Journal of Microbiology and Biotechnology
Vol. 7. No. 3, 174-179, 1997

Bifidogenic Effect of Glucooligosaccharide Prepared from Glucose by Extrusion Process

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In order to investigate effect of glucooligosaccharide (GOS) prepared by extrusion process as a bifidogenic factor, cultivation of *Bifidobacterium* sp., *Bacteroides fragilis* and *Clostridium perfringens* was done and analyzed. *B. fragilis* and *C. perfringens* were able to utilize only 16% and 11% of the oligosaccharides in GOS, respectively, whilst *Bifidobacterium* sp. FBD-22 could utilize 38%. Especially, many kinds of oligosaccharides in GOS were able to be utilized selectively only by *Bifidobacterium* sp.. In case that GOS, as a carbon source, was used in the co-cultivation by *Bifidobacterium* sp., *B. fragilis* and *C. perfringens*, growth of *Bifidobacterium* sp. was not influenced by the existence of *B. fragilis* and *C. perfringens*. *Bifidobacterium* sp. showed advantage on carbon source competition for GOS with *B. fragilis*. Acetic acid, antimicrobial agent in the intestine, was produced two times more from GOS than glucose in co-cultures of three strains. Therefore, it is suggested that GOS can be a potent bifidogenic factor which proliferates the population of *Bifidobacterium* sp. and may finally improve the intestinal environments of human.

Bifidobacteria has been known as one of the beneficial microorganisms in the human intestine. The beneficial effects of bifidobacteria such as anticancer activity (21), stimulation of immune systems (8), lowering plasma cholesterol (16), prevention of constipation (9) etc., have been reported. So it seems to be important to increase and maintain the high population of bifidobacteria in the intestine for ensuring good health and longevity in humans.

Generally, there may be no sufficient nutrients in the intestine for all microorganisms and only limited non-digestive dietary component can affect the selective growth of *Bifidobacterium* sp. (5, 18). Glucose and other digestible sugars are thought to exist in a low concentration and it may be rapidly used up (3). Therefore, it is required to take regularly bifidogenic nutrient factor which can be used only by *Bifidobacterium* sp. for the selective proliferation.

Nowadays many bifidogenic factors have been found. Glucosamine-derivatives (10, 22), peptides, pantetine-derivatives (20, 24) and non-digestive oligosaccharides such as lactulose (19), raffinose (1), fructooligosaccharide and galactooligosaccharide (7, 11) were reported. However, they are limited to use as a factor because of high cost and much effort for preparation.

In this research, we obtained glucooligosaccharide (GOS) by the extrusion process of glucose. Compared to the commercial oligosaccharides, GOS by the method was able to be made by a simple process and in a low price.

The principal aim of this study was to evaluate the effect of GOS as a bifidogenic factor on *Bifidobacterium* sp.. Growth effects toward *Clostridium perfringens*, pathogenic species, and *Bacteroides fragilis*, one of the predominant species in the human intestine were also determined.

MATERIALS AND METHODS

Bacterial Strains

Bifidobacterium sp. FBD-22 was isolated from a healthy Korean feces by the method of Ji *et al.* (14), which showed amylase activity. *Clostridium perfringens* ATCC 13124 and *Bacteroides fragilis* ATCC 25285 were purchased from American Type Culture Collection.

Media and Culture Condition

All media and cultures were prepared under anaerobic

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Key words: bifidogenic factor, glucooligosaccharide, extrusion process, *Bifidobacterium* sp.

condition as the method used commonly (23). For the broth cultivation, modified PYF medium was used. It contained yeast extract 10 g/l, proteose peptone No.3 (Difco) 5 g/l, L-cysteine HCl 0.5 g/l, salt solution 40 ml/l ($CaCl_2$ 0.2 g/l, $NaHCO_3$ 10 g/l, $MgSO_4$ 0.2 g/l, NaCl 2 g/l, K_2HPO_4 1 g/l). Carbon sources were filtrated using 0.2 μm pore size filter (Satorius, Germany) and added to autoclaved PYF broth. NN medium (15), (Table 1) and VA medium (13), (Table 2) were used for the selective viable count of *C. perfringens* and *B. fragilis*, respectively. For the selective enumeration of *Bifidobacterium* sp. FBD-22 from mixed culture, modified TP (MTP) medium was used (14) (Table 3).

To count total number of bacteria, BHI Medium (Difco, U.S.A.) supplimented with 1% (w/v) glucose was used. Samples (1 ml) taken from the culture broth were diluted serially with anaerobic dilution solution (KH_2PO_4 4.5 g/l, Na_2HPO_4 6.0 g/l, L-cysteine HCl 0.5 g/l, Bacto agar 0.5 g/l) and spreaded on each selective medium (BHI, MTP, NN and VA medium). Plates were incubated in the anaerobic glove box (COY laboratory product Inc., U.S.A.) at 37°C for 48 h.

Preparation of Glucooligosaccharide

In this research, glucooligosaccharide was manufactured by extrusion process of glucose. The extrusion reactor was employed to induce the polymerization of glucose. Glucose powder (Sam Yang Co., Korea) mixed with citric acid (99 : 1, w/w) was extruded at 180°C by corotation intermeshing twin screw extruder (Biex-DNDL 44, Buhler Brothers Co., Switzerland) with the L/D (length/diameter) ratio of 40.

Analytical Methods

Organic acids were determined by spectrophotometry at 340 nm using biochemical analysis kit (Behringer

Table 1. Composition of NN agar medium.

Component	Amount
Peptone (Difco)	40 g
Na_2HPO_4	5 g
KH_2PO_4	1 g
NaCl	2 g
$MgSO_4$	0.1 g
Glucose	2 g
Agar	25 g
D.W.	1000 ml
Egg york solution[a] (50% w/w)	100 ml
Neomycin sulfate (2% solution)	10 ml
	pH 7.6

[a]Yorks were separated aseptically from fresh eggs and mixed with the equal amount of sterilized saline. Egg york solution and neomycin sulfate solution were added to the autoclaved medium seperately.

Table 2. Composition of VA agar medium.

Component	Amount
Lab-lamco powder (Oxoid)	2.4 g
Proteose peptone No. 3 (Difco)	10.0 g
Yeast extract	5.0 g
Na_2HPO_4	4.0 g
Glucose	1.5 g
Starch, soluble	0.5 g
L-cystine	0.2 g
Silicon antifoamer	1 ml
Bacto agar	15.0 g
L-cysteine·HCl	0.5 g
Horse blood	50 ml
Vancomycin solution[a]	1 ml
D.W.	1000 ml
	pH 7.6~7.8

[a]1.5 mg of Vancomycin (Sigma) was dissolved into 1 ml of D.W. and sterilized by filtration and added to the autoclaved medium.

Table 3. Composition of MTP agar medium.

Component	Amount
Trypticase (BBL)	10 g
Proteose peptone NO. 3 (Difco)	5 g
Ammonium sulfate	3 g
Potassium phosphate, monobasic	2 g
Potassium phosphate, dibasis	1 g
L-cysteine·HCl	0.5 g
Magnesium sulfate	0.2 g
Sodium propionate	15 g
Oligosaccharides mixture[a]	50 ml
Agar	15 g
D.W.	1000 ml
	pH 7.0

[a]Oligosaccharides mixture contained fructooligosaccharide 20% (w/v) and galactooligosaccharide 20% (w/v), and was sterilized by filtration and added to the autoclaved medium.

Table 4. Analysis conditions for gel permeation chromatography (Waters, U.S.A.).

System	:	Waters 410
Detector	:	RI detector
Column	:	Ultrahydrogel 120, 250
Eluent	:	0.1 M $NaNO_3$
Flow rate	:	0.8 ml/min
Temperature	:	Room temperature

Table 5. Analysis conditions of GOS with ion chromatography (Dionex Co., Sunnyvale, U.S.A.).

System	:	DX-600
Detector	:	Pulsed Amperometric Detector (E1: 0.1 V, E2: 0.6 V, E3: -0.8 V)
Eluent A	:	100 mM NaOH
B	:	100 mM NaOH+500 mM NaOAc
Temperature	:	Room temperature

Manheim, Germany).

Molecular weight distribution of GOS was determined by gel permeation chromatography (Waters LC Module I/ M410 RI detecter/Millennium 2010, U.S.A.) and its analysis condition was shown in Table 4. The composition of oligosaccharides in GOS was analysed by ion chromatography (Dionex DX-600, U.S.A.) and analysis condition was shown in Table 5.

RESULT

Molecular Weight of Glucooligosaccharide (GOS)

GOS was composed of 24.99% (w/w) glucose and 75.01% (w/w) oligosaccharide, and its average molecular weight was 523 dalton equivalent to the polymerized product of three molecules of glucose. However, oligosaccharides equivalent to 2 to 6 glucose molecules existed in GOS (Fig. 1).

Utilization of GOS by *Bifidobacterium* sp., *B. fragilis* and *C. perfringens*

To investigate utilization patterns of GOS by *Bifidobacterium* sp., *B. fragilis* and *C. perfringens*, each strain was inoculated in the PYF broth containing GOS as a carbon source. After they were incubated for 24 h, residual oligosaccharides in the culture broth were analyzed by ion chromatography (Fig. 2, Table 6). There were several unknown oligosaccharide peaks in GOS. Peak G was identified as glucose whileas peak a, b and c were not identified in this experiment. Peak G, a, b and c among those peaks, which were main portion of GOS, showed big changes after incubation. Glucose was used up by those strains and the oligosaccharide a was utilized only by *C. perfringens*. Oligosaccharide b and c were utilized by neither *C. perfringens* nor *B. fragilis*. However, *Bifidobacterium* sp. used them exclusively. About 38% of oligosaccharides in GOS were utilized by *Bifidobacterium* sp., which was more than that 11% of *C. perfringens* and 16% of *B. fragilis*. These facts indicated that GOS would give a good selective growth on *Bifidobacterium* sp. than others in case of a carbon source deficiency like in the intestine.

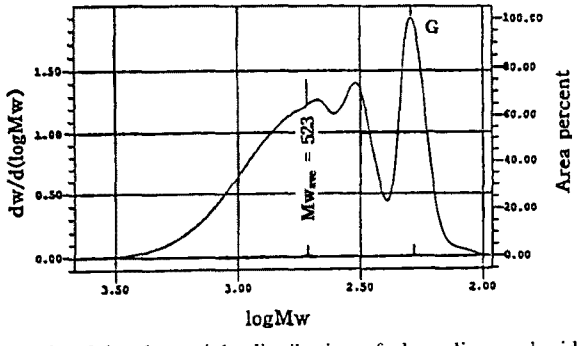

Fig. 1. Molecular weight distribution of glucooligosaccharide.
Contents of components were calculated from peak area. GOS was composed of 24.99% (w/w) glucose and 75.01% (w/w) oligosaccharides. G indicates glucose. Mw_{ave} means average molecular weight.

Growth of *Bifidobacterium* sp., *B. fragilis* and *C. perfringens* in Single Culture and Mixed-culture Containing GOS

To know how well GOS was used by *Bifidobacterium* sp., *B. fragilis* and *C. perfringens*, growth of each strain was observed by viable cell during incubating for 48 h in PYF broth containing 1% (w/v) GOS as a carbon source. All three strains showed good growth in the medium with GOS so that their population increased from 10^6 to 10^{8-9} cfu/ml in the single cultures (Fig. 3). *Bifidobacterium* sp., *B. fragilis* and *C. perfringens* were again co-cultivated (Fig. 4). Growth of *Bifidobacterium* sp.

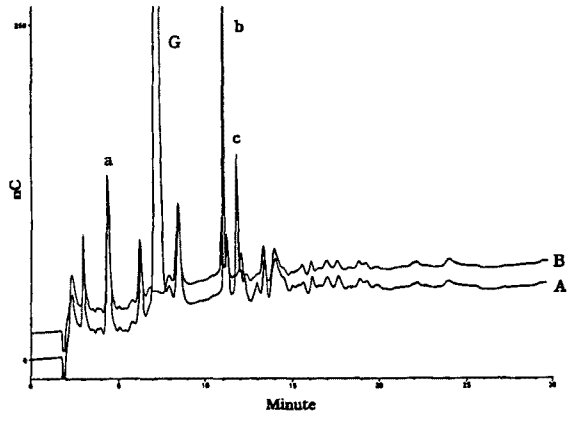

Fig. 2. Analyses of utilization patterns of glucooligosaccharide by *Bifidobacterium* sp.
Before inoculation of *Bifidobacterium* sp. FBD-22, oligosaccharides composition in GOS was analysed (chromatogram A). After *Bifidobacterium* sp. was incubated for 24 h in the PYF medium containing GOS, oligosaccharides which remained in the medium were analysed (chromatogram B). Peak G, a, b and c were major component of GOS. Peak a, b and c indicated unknown oligosaccharides, and peak G was identified as glucose.

Table 6. Utilization pattern of glucooligosaccharide by *Bifidobacterium* sp., *Bacteroides fragilis* and *Clostridium perfringens*.

Microorganism	Total[1] oligosaccharide	Oligosaccharide		
		a[2]	b[3]	c[4]
Control[5]	100	13.2	20.3	7.10
Bifidobacterium sp.	62	13.1	3.10	0.41
B. fragilis	84	12.7	16.6	7.20
C. perfringens	89	0.36	20.8	7.90

After each strain was incubated at 37°C for 24 h, quantitative ananlysis of oligosaccharides in GOS was performed by ion chromatography (Dionex DX-600, U.S.A.). Numbers were percentage of each components, which was calculated from the peak area in chromatogram. [1]Total oligosaccharide was calculated by subtracting content of glucose from GOS. [2,3,4]Unknown oligosaccharides in Fig. 2. [5]Intact GOS.

was not affected by the other members in the culture. The numbers of viable cell of *B. fragilis* started to decrease at about 4 h later. However, *C. perfringens* grew well and reached the maximum growth after 8 h incubation like in the single culture. Here, we noticed the fact that *C. perfringens* could grow in the sugar-free medium with amino acid or protein, which was tested (data not shown). This strain could utilize amino acid as a carbon source by deamination of amino acid (17). Therefore, growth of *C. perfringens* may not be affected by depletion of a carbon source.

To know the pattern of competition for uptake of carbon source more accurately, *Bifidobacterium* sp. and *B. fragilis* were co-cultivated in PYF broth containing glucose or GOS. Though growth of *Bifidobacterium* sp. was not influenced by the sugars, growth of *B. fragilis* was affected. The maximun number of viable cell of *B. fra-*

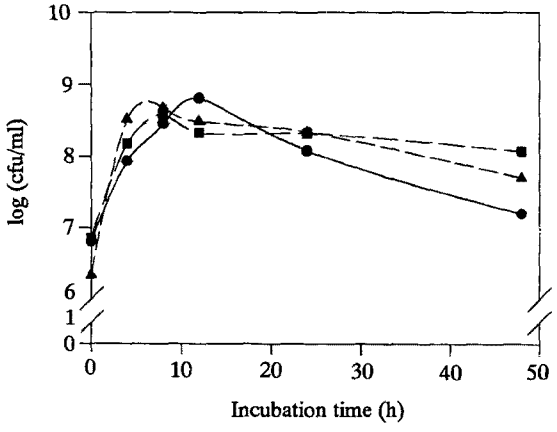

Fig. 3. Growth of *Bifidobacterium* sp., *Bacteroides fragilis* and *Clostridium perfringens* in medium with glucooligosaccharide.

Each strain was inoculated separately into three PYF media containing GOS as a carbon source and incubated at 37°C for 48 h. Symbols: —●—, *Bifidobacterium* sp. FBD-22; —■—, *Bacteroides fragilis*; —▲—, *Clostridium perfringens*.

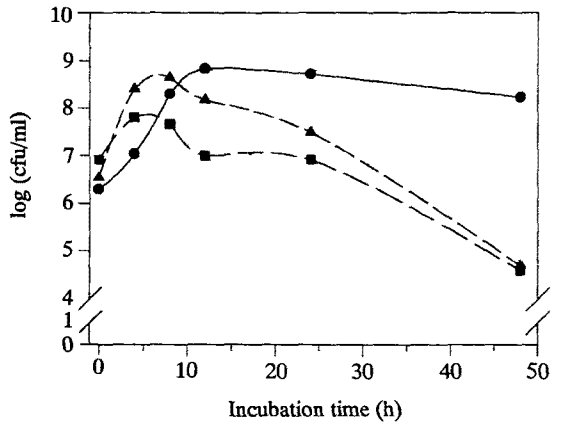

Fig. 4. Growth of *Bifidobacterium* sp., *Bacteroides fragilis* and *Clostridium perfringens* in co-cultivation with glucooligosacchride.

Each strain was inoculated together into a PYF medium containing GOS as a carbon source and incubated at 37°C for 48 h. Symbols: —●—, *Bifidobacterium* sp. FBD-22; —■—, *Bacteroides fragilis*; —▲—, *Clostridium perfringens*.

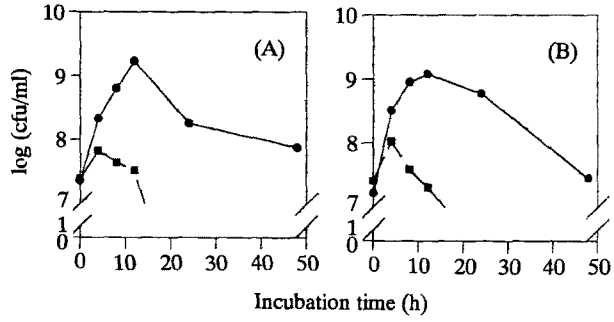

Fig. 5. Growth pattern of *Bifidobacterium* sp. and *Bacteroides fragilis* in co-cultivation with glucooligosaccharide (A) and glucose (B).

Each strain was inoculated together into the PYF medium with GOS and the medium with glucose, as a carbon source, and incubated at 37°C for 48 h. Symbols: —●—, *Bifidobacterium* sp. FBD-22; —■—, *Bacteroides fragilis*.

Table 7. Acetic and lactic acid production of *Bifidobacterium* sp., *Bacteroides fragilis* and *Clostridium perfringens*.

Incubation time (h)	Organic acid (mM)	*Bif.* sp FBD-22[a]	*Bacteroides fragilis*[b]	*Clostridium perfringens*[c]	Co-cultivation[d]	
					GOS	glucose
4	Acetic acid	19.8	0.93	3.16	11.2	12.3
	Lactic acid	8.51	ND[e]	ND	2.30	3.90
8	Acetic acid	24.1	5.30	8.83	18.4	19.6
	Lactic acid	9.46	ND	ND	3.80	13.3
12	Acetic acid	30.8	8.00	10.9	31.0	23.7
	Lactic acid	15.1	ND	ND	7.20	17.4
24	Acetic acid	46.5	12.7	14.6	51.3	24.5
	Lactic acid	18.6	ND	ND	11.5	21.6

[a,b,c] A carbon source for each strain was GOS. [d] *Bifidobacterium* sp. FBD-22, *B. fragilis* and *C. perfringens* were inoculated together into a PYF medium containing GOS or glucose and incubated at 37°C for 24 h. [e] Not detected.

gilis in the medium with GOS was much less than that in the medium with glucose (Fig. 5). It meant that GOS was not a favorate carbon source for *B. fragilis* growth. Similar to the previous result (Fig 4), its growth was inhibited after 4 h. It might be due to lack of a carbon source available by itself.

Production of Organic Acids by *Bifidobacterium* sp., *B. fragilis* and *C. perfringens*

The amount of acetic acid and lactic acid in the cultures was determined. *Bifidobacterium* sp. produced organic acid more than *B. fragilis* and *C. perfringens*. *Bifidobacterium* sp. produced 46.5 mM acetic acid from GOS, while *C. perfringens* and *B. fragilis* produced only 12.7 mM and 14.6 mM acetic acid, respectively. In addition, lactic acid was produced only by *Bifidobacterium* sp. (Table 7). In co-cultivation, GOS was a better carbon source for production of acetic acid than glucose, which was known as a antimicrobial agent.

DISCUSSION

In present research, we obtained glucooligosaccharide (GOS) manufactured by the extrusion process of glucose (12). In order to know whether GOS can be used as a bifidogenic factor which promotes the growth of *Bifidobacterium* sp., GOS was cultured by 10 *Bifidobacterium* sp. from the Culture Collection's. However, almost *Bifidobacterium* sp. which did not have activity of amylase utilized limitedly the oligosaccharides of GOS. So we isolated five *Bifidobacterium* sp. showing amylase acitivity from Korean feces, which grew well in the GOS broth. Among the isolated strains, we chose *Bifidobacterium* sp. FBD-22 that could utilize well many kinds of monosaccharides and oligosaccharides such as fructooligosaccharide, galactooligosaccharide and isomaltooligosaccharide. This strain also showed high acivities of α-glucosidase and β-glucosidase in comparison with other non-amylolytic *Bifidobacterium* sp. (data not shown here).

For the co-culture, *B. fragilis* was chosen, because it might be the highest population among the microflora of human intestine. *C. perfringens* was reported to be a representative pathogen producing the harmful metabolite in human gut (4). The effect of GOS on growth of each strain was investigated. *B. fragilis* did not grow as much as the the other strains. It said that GOS did not work as a good growth factor for *B. fragilis*. In fact, Table 6 showed that *B. fragilis* was hardly able to utilize oligosaccharides in GOS. Growth of *C. perfringens* seemed not to be affected by depletion of a carbon source as long as nitrogen source remained. Growth of *Bifidobacterium* sp. was not affected by co-cultivation. When glucose was used up, *Bifidobacterium* sp. might utilize oligosaccharides that were not utilized by the other two strains.

It was reported that molar ratio of acetic acid and lactic acid, which were produced during incubation of bifidobacteria, could be changed according to the carbon sources (2). In our result, GOS was a better carbon source than glucose for the production of acetic acid. Generally, as it have been known that antimicrobial activity of acetic acid would be important in the intestine (6), GOS was thought to be more advantageous carbon source to inhibit the undesirable microorganisms in it. The acetic acid seemed to have something to do with viability of *B. fragilis* and *C. perfringens* at the late stationary phase (Fig. 3 and 4).

In conclusion, we showed that GOS could be a potent bifidogenic factor for amylolytic bifidobacteria and might give some inhibitory effect of *C. perfringens* and *B. fragilis*. We can expect that daily uptake of GOS will cause the selective proliferation of some *Bifidobacterium* sp. in the gut. However, further work to verify the effect *in vivo* is required and human administration is also needed.

Acknowledgement

The present studies were supported partially by a grant from Korea Ministry of Science and Technology.

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(Received February 14, 1997)