Q. Scientific Inc., U.S.A.) and total acidity was titrated according to the AOAC method [2]. Ten ml of sample was mixed with an equal volume of distilled water and the titrable acidity was determined by titrating with 0.1 N NaOH to an end point of pH 8.3. The % lactic acid in the sample was calculated by multiplying the volume of NaOH solution (ml).

Microbial Analysis

For viable cell counting of microorganisms during milk fermentation, MRS and phenylethanol agars (Difco, U.S.A.) were used with 2% sucrose (PES, [20]). Each sample was serially diluted with 0.85% (w/v) physiological saline. The number of total lactic acid bacteria was determined by spread-plating onto MRS agar and incubating at 28°C for 48 h. The genus *Leuconostoc* was counted by spread-plating onto PES agar after incubation at 20°C for 48 h [6].

Sugar Analysis

Sugar analysis was carried out using a high-pressure ion-exchange chromatography (HPIC, Dionex Corp., Sunnyvale, CA, U.S.A.) and Whatman K5 TLC plates (Merck, Darmstadt, Germany) by the method of Robyt and Mukerjea [26]. For removing impurities such as casein and fat in milk, 1.5 ml of the milk sample was boiled at 100°C for 5 min and centrifuged at 12,000 ×*g* for 1 min. The supernatants were filtered using a 0.45-μm syringe filter (Satorius AG, Goettingen, Germany) and subsequently used for qualitative sugar analysis using HPIC. For quantitative analysis of sugars, 1 μl of each sample was loaded onto the TLC plate and developed three times with acetonitrile/distilled water (85:15, v/v). The separated sugars were detected by dipping the plate in ethanol containing 0.5% (w/v) α-naphthol and 5% (v/v) sulfuric acid, followed by heating at 110°C for 5 min. Analysis of sugars was performed using the Sigmagel program (Sigma Inc., U.S.A.) [26].

RESULTS AND DISCUSSION

Milk Fermentation

Starter strains of *Lb. casei, Lb. delbrueckii* subsp. *bulgaricus*, *St. thermophilus*, and *Ln. citreum* were precultured in MRS medium, inoculated in milk (1×10^7 CFU/ml each) with sugars (Table 1), and then incubated at 15, 25, or 35°C, respectively. The initial pH and total acidity of milk samples were immediately measured after mixing the

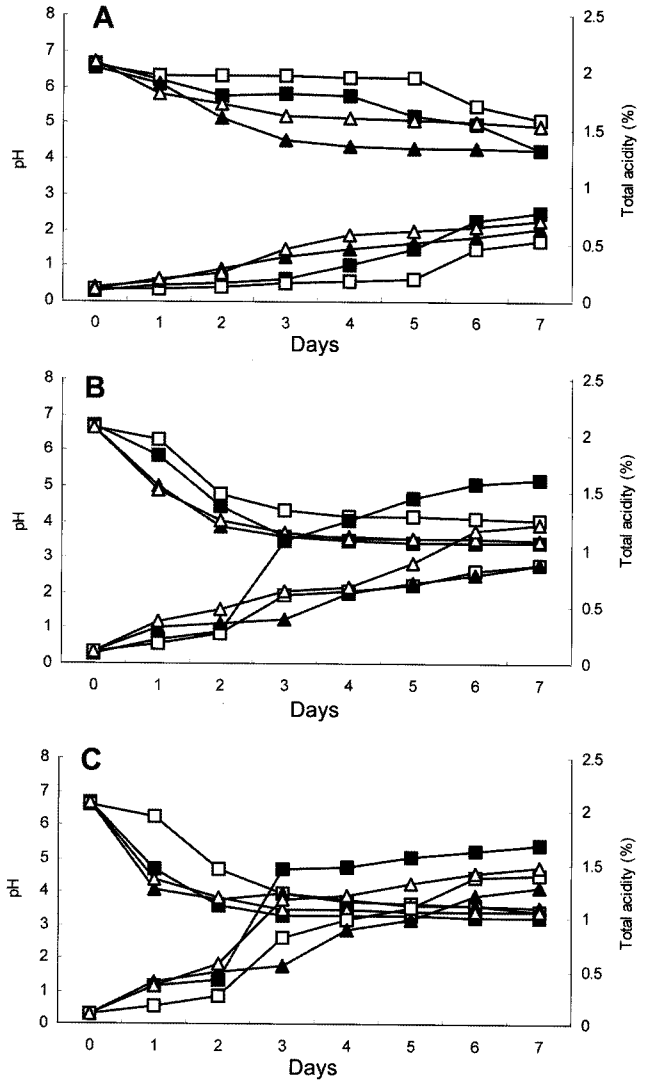

Fig. 1. Profiles of pH and total acidity changes during milk fermentation at 15 (**A**), 25 (**B**), and 35°C (**C**).
■, Control, starters for yogurt (*Lb. casei*, *Lb. delbrueckii* subsp. *bulgaricus*, and *St. thermophilus*) were added; □, fermented milk sample I, *Ln. citreum* was added in addition in control starters; ▲, fermented milk sample II, 4% sucrose was added to sample I; △, fermented milk sample III, 2% sucrose and 2% maltose were added in sample I.

starter strains, and they were pH 6.8 and 0.23%, respectively. Fig. 1 indicates the profiles of pH and total acidity changes for 7 days of fermentation period at 15, 25, and 35°C (A, B, and C, respectively). As the temperature rose (Figs. 1A to 1C), the total acid contents increased and the pH dropped rapidly. Regardless of temperatures, the sample I cocultured with *Ln. citreum* without sugars showed very slow changes in pH and total acidity, possibly resulted by heterolactate fermentation of leuconostoc. However, the control milk inoculated with yogurt starters excreted larger quantity of acids than milk samples I, II, and III at above 25°C, converting most carbon sources into lactic acids. Indeed,

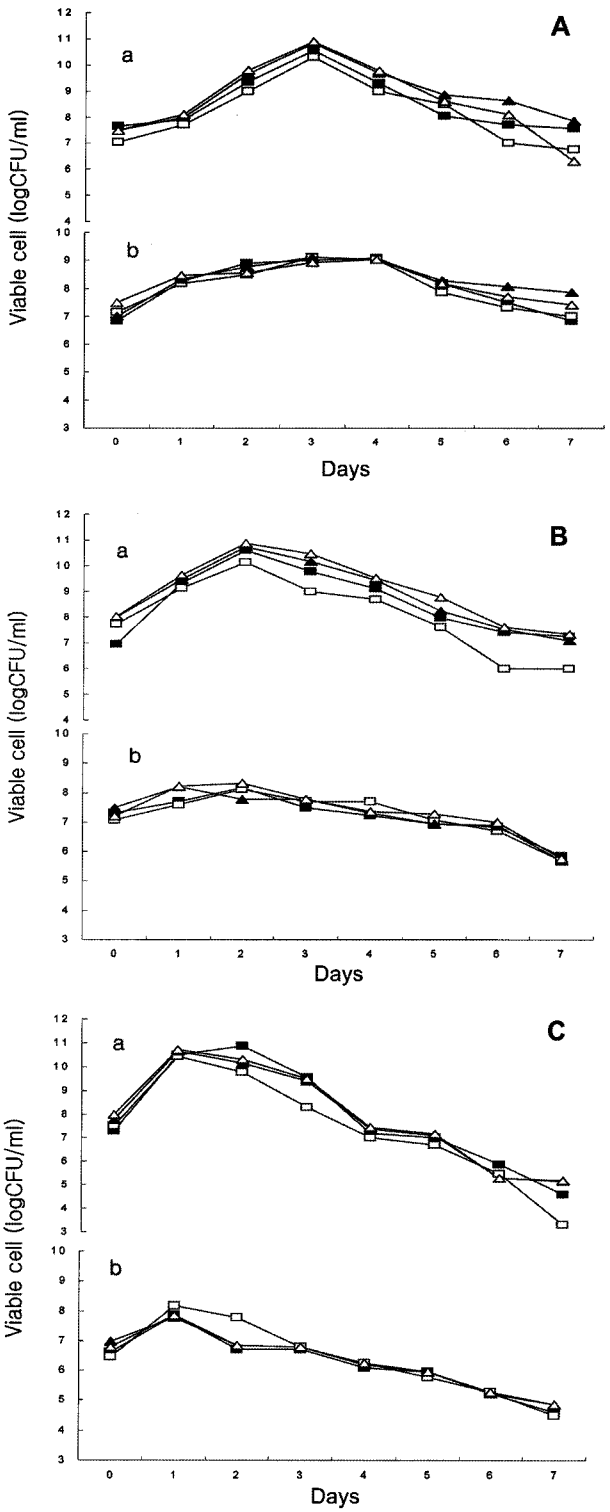

Fig. 2. Changes of viable cell counts during milk fermentation at 15 (**A**), 25 (**B**), and 35°C (**C**).
a: Total lactic acid bacteria; b: *Ln. citreum*. ■, Control, starters for yogurt (*Lb. casei*, *Lb. delbrueckii* subsp. *bulgaricus*, and *St. thermophilus*) were added; □, fermented milk sample I, *Ln. citreum* was added in addition to control starters; ▲, fermented milk sample II, 4% sucrose was added to sample I; △, fermented milk sample III, 2% sucrose and 2% maltose were added to sample I.

these three species of yogurt starters belong to the homofermentative lactic acid bacteria [11]. The interesting result is that addition of sucrose and maltose in the milk sample III did not affect notably on lactic acid production as much as the control. Generally, sugar addition in lactate fermentation is not favored because of the possibility of overacidification of foods and less organoleptic preference. However, in this experiment, most sugars added were supposed to be converted into other forms (possibly oligosaccharides) and also metabolized by *Ln. citreum* into other chemicals as well as lactic acid.

Fig. 2 represents the cell growth profiles of starters according to the passage of fermentation. The initial cell counts of lactic acid bacteria at all temperatures were approximately 10^7–10^8 CFU/ml. The bacterial growth patterns at various temperatures (15, 25, and 35°C) were different from each other, reaching the maximum cell count levels after 3, 2, and 1 day, respectively. In the cases of 15 and 25°C (Figs. 2A and 2B), starters showed a slow cell division. However, at 35°C (Fig. 2C), both total lactic acid bacteria and leuconostoc made fast growth to reach the top levels in a day and rapidly declined to the bottom level because of the accumulation of lactic acid in the milk. Accordingly, in the dairy manufacturing process, the relationship between temperature and growth rate of bacterial cultures should be carefully considered. When maximum cell counts are reached, the fermentation temperature should drop down to 0°C to stop the cell growth and preserve fermented milks. When we compared cell growth patterns among the four groups (control, samples I, II, and III), no remarkable difference was found, and this fact implies that sugar addition in milk did not give an undesirable effect on cell growth as well as the acidity of fermented milk.

Synthesis of Oligosaccharides in Fermented Milk

Robyt and Eklund [27] carried out a series of reactions with *Ln. mesenteroides* B-512F dextransucrase using different acceptors at a 1:1 acceptor-to-sucrose ratio, and then they measured the amount of oligosaccharides formed in the reaction. Among sixteen other acceptors compared

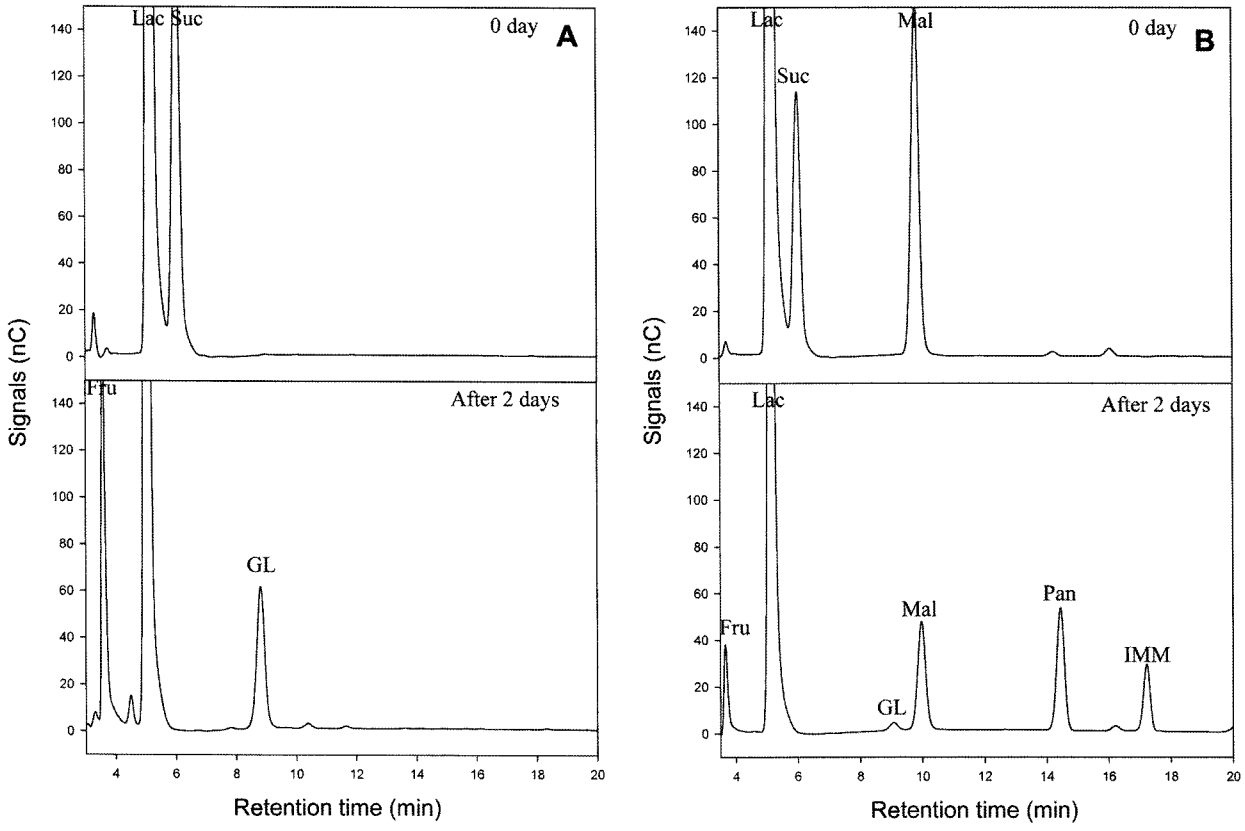

Fig. 3. Analysis of sugar concentration in fermented milk using high-pressure ion-exchange chromatography (HPIC).
CarboPac PA-1 column was used for the separation of oligosaccharides. After the column was equilibrated with 150 mM of NaOH solution, milk sample (200 μl) was loaded and then eluted with 600 mM of Na-acetate solution by the gradient mode with 150 mM NaOH. The pulsed amperometric detection method and Chromate Window v.3.0 were used for detection and analysis of sugars. Graph A shows peaks in milk sample II containing 4% sucrose before (top) and after (bottom) 2 days of fermentation. Graph B shows peaks of sample III containing 2% sucrose and 2% maltose before (top) and after (bottom) 2 days of fermentation at 25°C. After acceptor reaction of lactose and maltose, several oligosaccharides synthesized in milk are shown. (Fru, fructose; Lac, lactose; Suc, sucrose; Mal, maltose; GL, glucosyl-lactose; Pan, panose; IMM, isomaltosyl-maltose).

on a relative scale, with maltose defined as 100%, the next best acceptor was isomaltose (89%), followed by nigerose (58%), methyl-α-D-glucopyranoside (52%), D-glucose (17%), turanose (13%), lactose (11%), cellobiose (9%), and D-fructose (6.4%). As such, these results indicate a way of producing various oligosaccharides in foods based on the transglycosylation of dextransucrase with the above acceptor compounds. Therefore, we attempted to apply the transglycosylation reaction in fermented milk with the benefit of inherent lactose (4%) in milk. For that purpose, 4% of sucrose was added in milk (sample II) as donor, or 2% sucrose with 2% maltose were added (sample III) as donor-acceptor molecules. After inoculation of starters in milk, changes of sugar composition during fermentation were analyzed (Fig. 3A) and the profile is depicted in Fig. 4. As expected, the transglycosylation reaction of dextransucrase occurred, transferring the glucose residue from sucrose to lactose, thereby producing glucosyl-lactose as product and releasing fructose as a free residue (Fig. 3A). As seen in Fig. 4, sucrose was rapidly digested within three days at 15 or 25°C and, during the same period, lactose (<1%) was consumed as acceptor molecules. Concentrations of glucosyl-lactose increased up to 1% (w/v) after 1–2 days and these levels maintained for the next period without any remarkable decomposition. When milk sample II was fermented at 35°C, digestion of sucrose was relatively slow and the amount of glucosyl-lactose was less than 0.5%. The sudden decline of leuconostoc counts at 35°C, observed in Fig. 2C, might result in the low dextransucrase activity at this condition. The maximum concentrations of glucosyl-lactose at 15, 25, and 35°C were 1.0, 0.9, and 0.5%, respectively. Hence, 15 and 25°C were considered as the proper temperatures for the glucosyl-lactose synthesis reaction.

As an alternative way to synthesize oligosaccharides in milk, we added another disaccharide, maltose, which was proved as the best acceptor molecule for the transglycosylation of dextransucrase [27]. Since the equimolar addition of sucrose and maltose gave the best result for higher production of panose in previous experiment [10], 2%

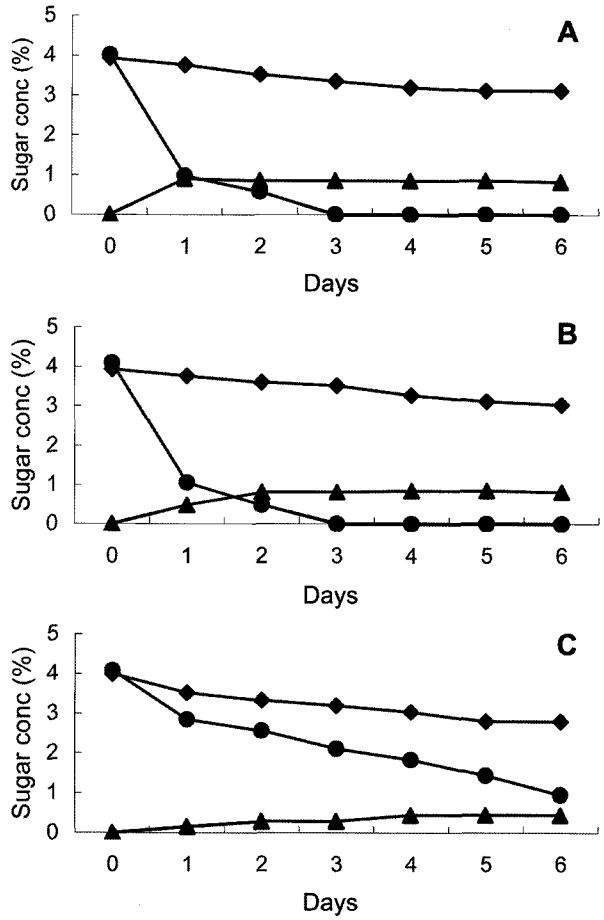

Fig. 4. Profiles of sugar changes in milk sample II containing 4% sucrose and 4% lactose during milk fermentation at 15 (**A**), 25 (**B**), and 35°C (**C**).
●, Sucrose; ◆, lactose; ▲, glucosyl-lactose.

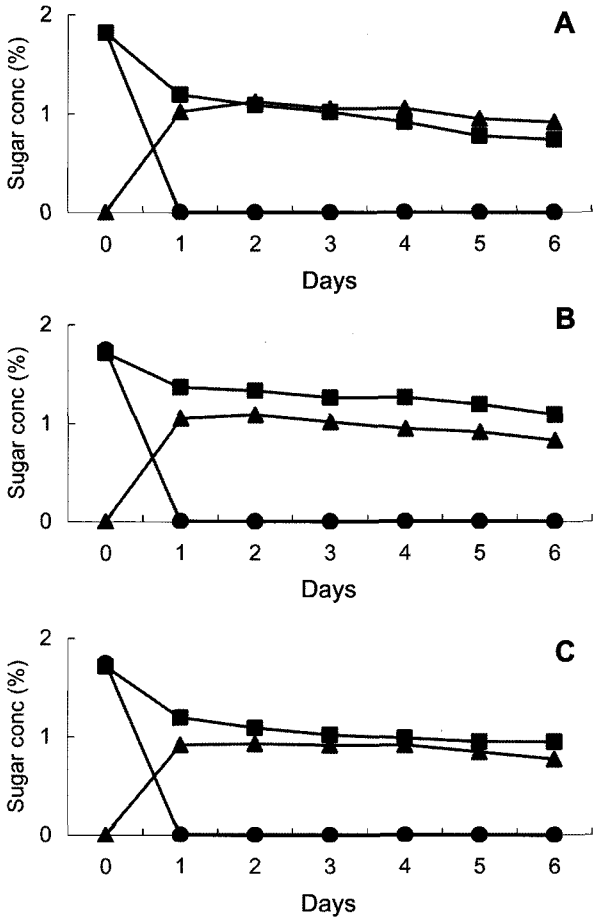

Fig. 5. Profiles of sugar changes in milk sample III containing 2% sucrose and 2% maltose during milk fermentation at 15 (**A**), 25 (**B**), and 35°C (**C**).
●, Sucrose; ■, maltose; ▲, panose.

sucrose and 2% maltose were added in milk. Changes in the sugar composition during fermentation were analyzed in Fig. 3B. Similar to the above experiment, transfer of the glucose residue from sucrose to maltose occurred consecutively, thereby isomaltooligosaccharides were synthesized and panose was the major component among them. After the reaction, sucrose and maltose peaks diminished along with synthesis of oligosaccharides. In Fig. 5, profiles of sugar changes at various temperatures are shown (here, only panose concentration is depicted). Sucrose (2%) was rapidly consumed in a day and about half the amount (1%) of maltose was used at all temperature conditions. Concentrations of panose were highest after 1–2 days and these levels were maintained for the next period. There was no remarkable difference between reaction profiles depending on reaction temperatures, and perhaps this was caused by the fast acceptor reaction between sucrose and maltose. Even though lactose (4%) was in milk, glucosyl-lactose was synthesized as low as <0.1% being due to the comparative reactivity as acceptor molecule between maltose and lactose (100% vs. 11%) [27]. The maximum concentration of panose at 15, 25, and 35°C are comparable (1.15, 1.05, and 1.00%, respectively) and this result illustrates that those conditions can be used practically for panose synthesis during milk fermentation. In addition, a preliminary experiment showed that the maltose-syrup is an economically feasible source of maltose and contains about 50% maltose, giving a suitable yield of panose.

The control fermented milk was a typical yogurt with regard to its starter composition; samples I, II, and III cocultured with *Leuconostoc* sp. were rather close to kefir [32], which is a traditional Russian fermented milk with a consortium of lactic acid bacteria. The experimental results in Fig. 2 reveal that the fermentation temperature for samples II and III is not necessarily different from yogurt, hence 35°C is preferred because it reached the maximum bacterial number in a day. However, for the synthesis of glucosyl-lactose, temperatures lower than 35°C are recommended (Fig. 4).

Lactose is one of the very important sugars in nature because of its abundance in the milk of humans and domestic animals. Most of the oligosaccharides derived from lactose are resistant to digestion in the stomach and small intestine because of its nondigestibility. Thereby, it may be selectively utilized by beneficial intestinal microflora such as *Bifidobacterium* species, resulting in significant induction of growth of these bacteria in the colon. The various oligosaccharides that are reported as prebiotics are fructooligosaccharides and isomaltooligosaccharides [22]. The oligosaccharides associated with lactose are as follows: (1) glucosyl-sucrose is produced by transfer of the glucosyl residue in lactose as a donor to sucrose as an acceptor, (2) lactosucrose is produced from a mixture of lactose as an acceptor and sucrose as a fructosyl donor using β-fructofuranosidase, and (3) sialyllactose, which is an oligosaccharide formed from lactose and sialic acid, is only found in human milk [18].

In conclusion, we attempted the synthesis of oligosaccharides during milk fermentation by inoculating *Ln. citreum* with *Lb. casei*, *Lb. delbrueckii* subsp. *bulgaricus*, and *St. thermophilus*. Dextransucrase of *Ln. citreum* was used as a catalyst for the transglycosylation reaction of sugars; when sucrose was added to the milk, glucosyl-lactose was synthesized, whereas when sucrose and maltose were added, panose with isomaltooligosaccharides was produced. Growth patterns of lactobacilli and streptococci starters were not affected by the coculture of the leuconostoc starter, but the rate of acid synthesis was slightly slowed at every temperature. Addition of sugars in milk did not give adverse effect on the lactate fermentation. Accordingly, the use of a *Leuconostoc* starter and addition of sugars in milk allow the production of oligosaccharides-containing fermented milk, and further development of this method will permit economically feasible production of synbiotic products.

Acknowledgments

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