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In Vitro Bifidogenic Effect of Nondigestible Oligosaccharides Isolated from Red Ginseng Marc

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Abstract The purpose of this research was to investigate the effects of nondigestible oligosaccharides (NDO) from red ginseng marc on the growth of *Bifidobacterium* spp. Red ginseng marc, a fibrous byproduct of ginseng extract from processing, was destarched by α-amylase and amyloglucosidase treatment, and then treated with a commercial pectinase to produce NDO. The bifidogenic effects of NDO on *B. adolescentis*, *B. animalis*, *B. breve*, and *B. longum* were investigated *in vitro*. NDO significantly promoted the growth of *Bifidobacterium* spp. The growth, decrease of pH, and organic acid formation (acetate, lactate, formate) were markedly different among the species. *B. adolescentis* showed the best growth and produced the greatest amount of organic acids. When NDO was used as a carbon source in the cocultivation of *Bifidobacterium* spp. and *Clostridium perfringens*, the growth of *Bifidobacterium* spp. was not influenced by the existence of *Cl. perfringens*. The result strongly suggested that NDO from red ginseng marc could be used as a potential bifidogenic source.

Key words: Ginseng fiber, oligosaccharides, bifidogenic effect, *Bifidobacterium* spp.

Bifidobacteria are predominant bacteria of the normal human intestinal microflora and are thought to play an important role in maintaining good health. The potential benefits ascribed to bifidobacteria include maintenance of a healthy microflora, inhibition of pathogenic bacteria, reduction of serum cholesterol, reduction of blood ammonia concentration, synthesis of vitamins and anticarcinogenics, stimulation of immune systems, prevention of constipation, and anticancer activity [13, 7, 8, 9, 15].

Ginseng marc, which is a residue remaining after manufacturing ginseng extract with hot water or 70% of alcohol, contains 50–60% of starch [10] and a large quantity of acidic polysaccharide [5]. The bifidogenic effects of solvent extracts from ginseng (*Panax ginseng* C.A. Meyer) [2] and conditions for enzymatic extraction of the polysaccharide from ginseng marc have been investigated [6]. However, ginseng marc as a functional dietary fiber or oligosaccharides has not been utilized. Currently, industrial strategies have been focused on the selective stimulation of growth and activity of bifidobacteria in the intestine by supplementation of the host's diet with specific, nondigestible carbohydrates [3].

The aim of this study was to investigate the potential utilization of NDO from red ginseng marc for different species of *Bifidobacterium*.

MATERIAL AND METHODS

Bacterial Strains

The following strains were used in this study: *B. adolescentis* KFRI 740 (ATCC 15703), *B. animalis* KFRI 741 (ATCC 25527), *B. breve* KFRI 744 (ATCC 15700), *B. longum* KFRI 893 (ATCC 15707), and *Clostridium perfringens* KFRI 752 (ATCC 13124). All strains were purchased from Korea Food Research Institute (Sungnam, Korea).

Media and Culture Conditions

All media and cultures were prepared under anaerobic conditions and cells were routinely cultured in a KFRI 25 medium (RCM; Oxoid CM 149). The culture was carried out by using a PYF medium (pH 7.5) which contained 1% yeast extract, 0.5% proteose peptone No. 3 (Difco), 0.5% tryptone peptone, 0.05% L-cysteine · HCl, and 4% salt solution (0.02% $CaCl_2$, 1% $NaHCO_3$, 0.02% $MgSO_4$, 0.2% NaCl, 0.1% K_2HPO_4). NDO as a carbon source was added to autoclaved PYF medium to give a final concentration of 1% (w/v). Cultures were performed anaerobically in a N_2 atmosphere at 37°C for 48 h without any pH control. Stock

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cultures were maintained at -70°C in a culture medium. Growth was followed by measuring the optical density at 660 nm, determining the dry cell weight (DCW), and measuring the decrease of pH. A calibration curve for each bacterial strain was established by the relation between absorbance and DCW. Specific growth rates were calculated by using the equation $\mu=dX/dt \times X^{-1}$, where X is the biomass (DCW) at time t, and μ is the specific growth rate (h^{-1}). BS [14] and NN [12] media were used for the selective viable cell count of *Bifidobacterium* spp. and *Clostridium perfringens*, respectively. To count the total number of bacteria, BHI medium (Difco, U.S.A.) supplemented with 1% (w/v) glucose was used. Samples (1 ml) taken from the culture broth were diluted serially with anaerobic dilution solution (0.45% KH_2PO_4, 0.6% Na_2HPO_4, 0.05% L-cysteine · HCl, 0.05% Bacto agar) and spread on each selective medium (BHI, BS, NN medium). Plates were incubated in the anaerobic gaspack jar system (BBL, U.S.A.) at 37°C for 48 h.

Enzymes

α-Amylase (Termamyl 120L, Novo Nordisk A/S, Denmark) and amyloglucosidase (AMG E, Novo Nordisk A/S, Denmark) were used to destarch red ginseng marc. Commercial food grade pectinase (Pectinex Ultra SP-L, Novo Nordisk A/S, Denmark) and hemicellulase (Econase HC 400, Econase Co., Finland) were used to hydrolyze the nonstarch water-soluble polysaccharides from ginseng marc.

Preparation of Nondigestible Oligosaccharides (NDO)

Red ginseng marc (100 g) was grinded well and destarched with Termamyl 120-l (α-amylase, 95°C, 2 h, pH 6.0) and AMG E (amyloglucosidase, 60°C, 2 h, pH 6.0). The water-soluble fraction was discarded by centrifugation and the insoluble fraction was washed several times with water, followed by enzymatic hydrolysis with 1:1 (w/w) mixture of Pectinex Ultra SP-L and Econase HC 400 (45°C, 4 h, pH 4.5, 10 mM acetate buffer). The hydrolysates were treated with four times (v/v) volume of isopropanol to remove high molecular weight fractions, and the supernatant was evaporated in a rotary evaporator (Heidolph Elckito, Lauda Vacuubtaqnd, Germany). The low molecular weight sugars of the concentrate were separated by ultrafiltration with MWCO 500 membrane (Millipore, U.S.A.) and the retentate was freeze-dried as NDO.

Analytical Methods

The molecular weight distribution of NDO was determined by a gel permeation chromatography (Waters Alliance 2690, U.S.A.) equipped with an ultrahydrogel linear (7.8×300 mm, 6–13 μm) column and ultrahydrogel 500 (7.8×300 mm, 7 μm) column. The columns were operated with 0.1 M $NaNO_3$ with a flow rate of 1.0 ml min^{-1}, and the eluent was monitored with a refractive index detector (2410, Waters, U.S.A.). For analyzing the sugar constituents, NDO was hydrolyzed with 72% H_2SO_4 (3 h, 100°C) which was then analyzed by a Bio-LC system (Dionex Co., Sunnyvale, U.S.A.) equipped with a Dionex Carbopac PA-100 column (4×250 mm). The neutral sugar was analyzed by using a 22.6 mM NaOH eluent with a flow rate of 0.3 ml min^{-1}, and monitored by using an electronic chemical detector. Galacturonic acid was determined by the method of Bluemkrantz and Asboe-Hansen [4]. The concentration of organic acids in the culture broth was determined periodically by HPLC (Younglin, Korea) with a UV detector (215 nm). The organic acid analysis column (Aminex HPX-87H ion exclusion, Bio-Rad, U.S.A.) was operated with a flow rate of 0.5 ml min^{-1} of 0.01 N H_2SO_4.

RESULT

Preparation and Characteristics of NDO

As described in Material and Methods, nondigestible oligosaccharides (NDO) in this research were prepared by destarching and enzymatic hydrolysis of red ginseng marc. Production yield of NDO from destarched ginseng fiber was determined to be 8.2% (w/w). The sugar composition of NDO was galactose 27.1%, arabinose 26.2%, glucose 25.6%, rhamnose 5.6%, mannose 4.0%, and galacturonic acid 11.5%. The GPC analysis showed the average molecular weight of NDO to be 807 dalton.

Growth of *Bifidobacterium* spp. Containing NDO

To investigate growth patterns of NDO by *Bifidobacterium* spp., each strain was inoculated in the PYF broth containing 1% (w/v) NDO as a carbon source. All strains of *Bifidobacterium* tested grew in a culture with NDO (Fig. 1). The growth rates of *B. adolescentis*, *B. animalis*, *B. breve*, and *B. longum* were 0.35 h^{-1}, 0.11 h^{-1}, 0.92 h^{-1}, and 0.23 h^{-1}, respectively. No growth was observed on carbohydrate-free medium (data not shown). Among the bacterial strains tested, *B. adolescentis* grew the best, reaching a dry cell weight of up to 0.96 g/l, which was 2–3 times of that obtained for the other *Bifidobacterium* species (Fig. 1Ia). Growth profiles of *Bifidobacterium* species showed that the maximal biomass was reached after approximately 12 h of culture. Drop of pH of the growth media was known to happen, mainly because of the production of organic acid, i.e. acetate, lactate, and formate (Fig. 1II). Acidification correlated with the degradation of NDO, since the final pH was the lowest and degradation potential the highest with *B. adolescentis* (Fig. 1IIa).

Production of Organic Acids by *Bifidobacterium* spp. and *Cl. perfringens*

B. adolescentis, *B. animalis*, *B. breve*, and *B. longum* produced 23.77, 8.90, 6.90, and 16.10 mM acetate and 11.83, 1.23, 0.65, and 7.17 mM lactate, respectively. *Cl. perfringens*

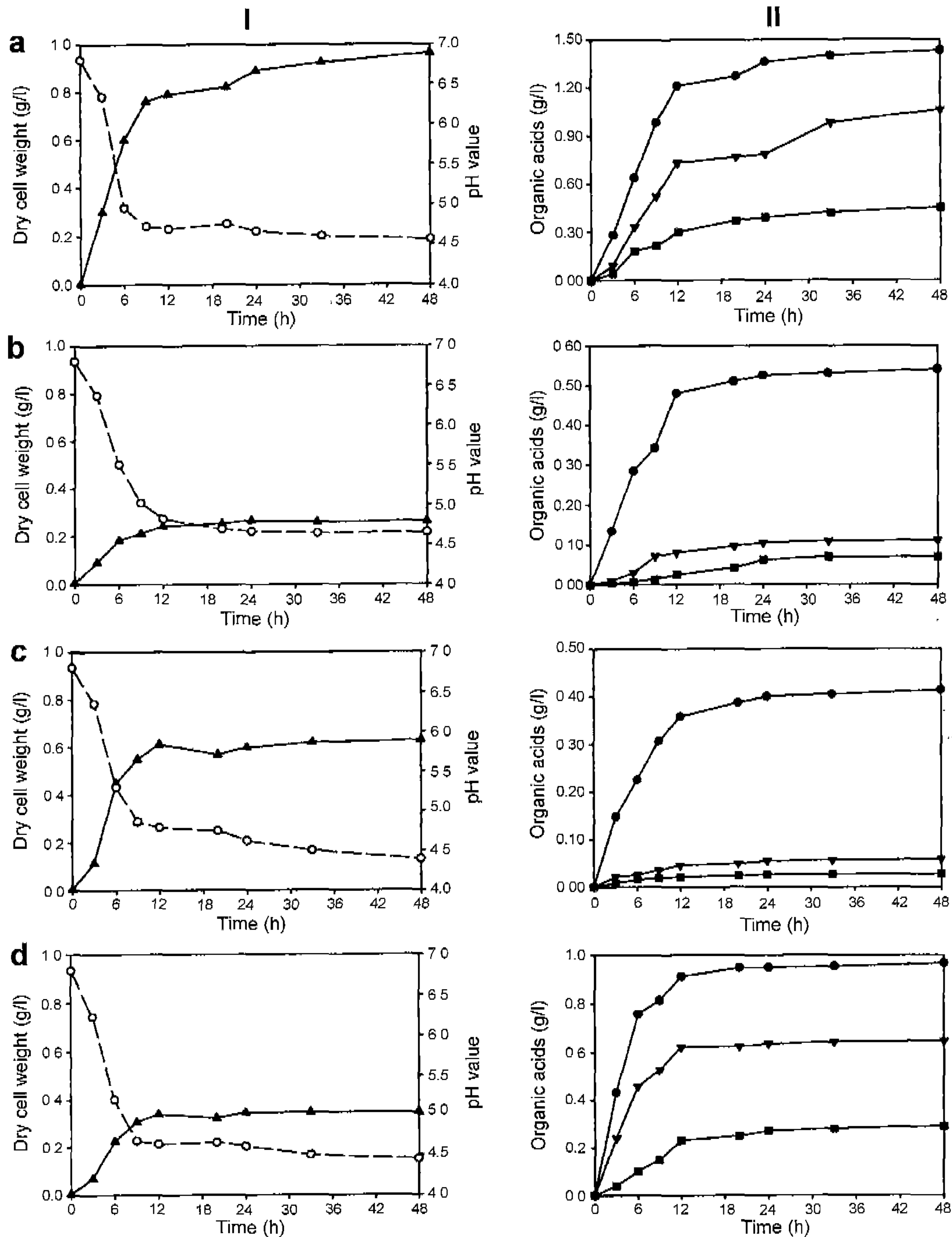

Fig. 1. Growth and acidification (column I) and production of organic acids (column II) of different *Bifidobacterium* species in batch cultures with NDO (1%) as a substrate.

Bifidobacterium adolescentis (a), *B. animaleis* (b), *B. breve* (c) and *B. longum* (d). (▲) dry cell weight: (○) pH value: (●) acetate: (▼) lactate: (■) formate.

produced 20.45 mM acetate but did not produce lactate (Table 1). The molar ratio of acetate to lactate varied markedly among the species and was dependent on the substrate. *B. adolescentis* produced the highest amounts of acetate and lactate among the *Bifidobacterium* spp. but the molar ratio of acetate to lactate was the lowest, while *B. breve* produced the least organic acid but the molar ratio was the highest. The total amount of organic acid was always higher in the control culture, with glucose as a carbon source. However, in all cultures with NDO, the acetate to lactate ratio was markedly increased when compared with the control cultures (Table 1).

Table 1. Organic acid produced by different species during culture using NDO or glucose (1%) as substrate.

		NDO		Glucose	
		mM	A:L	mM	A:L
	Acetate	23.77		38.77	
B. adolescentis	Lactate	11.83	2.01:1	28.63	1.35:1
	Formate	9.74		1.52	
	Acetate	8.90		20.4	
B. animalis	Lactate	1.23	7.23:1	3.21	6.36:1
	Formate	1.05		7.17	
	Acetate	6.90		29.45	
B. breve	Lactate	0.65	10.54:1	6.10	4.83:1
	Formate	0.60		18.04	
	Acetate	16.10		33.11	
B. longum	Lactate	7.19	2.24:1	26.52	1.25:1
	Formate	6.30		4.13	
	Acetate	20.45		18.00	
Cl. perfringens	Lactate	ND		ND	
	Formate	7.22		3.70	

A, acetate; L, lactate; ND, not detected.

Growth of *Bifidobacterium* spp. and *Cl. perfringens* in Single Culture and Mixed Culture Containing NDO

The growth inhibition effect of *Bifidobacterium* spp. against *Cl. perfringens* was investigated. Growth of each strain was observed by viable cell counting during culture for 48 h in PYF broth containing 1% (w/v) NDO as a carbon source. All strains showed good growth in the medium with NDO, and as a result, their population increased from 10^{4-5} to 10^{7} cfu/ml in the single culture (Fig. 2a). *Bifidobacterium* spp. and *Cl. perfringens* were then cocultivated (Fig. 2b). Growth of *Bifidobacterium* spp. was not affected by *Cl. perfringens*. *Cl. perfringens* grew well and reached the maximum growth after 8 h of culture, similar to the single culture. This strain could utilize amino acid as a carbon source after deamination [14], therefore, the growth of *Cl. perfringens* may not be affected by depletion of carbon source. However, the number of viable *Cl. perfringens* cells started to decrease after about 12 h.

DISCUSSION

Oligosaccharides are considered as a functional dietary fiber, and have been the focus of intensive research [7]. They attracted much attention as soluble dietary fibers which specifically support the growth and activity of beneficial microorganisms in the colon, i.e. bifidobacteria. The physiological effects of soluble dietary fibers depend largely on their metabolism in the colon, and it is very likely that variations in the chemical nature of different oligosaccharides will also affect their fermentability [11].

In the present research, sufficient amount of substrate for all experiments was produced by commercial enzyme treatment of red ginseng marc. The mixture of pectinase and hemicellulase was the best for the hydrolysis of ginseng marc to produce NDO. Treatment with enzyme mixtures was more effective than a single treatment (data not shown): enzymatic degradation of ginseng marc involved a number of different enzyme systems, e.g. exo- and endo-hydrolases.

In order to find out whether NDO from red ginseng marc can be used as a bifidogenic factor which promotes the growth of *Bifidobacterium* spp., NDO was added to *Bifidobacterium* spp. cultures. In this study, it was demonstrated for the first time that different *Bifidobacterium* strains were capable of metabolizing NDO, that is, when supplied as a sole carbon source. *B. adolescentis* showed the best growth and the highest degree of acidification (Fig.

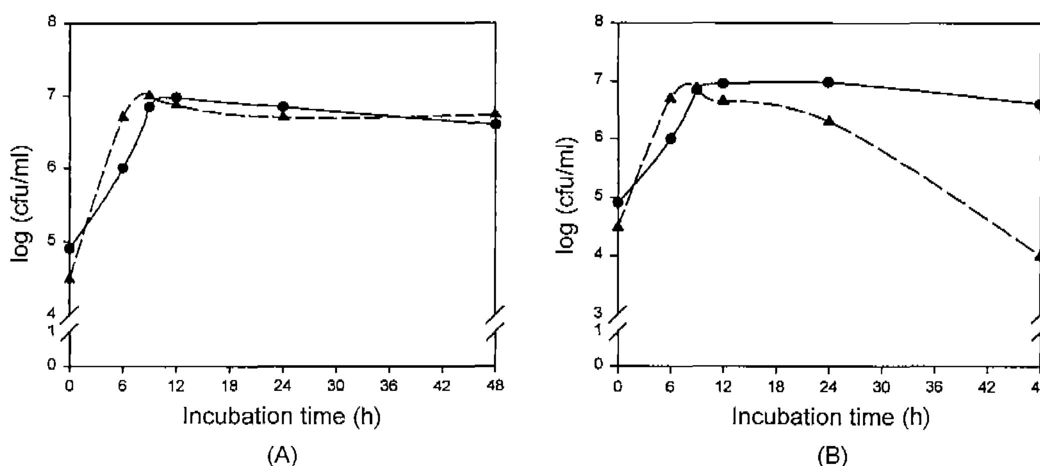

Fig. 2. Growth of *Bifidobacterium* spp. and *Clostridium perfringens* in medium with NDO. Each strain was incubated separately (A) or cocultivated (B) with PYF media containing NDO as a carbon source and incubated at 37C for 48 h.
(●) *Bifidobacterium* spp., (▲) *Clostridium perfringens*.

1). Discrepancy in the capability of different *Bifidobacterium* species to metabolize NDO *in vitro* might have been caused by differences in the expression of hydrolyzing enzymes.

One of the major health-promoting properties of bifidobacteria is the production of short chain fatty acids (SCFA) during the fermentation process. It has been reported that the molar ratio of acetic acid to lactic acid, which are produced during incubation of bifidobacteria, can be changed depending on the carbon sources [11]. According to our result, the molar ratio of acetate to lactate varied markedly among *Bifidobacterium* species, and it was higher with NDO as a carbon source than glucose. In conditions of carbon limitation, bifidobacteria can synthesize extra ATP by phosphoroclastically splitting pyruvate to formate and acetyl CoA, which is partially used for ATP synthesis. However, when carbohydrate is not a limiting factor, ATP is freely available, and pyruvate can be reduced to lactate [11]. It is considered that, when the molar ratio of acetate to lactate is higher, it indicates more of its metabolization as a carbon source. Therefore, NDO can be a better carbon source than glucose (Table 1).

In cocultivation of *Bifidobacterium* spp. and *Cl. perfringens*, the growth of *Bifidobacterium* spp. was not affected. *Cl. perfringens* seemed not to be affected by depletion of a carbon source as long as the nitrogen source remained [1]. It was shown that NDO could be a potent bifidogenic factor for *Bifidobacterium* spp. and it might have an inhibitory effect on *Cl. perfringens*. The acetic acid seemed to inhibit the growth of *Cl. perfringens* at the late stationary phase (Fig. 2). Therefore, NDO was thought to be a more advantageous carbon source to inhibit undesirable microorganisms in human intestine.

Based on the present results, it can be expected that daily uptake of NDO from ginseng marc will induce selective proliferation of some *Bifidobacterium* spp. in human intestine. However, to facilitate development of new prebiotic oligosaccharides, further studies on the identification and characterization of the enzyme(s) responsible for the hydrolysis of ginseng marc and increased yield of NDO product should be the main focuses in this laboratory.

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