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Glucooligosaccharides from *Leuconostoc mesenteroides* B-742 (ATCC 13146): A potential prebiotic

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There is an emerging market for functional oligosaccharides for use in foods. Currently, technology for the production of oligosaccharides is limited to extraction from plant sources, acid or enzymatic hydrolysis of polysaccharides or synthesis by transglycosylation reactions. Oligosaccharides can also be produced using a *Leuconostoc* fermentation and restricting the polymer size by addition of maltose. Maltose limits the dextransucrase reaction, yielding high concentrations of α-glucooligosaccharides. Branched oligomers produced by this process were readily catabolized by bifidobacteria and lactobacilli but were not readily utilized by either *Salmonella* sp. or *Escherichia coli*, pointing toward their use in intestinal microflora modification.
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Introduction

In recent years, use of functional food products, especially those containing live cultures of *Bifidobacterium* and *Lactobacillus*, called probiotics, has increased [19]. These bacteria reside naturally in the large intestine. Although the mechanism of action is not fully understood, the available evidence indicates ingestion of probiotic bacteria may improve several of the host's physiological processes. These include the alleviation of lactose intolerance, immune enhancement, and a reduction in mutagenic enzymes such as β-glucuronidase, nitroreductase and choloylglycine hydrolase [5,22]. To be effective, dietary probiotics must reach the large intestine. Barriers in the human body, which include acidic pH in the stomach, degradative enzymes and bile in small intestine, often prohibit effective ingestion of probiotics. Researchers have attempted to isolate bacterial strains resistant to bile and acidic conditions [4,12] and have also tried to develop microencapsulation technology that would protect these bacteria on their way to a target location [7].

It is believed that the ability of these probiotics to catabolize oligosaccharides (2 to 10 monosaccharide units linked with glycosidic bonds) is the key factor in their bestowing beneficial health effects. Certain carbohydrates, called prebiotics, can escape metabolism and adsorption in the small intestine and ultimately influence the composition of microflora in the large intestine [14]. They are generally nondigestible by humans. Oligosaccharides are used widely in foods such as soft drinks, cookies, cereals, candies and dairy products. Other applications for oligosaccharides such as an anticariogenic agent [17] or a low-sweetness humectant [24] have been explored.

Antibiotic resistance among known pathogens such as *Salmonella* and *Escherichia coli* is expanding due to the wide use of these compounds in areas ranging from medicine to animal feed. The pressure to remove antibiotics from animal feeds has left a need for safe alternatives that can reduce levels of these bacteria in animals [8,9]. Selected fructooligosaccharides (FOS) and glucooligosaccharides (GOS) have shown potential as alternatives to antibiotics [15]. Those oligosaccharides with the greatest antibacterial effects are either fructans or branched glucans [1,14,23].

Prebiotic oligosaccharides are currently produced either by extraction from plant sources, acid or enzymatic hydrolysis of polysaccharides or enzymatic synthesis by transglycosylation reactions [3,16]. Glucooligosaccharides can be produced by restricting polymer size during the fermentation process [13]. Dextransucrase (EC 2.4.1.5), an enzyme usually produced by species of *Leuconostoc* and *Streptococcus*, catalyzes the synthesis of high molecular weight glucans (dextrans) according to the following reaction:

$$\underset{\text{(sucrose)}}{\text{n G}-\text{F}} \xrightarrow{\textit{dextransucrase}} \underset{\text{dextran}}{\text{G}_\text{n}} + \underset{\text{fructose}}{\text{nF}}$$

In the presence of sucrose, dextransucrase produces a linear backbone of D-glucopyranosyl units, linked α-1,6 that can have variable amounts of α-1,2-, α-1,3-, or α-1,4-branched side chains. The actual degree of branching depends on the specific strain of microorganism [20]. When an efficient chain-ending acceptor such as maltose or isomaltose is present in high concentration, dextransucrase will catalyze the synthesis of α-glucooligosaccharide [13,18,21]. This process applied to a *L. mesenteroides* NRRL B-742 fermentation produces branched GOS that can function as prebiotics.

Materials and methods

Organism, culture medium and inoculum preparation

All strains of bacteria used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA). They were maintained on agar slants, at 4°C and transferred monthly. Anaerobes were subcultured weekly. *Salmonella typhimurium*

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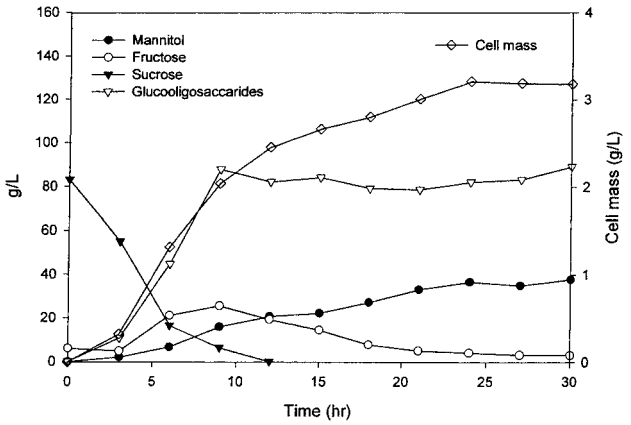

Figure 1 Glucooligosaccharide production by *L. mesenteroides* ATCC 13146 from sucrose as a function of time.

ATCC 14028 and *E. coli* B ATCC 23226 were maintained on tryptic soy agar (Difco, Detroit, MI). *Bifidobacterium bifidum* ATCC 35914, *Bifidobacterium longum* ATCC 15708, *Lactobacillus johnsonii* ATCC 33200 and *Leuconostoc mesenteroides* ATCC 13146 were maintained anaerobically on *Lactobaccilli* MRS slants (Difco) containing 0.05% (w/v) cystein.

Preparation of oligosaccharides

Batch fermentations were conducted in a 2-l BioFlo II fermentor (New Brunswick Scientific, New Brunswick, NJ) with a working volume of 1.0 l. The medium had the composition (g/l): sucrose, 100; maltose, 50; yeast extract, 5; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; NaCl, 0.01; $MnSO_4 \cdot 7H_2O$, 0.01; $CaCl_2$, 0.05; KH_2PO_4, 3. Fermentors were inoculated from late log-phase flask cultures at 1.0% of working volume. Fermentations were conducted at pH 6.5, 28°C, and 200 rpm. After harvesting, cells were removed by centrifugation at 10,400×*g* for 20 min. Activated charcoal (5 g/l, Sigma Chemical, St. Louis, MO) and Celite 545 (1 g/l, Fisher Scientific, Fair Lawn, NJ) were added to cell-free culture broth and mixed at 50°C for 20 min. The broth was then filtered through No. 6 filter paper (Whatman International, Maidstone, England) to remove the carbon. Filtered broths were desalted using ion-exchange columns filled with an anion-exchange resin in the hydroxide form and a cation-exchange resin in the hydrogen form (Rohm and Haas, Philadelphia, PA). The eluents were concentrated by vacuum evaporation (Brinkmann Instrument, Westbury, NY) to 65% solids. Mannitol crystallized upon cooling the concentrates and was removed by decantation. Oligosaccharides were separated from the mannitol-free concentrates using a cation-exchange column (calcium form), The oligosaccharide fractions were concentrated by vacuum evaporation.

Analytical methods

Bacterial growth was measured by turbidimetry at 650 nm, calibrated against cell dry weight. Cells from a known volume were harvested by centrifugation at 10,400×*g* for 2 min, washed with deionized water, resuspended in a minimum volume of water and dried (initially overnight at 95°C and then at 105°C) to constant weight. An absorbance of 1.0 at 650 nm was equivalent to 0.51 g of dry matter·l^{-1}.

Separation and qualitative identification of oligosaccharides was done using thin-layer chromatography (TLC). Whatman K6F silica gel plates (10×20 cm) were obtained from Fisher Scientific (Chicago, IL). A homologous series of isomaltodextrins (DP 1–10) was kindly donated by Dr. Doman Kim (Chonnam National University, Kwangju, Korea). Pannose, maltopentose, maltohexose and maltoheptose (Sigma) were used as standards. Aliquots (1–2 μl) of the solutions to be analyzed were applied 20 mm from the bottom of the TLC plates with a 10-μl microsyringe pipet. The plates were developed at ambient temperature, using a mixture of solvents (acetonitrile, ethyl acetate, propanol and water in volume (ml) proportions of 85:20:50:70). After development was complete, the plates were dried, and the carbohydrates visualized using a spray of an ethanol solution containing 0.3% (w/v) α-naphthol and 5% (v/v) H_2SO_4. After air-drying the plate, spots were developed by heating in an oven for 10 to 20 min at 100°C. GOS were identified by comparing their chromatographic behavior with those of the standards.

High-performance ion chromatography was used for quantitative analysis of glucose, fructose, sucrose, mannitol and maltose concentrations in solution using a CarboPac MA1 column (Dionex,

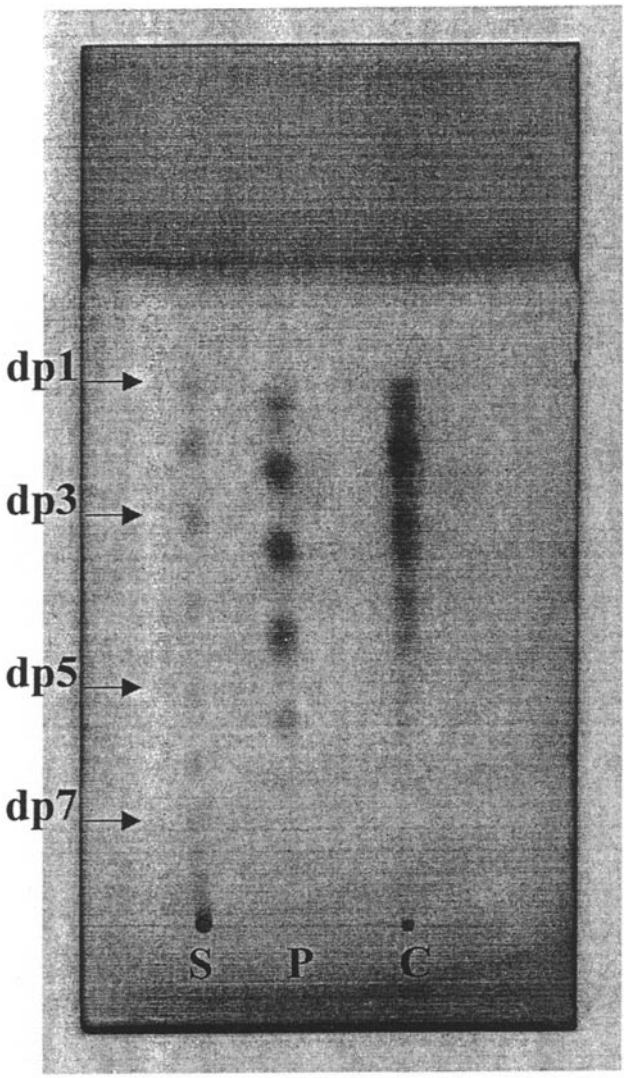

Figure 2 TLC of branched α-glucooligosaccharides of *L. mesenteroides* ATCC 13146. DP, degree of polymerization; S, isomaltodextrins; P, glucooligosaccharide product; C, commercial isomaltooligosaccharides (Wako Pure Chemical Industry, Osaka, Japan).

Table 1 Growth comparison on the glucooligosaccharide preparation

Organism	Growth rate at exponential growth phase ([absorbance unit×100]·h^{-1})			Relative growth rate on GOS[a]
	Glucose	GOS	FOS	
S. typhimurium	9.89	3.64	3.48	36.8
E. coli	9.35	2.68	2.44	28.7
B. bifidum	13.30	9.81	13.10	73.8
L. johnsonii	11.06	10.74	10.70	97.1
B. longum	11.72	11.69	11.70	99.7

GOS, glucooligosaccharide product; FOS, commercial fructooligosaccharides (Samyang Genex, Seoul, Korea).
[a]Growth rate at exponential growth phase on glucose was calculated as 100.

Sunnyvale, CA) and a pulsed amperometric detector (PAD, Dionex). Samples were eluted at 0.4 ml·min^{-1} with a 0.48 M NaOH solution. Oligosaccharide concentrations were calculated from peak areas on high-performance liquid chromatography on an Aminex-HPX-87K Bio-Rad column (Bio-Rad Laboratories, Hercules, CA) run at 85°C with 0.02 M K_2HPO_4 as eluent, at a flow rate of 0.5 ml·min^{-1}, using glucose as a standard.

Oligosaccharide utilization by selected microorganisms

Growth of selected bacteria in the presence of oligosaccharides was compared by measuring absorbances at 660 nm. The medium used for both the *Bifidobacterium* sp. and *L. johnsonii* had the same composition as *Lactobacillus* MRS broth with 0.05% (w/v) cystein except the carbon source was replaced by oligosaccharide preparations. The growth media for *S. typhimurium* and *E. coli* was tryptic soy broth with the carbon source replaced by oligosaccharide preparations. All carbon sources were filter sterilized (0.2 μm). The following carbon sources were compared: glucose (Sigma), commercial fructooligosaccharides (FOS; >97.5%, Samyang Genex, Seoul, Korea) and oligosaccharide preparations. Individual culture, anaerobic growth tests were conducted in sealed glass test tubes. Each tube was inoculated from an overnight culture with either *S. typhimurium* or *E. coli* and a 24- to 48-h culture of a *Bifidobacterium* sp. or *L. johnsonii*. The experiments with *Bifidobacterium* sp. and *L. johnsonii* were conducted under anaerobic conditions using anaerobic jars (BBL, Cockeysville, MD). MRS broth containing 0.05% (w/v) cystein with oligosaccharides as a carbon source was used for mixed cultures of *S. typhimurium* and *L. johnsonii*. Total viable counts were conducted on MRS agar and the cell numbers of *S. typhimurium* were determined from growth on MacConkey agar plates (Difco). Cell numbers for *L. johnsonii* were obtained as the difference between total viable count and *S. typhimurium* numbers.

Results

GOS: production and composition

GOS production by fermentation was complete by late log phase, about 10 h postinoculation, and levels did not drop thereafter (Figure 1). Sucrose disappeared rapidly during the log phase with depletion corresponding to the transition to stationary phase. Fructose accumulation peaked at 9 h. Mannitol production occurred primarily in the stationary phase and was linked to the fructose concentration, where the rate of fructose disappearance was the inverse of the rate of mannitol formation. Upon completion of the fermentation, the cell mass was 3.2 g/l. The weight percent yield of oligosaccharide (product produced×100/[160×mol of sucrose consumed)+(342×mol of maltose consumed)]) was 82% of theoretical and the conversion of fructose to mannitol was 71% of theoretical. Chromatography showed that the GOS produced were branched polymers ranging in size from DP (degree of polymerization) 2 to 7 (Figure 2). By weight, there was 6.9% DP 2, 28.4% pannose, 36.7% branched DP 4, 19.1% branched DP5, 7.4% branched DP 6 and 1.2% branched DP 7. There was only a trace amount of monosaccharide (<0.2%) present and no polysaccharides larger than DP 7.

GOS as a carbon source for microbial growth

Individual cultures: Growth of selected bacteria on *L. mesenteroides* B-742 GOS was compared with growth on a commercial fructooligosaccharide (FOS) mixture. Both types of oligosaccharides were utilized by *S. typhimurium* and *E. coli* more slowly than glucose. There was no significant difference between growth rate on either of the oligosaccharide preparations. The growth suppression of *E. coli* in the presence of oligosaccharides was marginally greater than that of *S. typhimurium* (Table 1). The growth of selected probiotic strains on GOS was also compared. GOS supported the growth of *B. longum* and *L. johnsonii* and showed no significant difference compared to glucose as carbon source. Utilization of the GOS product by *B. bifidum* was less rapid than utilization of commercial FOS or glucose.

Mixed cultures: To test for a prebiotic effect of GOS, mixed cultures of *S. typhimurium* and *L. johnsonii* were grown on the oligosaccharides. When the medium pH was above 5.0, both

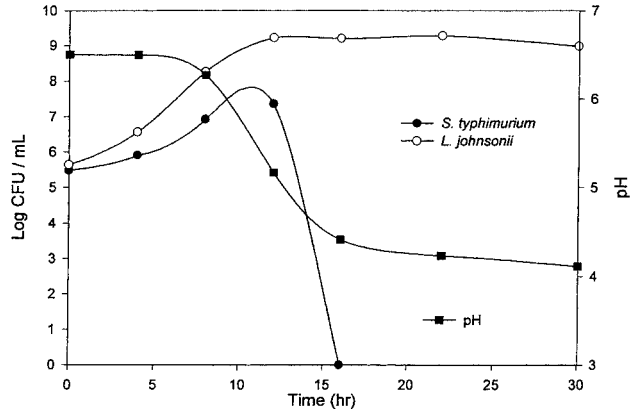

Figure 3 Growth of mixed cultures of *S. typhimurium* and *L. johnsonii* on the glucooligosaccharide preparation.

organisms grew; however, *S. typhimurium* grew more slowly than *L. johnsonii*. As the population of *L. johnsonii* increased, the pH dropped (Figure 3). When the pH was below 5.0, viable *S. typhimurium* numbers decreased until they were below detection level (<1).

Discussion

Current technology for the production of oligosaccharides is limited to extraction from plant sources, acid or enzymatic hydrolysis of polysaccharides or synthesis from starch by transglycosylation reactions [3,16]. These procedures are costly, limiting use of oligosaccharides to high value products. Conventional fermentations are the most practical means for industrial manufacture of carbohydrate polymers. Use of a chain-shortening acceptor [25] and a microbial strain that primarily produces highly branched polymers in a dextran fermentation resulted in production of selected α-glucooligosaccharides. These oligosaccharides are branched polymers between DP 2 and 8.

DP for most prebiotic oligosaccharides falls in the range of DP 2 to 8 [14]. Those oligosaccharides larger than DP 3 produced in this fermentation were branched and ranged from DP 2 to 7. Dextrans from *L. mesenteroides* ATCC 13146 show a high degree of branching and exhibit a comb-like structure that is resistant to enzyme hydrolysis. The branches are single glucose molecules in length [6,18]. Oligosaccharides synthesized by dextransucrase from this bacterium had an α-1,6 backbone with α-1,3- and/or α-1,4-branched side chains when maltose was used as acceptor [18].

Growth of *S. typhimurium* or *E. coli* on ATCC 13146 oligosaccharides was less than 40% of the growth on an equivalent amount of glucose and similar to growth on commercial FOS. *L. johnsonii* and *B. longum* showed no difference in growth rate on glucose or the oligosaccharide preparations. When *L. johnsonii* and *S. typhimurium* were grown together on the oligosaccharide preparations, the oligomers stimulated the growth of the *Lactobacillus* but were not readily utilized by the pathogenic organism. It appears that these oligosaccharides are utilized preferentially by probiotic strains.

Use of these oligosaccharides as prebiotics should lead to the production of intestinal lactic acid, increases in short-chain fatty acid production and lower pHs in large intestine. With appropriate application, they may be a useful food additive to help prevent establishment of pathogenic organisms [10,11]. Similar effects have been seen in studies on the effect of FOS in feed trials with broilers. Oligosaccharides reduced the susceptibility to *Salmonella* colonization of the intestine of chickens, [2] increased *Bifidobacterium* levels and reduction in the level of *Salmonella* present in the caecum. These studies do not allow direct prediction of *in vivo* effects but indicate that this type of oligomer can be a prebiotic for intestinal microflora.

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