JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Fermentation of β-Glucans Derived from Different Sources by Bifidobacteria: Evaluation of Their Bifidogenic Effect

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ABSTRACT: β-Glucans obtained from barley, seaweed, bacteria, and mushroom sclerotia were incubated with pure cultures of *Bifidobacterium infantis*, *Bifidobacterium longum*, and *Bifidobacterium adolescentis* for a 24 h batch fermentation to evaluate their bifidogenic effect with inulin as the positive control. The pH value in all culture media was decreased by 0.5−1.5 units. All β-glucans supported the growth of the three bifidobacteria with *B. infantis*, having a relatively larger increase in populations (3−4 $\log_{10}$ colony forming units). *B. infantis* produced almost double the amount of total short-chain fatty acids (SCFAs) than the other two bifidobacteria. The SCFA profile of *B. infantis* had a relatively higher proportion of propionic and butyric acid but less acetic acid than the other bifidobacteria. The utilization of all the β-glucans isolated from different sources regardless of their differences in glycosidic linkages and molecular weight by all three bifidobacteria was comparable to that of inulin.

KEYWORDS: β-glucan, bifidobacteria, fermentation, bifidogenic effect

INTRODUCTION

A large microbial population is present in the human colon at a level of $10^{10}-10^{11}$ colony-forming units (cfu)/g wet weight, with more than 50 genera and over 400 species of bacteria being identified in human feces.[1] The predominant species are non-spore-forming anaerobes belonging to the genera *Bacteroides*, *Eubacterium*, and *Bifidobacterium*.[2] Probiotics are living microorganisms, which upon ingestion in sufficient amount can exert health benefits beyond inherent basic nutrition.[3] These potential health effects include lowering serum cholesterol, increasing immune response, relieving constipation, exerting antimicrobial activity against pathogens, etc.[4] Bifidobacteria and lactobacilli are the major groups of probiotics found in humans. Prebiotics are nondigested food ingredients/components mainly consisting of nondigestible carbohydrates (NDCs) that affect the host by selectively stimulating the growth or activity of one or a number of bacteria (probiotics) in the colon that can improve the host's health.[5] To be classified as a prebiotic, a substance should be nondigestible by the mammalian enzymes and at least partially or completely fermented (anaerobic breakdown) by the colonic bacteria (probiotics) in a selective way.[6,7] NDCs should either have not been or cannot be hydrolyzed by human digestive enzymes in the small bowel. They are considered as prebiotics by acting as the substrates for fermentation in the colon by the probiotic bacteria.[8] Fermentation of NDCs involves a variety of specific enzymatic reactions and metabolic processes in the anaerobic microbial breakdown of organic matter, yielding metabolic energy for microbial growth and maintenance as well as other metabolic end products for use by the host.[2] The chief end products are short-chain fatty acids (SCFAs) together with gases including carbon dioxide, hydrogen, and methane.[8]

NDCs including nonstarch polysaccharides and oligosaccharides are the most common prebiotics.[9] Sources of prebiotic NDCs are mainly from the cell wall of higher plants including cereals and grains, fruits, and vegetables as well as legumes and seaweeds because they are resistant to hydrolysis by human alimentary enzymes.[10] Moreover, the most extensively investigated prebiotic NDC is inulin, which is a naturally occurring glycan consisting of a linear chain of fructose residues with β-(2→1) glycosidic linkages.[11] Other emerging prebiotics include galacto-oligosaccharides (GOS) and xylo-oligosaccharides (XOS) that have received much attention recently.[12,13] Prebiotic NDCs that are glucose-based are lesser known, with isomaltulose and β-glucans being the potential ones.[14] Some limited studies on β-glucans with mixed 1→3 and 1→4 β-glycosidic linkages from oat and wheat and gentio-oligosaccharides with β-(1→6) links have shown that glucose-based NDCs are also highly fermentable and can act as an alternative source of prebiotics.[15,16] Water-soluble β-glucans with different structural characteristics can be commonly found from different origins including algae (seaweed), cereal (barley), bacteria, and fungi (mushrooms). Laminarin from *Laminaria digitata* is an algal (seaweed) storage polysaccharide having a linear β-(1→3)-linked glucan backbone with β-(1→6)-linked branches (at the 3:1 ratio).[17] Curdlan is an exopolysaccharide produced by the nonpathogenic bacteria *Alcaligenes faecalis* and consists of a linear polymer with β-(1→3)-linked glucose residues; it is used as a gelling agent in food.[18] β-Glucan from barley is a common cereal NDC that consists of mixed linkages of β-(1→3) and β-(1→4) in the main chain.[19] Fungal β-glucans found in mushrooms usually have a β-(1→3) linked glucose main chain with different branching ratios of side chains having β-(1→6) linked glucose.[20] All of these different β-glucans have been shown to be bioactive (especially on immunomodulation) and have health benefits to humans.[21] Recently, mushroom β-glucans isolated from sclerotia (multihyphal structures developed by the aggregation of specialized mycelia) have been shown to promote the growth of probiotics and are potential prebiotic candidates.[22,23] β-Glucans from cereals (oat and barley) have also been evaluated for their effects to improve the viability of bifidobacteria strains in probiotic-containing yogurt recently.[24,25]

Received: February 14, 2011
Revised: May 5, 2011
Accepted: May 6, 2011
Published: May 06, 2011

dx.doi.org/10.1021/jf200621y | *J. Agric. Food Chem.* 2011, 59, 5986–5992

The present study evaluated the bifidogenic effect of β-glucans from different origins by comparing their in vitro fermentation by three bifidobacteria commonly found in the intestinal lumen of humans including *Bifidobacterium infantis* (in nursing infants) and *Bifidobacterium longum* and *Bifidobacterium adolescentis* (both in human adults). The parameters monitored during the fermentation included the changes in pH, microbial proliferation, and SCFA production. The differences found in the fermentation of these β-glucans by the three bifidobacteria were discussed.

MATERIALS AND METHODS

Sources of β-Glucans. Three commercial β-glucans were obtained for this study: laminarin from *L. digitata* (Sigma, St. Louis, MO), barley β-glucan (Megazyme International Ltd., Wicklow, Ireland), and Curdlan from *A. faecalis* (Megazyme). Inulin was obtained from dahlia tubers (Sigma).

Preparation of Mushroom β-Glucans. The procedures used were reported previously.[23] In brief, β-glucan-rich mushroom sclerotia from *Pleurotus tuber-regium* were supplied by the Sanming Mycological Institute in Fujian, China. After peeling, the sclerotia were milled to powder by a mechanical grinder using a 0.5 mm sieve. Twenty grams of mushroom sclerotial powder was refluxed with 300 mL of ethanol (80%) for an hour to remove simple sugars and other low molecular weight substances. After centrifugation at 10000*g* for 30 min, the pellets were soaked in NaOH (1 M) in a ratio of 1:50 for 48 h at ambient temperature. The mixture was then subjected to ultrasonication (Sonics VCX600) with amplitude 40% for 40 min in an ice bath, and the mixture was centrifuged at 10000*g* for 30 min. The supernatant was dialyzed to allow salt removal and was lyophilized to give the mushroom β-glucan.[23]

Composition Analysis of β-Glucans. The protein content of the β-glucans was determined by the AOAC Kjeldahl method,[26] whereas the levels of β-glucans were determined by a β-glucan assay kit (Megazyme).

Molecular Weight Determination of β-Glucans. A TSK gel G5000 PW size exclusion column (30 cm × 7.5 mm i.d., catalog no. 8-05764, Supelco) with a PWH guard column (7.5 cm × 1.5 mm i.d., catalog no. 8-06762, Supelco) were used to determine the molecular weight (M_w) profile of individual β-glucans in reference to that of pullulan standards (Shodex Standard, Showa Denko) with a molecular range between 5.9×10^3 and 788×10^3 Da. The flow rate of the eluent (0.2 M sodium chloride) was 0.8 mL/min, and the temperature of the column was controlled externally at 25 °C. The samples and standards were dissolved in 0.2 M sodium chloride, filtered by the 0.45 μm Millipore filter, passed through the size exclusion column, and then detected by a Waters 2414 refractive index detector. The M_w of β-glucans was estimated from a calibration curve obtained from the retention time of pullulan standards against the log value of their sizes.

Sugar Composition and Linkage Analysis of β-Glucans. All of the β-glucans were subjected to sequential acid hydrolysis (12 M sulfuric acid for 1 h at 35 °C and then 2 M sulfuric acid for 1 h in a boiling water bath). Alditol acetates of the neutral and amino sugars in the acid hydrolysate were prepared according to the method described by Blakeney et al.[27] with β-D-allose as the internal standard. Alditol acetates of the monosaccharides were quantified by an HP6890 series II gas chromatography, using an Alltech DB-225 capillary column (15 m × 0.25 mm i.d., 0.25 μm film) with the following oven temperature program: initial temperature, 170 °C; temperature rise at 2 °C/min to 220 °C, and final hold for 15 min. The carrier gas was helium, and detection mode was flame ionization.

The sugar linkages of the of β-glucans were analyzed by methylation study using the method described by Anumula et al.[28] and an analysis by gas chromatography−mass spectrometry (GC-MS) of the partially methylated alditol acetate (PMAA) derivatives. In brief, methylsulfinyl methyl sodium was prepared from sodium hydride and dimethyl sulfoxide (DMSO). β-Glucans that were insoluble in DMSO were premethylated by methylsulfinyl methyl sodium and methyl iodide prior to the normal methylation procedures. The PMAA samples were prepared from the methylated β-glucans by acid hydrolysis (2 M trifluoroacetic acid at 121 °C for 1 h), reduction of the hydrolyzed sugars by sodium borohydride, and acetylation by glacial acetic anhydride. The PMAA samples were analyzed by a GC (Agilent Technology, 6890N)-MS (Agilent Technology, 5973N). The GC conditions were as follows: Alltech DB-225 capillary column (15 m × 0.25 mm i.d., 0.25 μm film); helium as carrier gas at a flow rate of 1.0 mL/min; initial oven temperature at 160 °C, followed by a 4 °C/min rise to 220 °C and a final hold for 15 min; injector temperature at 280 °C; interface temperature at 280 °C. The MS conditions were as follows: ion source temperature at 250 °C, ionization energy at 70 eV, detector voltage at 1.5 kV, and mass range from 50 to 350. Each PMAA was identified by matching its mass spectrum with the NIST/EPA/NIT database in the computer.

In Vitro Fermentation of β-Glucans. In vitro fermentation of the different β-glucans derived from different origins was conducted by a batch fermentation system under strict anaerobic conditions with individual bifidobacteria including *B. infantis* (JCM 1222), *B. longum* (JCM 1217), and *B. adolescentis* (JCM 1275) obtained from the Japan Collection of Microorganism (JCM). The bacterial strain was activated by incubation in 2% reinforced clostridial broth (RCB, Oxoid, Hampshire, U.K.), and the fermentation of β-glucans was conducted in a medium for colonic bacteria (MCB),[29] which contained (per liter) 6.5 g of bacteriological peptone, 5.0 g of soy peptone, 2.5 g of tryptone, 3.0 g of yeast extract, 2.0 g of KCl, 0.2 g of $NaHCO_3$, 4.5 g of NaCl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.45 g of $CaCl_2 \cdot 2H_2O$, 0.2 g of $MnSO_4 \cdot H_2O$, 0.005 g of $FeSO_4 \cdot 7H_2O$, 0.005 g of $ZnSO_4 \cdot 7H_2O$, 0.4 g of cysteine−HCl, 0.005 g of hemin, 0.005 g of menadione, 0.5 mL of H_3PO_4, and 2 mL of Tween 80.[27] The β-glucans and inulin (as positive control) were used as the sole carbon source at a concentration of 0.5% (w/v) in the MCB. The pH of the medium was adjusted to 6.50 before autoclaving (210 kPa, 121 °C, 20 min). The activated bacteria were inoculated at a concentration of 0.8% (v/v) in the autoclaved MCB containing the β-glucan samples, and oxyrase was added at a concentration of 0.8% (v/v) to maintain the anaerobic environment. The fermentation mixtures (total volume of 30 mL) were put in 50 mL bottles flushed with argon to displace the air before being capped and placed on a gentle shaker at 37 °C for 24 h. At the end of the 24 h fermentation, the pH value in the fermentation broth was measured by a pH-meter (Φ240 pH/Temp Meter, Beckman). After 24 h of fermentation, an aliquot of 200 μL of the fermentation broth was transferred into tubes for culture viability determination. To determine cell viability, the culture samples taken were diluted serially with sterile 0.9% NaCl solution and pour-plated onto plate count agar (BD Difco). The agar plates were placed in anaerobic jars with oxyrase inside and incubated at 37 °C for 48 h before colony counting. Another aliquot of 600 μL was collected and stored at −20 °C for SCFA analysis.

Analysis of SCFA Produced in the Fermentation of β-Glucans. The SCFAs were analyzed by GC as described previously with slight modifications.[20] An aliquot of 600 μL of the fermentation broth was centrifuged (4800*g*) at 4 °C for 30 min, and then an aliquot of 350 μL of the upper supernatant was transferred into a new Eppendorf, followed by the addition of 82.5 μL of metaphosphoric acid (25%, w/v) to acidify the SCFAs and 67.5 μL of methylpentanoic acid (4.0 mg/mL) as internal standard (catalog no. 6220601, Alltech, Deerfield, IL). The SCFAs in the mixture were extracted twice with 500 μL of diethyl ether, and the upper layer of the organic fraction was pooled and dehydrated with anhydrous sodium sulfate before being filtered through a 0.45 μm membrane into GC vials. The SCFA content were quantified by a HP 6890 GC system equipped with a Quadrex 007-FFAP capillary column (30 m × 0.25 mm i.d.; 0.25 μm film). The condition of the oven was programmed at an initial temperature of 80 °C with a hold of 5 min,

Table 1. Normalized Percentage of Partially Methylated Alditol Acetates of β-Glucans Used in 24 h Fermentation by Bifidobacteria[a]

partially methylated sugar	barley[b]	bacteria	seaweed	mushroom	linkage type
2,3,4,6-Me_4-Glc	3	1	25	49	→1) Glc
2,4,6-Me_3-Glc	22	99	69	14	→1) Glc (3→
2,3,6-Me_3-Glc	75	nd	nd	18	→1) Glc (4→
2,3-Me_2-Glc	nd	nd	nd	11	→1) Glc (4→, 6→
2,3,4-Me_3-Glc	nd	nd	2	5	→1) Glc (6→
2,4-Me_2-Glc	nd	nd	4	3	→1) Glc (3→,6→

[a] Me, methyl residues; Glc, glucose residue; nd, not detected. [b] NDC is represented by the name of its source.

followed by a temperature rise of 5 °C/min to 180 °C with a final hold of 5 min at 200 °C. Two microliters of samples was injected into the GC with a split ratio of 14.3:1, and detection of samples was by flame ionization. A mixture of individual SCFA standards including acetic acid, propionic acid, butyric acid (SCFA standards kit, Alltech), and 4-methylpentaneoic acid was prepared in 25% metaphosphoric acid at a final concentration of 1.0 mg/mL for SCFA identification and quantitation. The amount of the SCFAs after 24 h of fermentation was expressed in millimoles per liter of culture medium (mmol/L) and individual SCFAs were expressed as normalized percentage of total SCFA concentration.

Statistical Analyses. All statistical analyses were performed by using the software SPSS Statistics 17.0 (SPSS, Chicago, IL). All of the data obtained were analyzed by one-way ANOVA, and tests of significant differences were determined by using Tukey's multiple comparison at $p < 0.05$.

RESULTS AND DISCUSSION

Chemical Composition and Structure of β-Glucans. The content of β-glucan in the NDCs prepared from the mushroom sclerotium of *P. tuber-regium* was 80.8% with some non-β-glucan carbohydrate (10%) and small amounts of proteins (1%) and ash (1%), whereas those of Curdlan, barley, and laminarin all have >95% β-glucan with small amounts of protein ranging from 0.45 to 3%.

All of the β-glucans contained almost purely glucose as their sugar components with only trace amounts of mannose (<2%) being found in laminarin (data not shown). From the results of the glycosidic linkage analysis on the β-glucans by methylation study, it was found that β-glucans from barley were a linear chain polysaccharide with mixed 1→3 and 1→4 β-linkages in the ratio of 1:3 (Table 1). Whereas β-glucans from both bacteria (Curdlan) and seaweeds (laminarin) had a β-(1→3) linked linear chain, Curdlan was unbranched and laminarin was highly branched (Table 1). β-Glucans from mushroom sclerotia had the most complicated glycosidic linkage composition when compared with the other β-glucans by having a highly branched main chain with mixed glycosidic 1→3, 1→4, and 1→6 β-linkages (Table 1).

The molecular weight (M_w) of the β-glucans also differed greatly, with that from barley having the largest M_w of 590×10^3 Da, followed by that of mushroom (96×10^3 Da) and that from seaweed (6×10^3 Da) (data not shown). The M_w of Curdlan could not be determined in the present study due to its poor water solubility, but its M_w had been reported to be about $(10-30) \times 10^3$ Da.[30] The M_w of inulin was about 5×10^3 Da according to the specification provided by the supplier (Sigma).

Changes in pH in the Fermentation System. In this study, the in vitro fermentation time was 24 h, which should be sufficient to imitate the microbial degradation of NSPs in vivo.[31] All of the in vitro fermentation systems showed a significant drop ($p < 0.05$) of at least 0.5 unit in their pH values after 24 h, which is most probably caused by the fermentation end products (SCFAs) (Table 2). For *B. infantis*, the largest drop in pH was found in inulin (decrease from 6.51 to 4.77), implying that inulin was most efficiently utilized by *B. infantis*. No significant differences in the pH value between different NDC substrates were found in the fermentation system containing *B. longum* and *B. adolescentis*. It had been reported there was only a modest drop of pH value (0.1−0.3 unit) during a 24 h fermentation of β-glucan rich oat samples with a β-glucan content of 5.2−7.7% using human fecal flora.[32] The lowering of pH during fermentation could exert beneficial effects for colonic environment, such as inhibiting the growth of pathogenic bacteria and increasing the growth of beneficial ones.[2,20]

Viability of Bifidobacteria during Fermentation of β-Glucans. Growth of bifidobacteria during the 24 h fermentation of β-glucans was monitored by viable count instead of by optical density (OD) measurement because the substrates in the fermentation broth caused interference to OD values. Among the three bifidobacteria, *B. infantis* increased in number by an average of 3 $\log_{10}$ cfu on all NDC substrates, with the highest increase of 4 $\log_{10}$ cfu in laminarin (Table 3). The increase in the populations of *B. longum* and *B. adolescentis* incubated with the different β-glucan substrates ranged from 1 to 1.5 $\log_{10}$ cfu and from 2 to 2.3 $\log_{10}$ cfu, respectively (Table 3). The multiplication of the populations of the three bifidobacteria in inulin was found to be similar to that of all the β-glucan substrates (Table 3). In contrast to our observations, it had been previously reported that *B. infantis*, *B. aldolescentis*, and *B. longum* did not grow in an in vitro fermentation system using barley β-glucan as carbon substrate.[33] Although there were many factors causing such differences in the results, the use of different strains of bifidobacteria (strains were obtained from VTT Biotechnology (Finland) in the previous study[33] compared to those obtained from JCM (Japan) in the present study) might be one of the contributing factors. The present results in the fermentation of inulin by the three bifidobacteria were consistent with a previous report that *B. adolescentis* and *B. longum* could grow in fructooligosaccharide (an inulin-type fructan).[34] In summary, *B. infantis* had the highest population after the 24 h of fermentation in the β-glucan substrates, which among many factors might be partly explained by having the shortest generation time (50 min) when compared to the comparatively longer generation time for *B. adolescentis* (69 min) and *B. longum* (111 min) as previously found.[35]

Production of SCFAs during Fermentation of β-Glucans. SCFAs are the major organic acids produced by colonic bacteria during the fermentation of polysaccharides, with acetic acid, propionic acid, and butyric acid being the principal components.[7] All three bifidobacteria produced various amounts of SCFAs as metabolites in the 24 h of fermentation of the β-glucans in this study. The total SCFA production in *B. infantis* was higher than that in *B. longum* and *B. adolescentis* for all of the β-glucans (Table 4). Compared with the β-glucans, *B. infantis* produced a significantly ($p < 0.05$) higher level of total SCFAs when incubated with inulin, which was also consistent with the largest drop in the pH value found (Table 2). The SCFA profile of

Table 2. pH Changes in Batch Cultures during 24 h of in Vitro Fermentation of Different β-Glucans by Bifidobacteria[a]

bifidobacteria	time (h)	dahlia tuber[b]	barley	bacteria	seaweed	mushroom
B. infantis	0	6.51 ± 0.00	6.52 ± 0.01	6.51 ± 0.01	6.50 ± 0.01	6.48 ± 0.01
	24	4.77 ± 0.01 a	6.04 ± 0.02 cd	5.96 ± 0.03 b	6.02 ± 0.00 c	6.05 ± 0.05 cd
B. longum	0	6.24 ± 0.02	6.28 ± 0.01	6.30 ± 0.01	6.28 ± 0.01	6.33 ± 0.01
	24	5.71 ± 0.05 a	5.81 ± 0.01 abc	5.83 ± 0.01 bc	5.78 ± 0.05 ab	5.85 ± 0.03 bc
B. adolescentis	0	6.50 ± 0.06	6.49 ± 0.01	6.51 ± 0.01	6.52 ± 0.00	6.52 ± 0.01
	24	5.73 ± 0.02 bc	5.69 ± 0.03 ab	5.74 ± 0.02 bcd	5.78 ± 0.01 cd	5.76 ± 0.01 cd

[a] Values are the mean ± standard deviation ($N = 3$). Values in the same row followed by different letters are significantly different (ANOVA Tukey's multiple-comparison test $p < 0.05$). [b] NDC is represented by the name of its source.

Table 3. Bifidobacterial Populations ($\log_{10}$ CFU/mL Batch Culture Fluid) in Batch Culture during 24 h of in Vitro Fermentation of Different β-Glucans[a]

bifidobacteria	time (h)	dahlia tuber[b]	barley	bacteria	seaweed	mushroom
B. infantis	0	5.22 ± 0.15	4.96 ± 0.26	5.17 ± 0.08	5.07 ± 0.16	5.07 ± 0.10
	24	8.60 ± 0.05 ab	8.63 ± 0.20 ab	8.77 ± 0.19 ab	9.25 ± 0.01 b	8.47 ± 0.12 a
B. longum	0	4.93 ± 0.04	4.66 ± 0.26	4.81 ± 0.05	4.59 ± 0.16	4.63 ± 0.46
	24	6.33 ± 0.17 a	6.02 ± 0.17 a	5.95 ± 0.07 a	5.89 ± 0.16 a	6.02 ± 0.17 a
B. adolescentis	0	4.40 ± 0.17	4.75 ± 0.05	4.52 ± 0.07	4.63 ± 0.06	4.40 ± 0.17
	24	6.77 ± 0.13 a	6.82 ± 0.08 a	6.84 ± 0.11 a	6.77 ± 0.06 a	6.66 ± 0.03 a

[a] Values are the mean values of ($\log_{10}$ viable count) ± standard deviations ($N = 3$). Values in the same row with different letters are significantly different (ANOVA Tukey's multiple-comparison test, $p < 0.05$). [b] NDC is represented by the name of its source.

Table 4. Production of Total SCFAs (mmol/L) and Their Normalized Molar Percentage in Batch Culture Fluid after 24 h in Vitro Fermentation of Different β-Glucans by Bifidobacteria[a]

bifidobacteria		dahlia tuber[b]	barley	bacteria	seaweed	mushroom
B. infantis	SCFA	3.86 ± 0.22 a	1.97 ± 0.33 b	2.60 ± 0.66 b	2.00 ± 0.03 b	2.24 ± 0.08 b
	acetic acid	95.06 ± 0.72 a	79.20 ± 1.04 b	80.48 ± 0.39 b	81.14 ± 1.77 b	78.70 ± 3.27 b
	propionic acid	2.82 ± 0.27 a	11.73 ± 0.08 b	11.21 ± 0.71 b	10.91 ± 0.79 b	11.78 ± 1.68 b
	butyric acid	2.12 ± 0.44 a	9.08 ± 0.99 b	8.32 ± 0.33 b	7.95 ± 0.98 b	9.52 ± 1.59 b
B. longum	SCFA	1.08 ± 0.10	1.03 ± 0.02	1.12 ± 0.06	1.00 ± 0.07	0.98 ± 0.01
	acetic acid	96.48 ± 0.21 a	96.32 ± 0.18 a	96.26 ± 0.15 a	96.29 ± 0.27 a	96.25 ± 0.06 a
	propionic acid	2.22 ± 0.12 a	2.34 ± 0.10 a	2.38 ± 0.24 a	2.33 ± 0.14 a	2.30 ± 0.01 a
	butyric acid	1.30 ± 0.10 a	1.34 ± 0.07 a	1.36 ± 0.09 a	1.38 ± 0.13 a	1.45 ± 0.06 a
B. adolescentis	SCFA	1.06 ± 0.05 a	1.18 ± 0.10 a	1.08 ± 0.11 a	1.08 ± 0.04 a	1.16 ± 0.02 a
	acetic acid	96.38 ± 0.05 a	96.24 ± 0.02 a	96.14 ± 0.38 a	96.32 ± 0.08 a	96.40 ± 0.16 a
	propionic acid	2.36 ± 0.04 a	2.43 ± 0.01 a	2.48 ± 0.20 a	2.35 ± 0.06 a	2.26 ± 0.07 a
	butyric acid	1.26 ± 0.09 a	1.34 ± 0.02 a	1.38 ± 0.22 a	1.33 ± 0.04 a	1.34 ± 0.09 a

[a] Values are the mean ± standard deviation ($N = 3$). Values in the same row with different letters are significantly different (ANOVA Tukey's multiple-comparison test, $p < 0.05$). [b] NDC is represented by the name of its source.

B. infantis was very different from that of *B. longum* and *B. adolescentis* for all of the β-glucans in that the ratio of acetic/propionic/butyric acid was 8:1:1. Acetic acid was predominantly produced in *B. longum* and *B. adolescentis*, accounting >96% of the total SCFAs, whereas propionic acid and butyric acid consitituted only about 2 and 1%, respectively, for all of the β-glucans (Table 4). The SCFA profile for inulin was also dominated by acetate (96%) and was the same among all three bifidobacteria (Table 4).

Comparison of the Fermentation of β-Glucans by Bifidobacteria. Laminarin, a water-soluble β-glucan from brown seaweed, is a linear polysaccharide with β-1,6 branches for every three glucose units that is used as a common substrate for laminarinase (EC 3.2.1.6) activity assays. Complete hydrolysis of laminarin should involve β-1,3 or β-1,6 glucanase and β-glucosidase. It had been reported that laminarin isolated from *L. digitata* could be extensively fermented by human fecal

Table 5. Summary of the Number of Proteins and Their Accession Numbers Identified in the Bifidobacterium Genome That May Play a Role in NSP Degradation[a]

protein	*B. infantis*	*B. longum*	*B. adolescentis*
β-glucosidase	YP_002322110.1 YP_002323355.1	YP_004221256.1 YP_004221261.1	YP_910057.1 YP_910150.1 YP_910473.1 YP_910474.1
glucan 1,3-β-glucosidase	YP_002322106.1	nil	nil
β-1,3-exoglucanase	nil	YP_004221257.1	YP_910065.1 YP_910067.1
glycogen/starch/α-glucan phosphorylase	YP_002321578.1	nil	nil
solute binding protein family 1	21	10	11

[a] Based on the information provided by NCBI database.[43] *B. infantis*, *Bifidobacterium longum* subsp. *infantis* ATCC 15697, complete genome; *B. longum*, *Bifidobacterium longum* subsp. *longum* JCM 1217, complete genome; *B. adolescentis*, *Bifidobacterium adolescentis* ATCC 15703 chromosome, complete genome.

inoculum to produce a total SCFA concentration of 85 mmol/L with >60% of the SCFAs being acetic acid after 24 h.[36] In the present study, all three bifidobacteria exerted high fermentability toward laminarin, implying that specific β-glucanases could be induced from these bifidobacteria to hydrolyze the laminarin.

As mentioned earlier, Curdlan is a linear β-1,3 glucan with almost no branches that is very insoluble in water. Despite such low water solubility, Curdlan produced by *A. faecalis* has been reported to markedly increase the in vitro proliferation of the same bifidobacteria used in the present study, with *B. adolescentis* having the largest increase in population.[37] Whereas the three bifidobacteria could utilize Curdlan to different extents, Curdlan seemed to be more efficiently degraded by *B. infantis* as shown by the highest proliferation (Table 3) and largest SCFA production (Table 4).

Water-soluble β-glucans from cereals including oat and barley contain β-1,3 and β-1,4 mixed linkages that require specific β-glucanases (lichenase) with both β-1,3 and β-1,4 cleavage activity to degrade them completely.[38] The present results had demonstrated that barley β-glucans with a M_w of 590×10^3 Da could be utilized by the three bifidobacteria, with *B. infantis* having the highest proliferation (Table 3) and largest SCFA production (Table 4), suggesting that lichenase-type enzymes could be induced in the bifidobacteria. However, previous in vitro experiments using human fecal microbiota to ferment barley derived β-glucans with M_w between 137×10^3 and 327×10^3 Da displayed no apparent prebiotic potential of these substrates.[38] More in vivo and in vitro investigations are required to ascertain the bifidogenic properties of cereal β-glucans in relationship to their physicochemical properties, especially their molecular size.

The most common chemical structure of β-glucans from mushrooms is a β-1,3 backbone with different degrees of β-1,6 and/or β-1,4 branching.[39] β-Glucans isolated from the sclerotia of *P. tuber-regium* in the present study had a mixed linkages of β-1,3, β-1,4, and β-1,6 (Table 1), which was consistent with our previous study.[20] In the present study, mushroom sclerotial β-glucan could be utilized similarly as the other β-glucans by pure cultures of bifidobacteria despite its complex linkages. It had been reported previously that β-glucans isolated from other mushroom sclerotia including *Polyporus rhinocerus* and *Poria cocos* could also stimulate the growth of *B. longum* and *Lactobacillus brevis* while inhibiting the growth of *Clostridium celatum*.[23] These results suggested that mushroom sclerotial β-glucans had the potential to be developed as novel prebiotics.

In vivo studies had shown that inulin could be selectively and efficiently utilized by bifidobacteria including *B. adolescentis* and *B. longum*.[40,41] The present results indicated that *B. infantis* could utilize inulin more efficiently than *B. longum* and *B. adolescentis* in pure cultures by having a lower pH value in the culture medium, a large increase in population, and larger amount of total SCFAs (Tables 2−4). These results were in agreement with the findings that bifidobacteria could degrade inulin and oligofructose by producing β-fructofuranosidase.[42]

The possible explanations for the present results can be inferred from the recent release of genome sequences of *B. infantis*, *B. longum*, and *B. adolescentis*, which would provide important information on the mechanisms of the fermentation of NSPs by these bifidobacteria in terms of their carbohydrate-modifying enzymes (carbohydrases).[43] Those proteins (enzymes) identified from the bifidobacterium genomes that are responsible for the utilization of NSPs are summarized in Table 5. The three bifidobacterium species all possess several glucan hydrolases, mainly glucosidases. Another interesting phenomenon is that there are 21 family 1 solute binding proteins (SBPs) in *B. infantis*, whereas there are only 10 and 11 family 1 SBPs in *B. longum* and *B. adolescentis*, respectively.[43] Family 1 SBPs are a component of the ATP-binding cassette (ABC) transport system, which is responsible for carbohydrate binding in the membrane.[44] The relatively larger number of SBPs found in *B. infantis* might in part explain its higher efficiency in the fermentation of the β-glucan substrates than the other two bifidobacteria. Moreover, in the case of *B. infantis*, the amount of total SCFA produced during the fermentation of inulin is almost twice the amount of that in the β-glucan substrates (Table 4), even though the bifidobacterial populations were similar (Table 3). This might be due to the fact that inulin has a relatively lower molecular weight and a higher proportion of oligosaccharides than those of the β-glucan substrates, allowing them to be more readily bound and transported by SBPs across the cell membrane for utilization. Other mechanistic models have been proposed to explain the molecular mechanism of the utilization of NDCs by colonic bacteria.[45] For example, *B. longum* possessed a multidomain glycanase, which includes one transmembrane domain to anchor in the cell membrane and two carbohydrate binding modules as a catalytic domain to capture NDCs such as xylan oligosaccharide.[45] In general, colonic bacteria such as bifidobacteria are capable of capturing and degrading NDCs in their surroundings and transporting the polysaccharides or their hydrolytic products into the cells as energy sources.[45]

In conclusion, all three bifidobacteria used in the present study are capable of utilizing all of the structurally diversified β-glucans

as a substrate of fermentation comparably to the known prebiotic inulin. *B. infantis* seems to be able to grow better than the other two bidfidobacteria in all of the β-glucans because of the larger number of relevant SBPs based on genome analysis of *Bifidobacterium* sp. in the literature. However, further biochemical characterization to confirm their mode of action and substrate specificity is required. Experiments are required to demonstrate the molecular mechanisms by which bifidobacteria can specifically degrade β-glucans from different sources that have different chemical structures by measuring the corresponding enzyme activity in the fermentation broth. Further detailed investigations are therefore needed to explain the apparent versatility of bifidobacteria to employ different strategies for utilizing different β-glucans. A proteomic study of the carbohydrases induced by these β-glucans in the bifidobacteria is underway. Moreover, in vitro and in vivo studies using human fecal flora[11] are necessary to evaluate more comprehensively the bifidogenic effect of these β-glucans.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper originally posted to the Web on May 13, 2011, with an error to the Results and Discussion section and errors to the Reference section. The corrected version was reposted with the Issue on June 1, 2011.