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Comparative Proteome Analysis of *Bifidobacterium longum* subsp. *infantis* Grown on β-Glucans from Different Sources and a Model for Their Utilization

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Supporting Information

ABSTRACT: Recent studies have demonstrated that β-glucans from different sources, which are considered as potential prebiotics, could enhance growth of bifidobacteria. To elucidate the metabolic pathway of β-glucans in the widely used probiotic *B. longum* subsp. *infantis*, a comparative proteomic analysis was carried out along with two-dimensional difference gel electrophoresis (2D-DIGE), real-time RT-PCR, and enzyme activity assay on samples obtained from cultures grown on β-glucans derived from barley, seaweed, and mushroom. Results showed that 77 spots were found to be differentially expressed among different cultures, and 17 of them were predicted to play a role in β-glucan catabolism, including ABC transporter for sugars, enolase, and phosphotransferase system protein. Among them, 6 genes encoding for 6 proteins were shown to be induced by β-glucans at the transcriptional level and had higher abundance. The enzyme activity assay detected intracellular glucanase activity present in the cultures grown on the β-glucans from seaweed and mushroom. On the basis of the above results, a model for catabolism of β-glucans in *B. infantis* is proposed as follows: β-glucan molecules in the medium are transported into the cell through the ABC (ATP-binding cassette) transport system and PTS (phosphotransferase system) proteins followed by hydrolysis through action of intracellular glucanase to glucose, which is subsequently incorporated into the central fermentative pathway 'bifid shunt'. This study for the first time reveals the possible degradation pathway of β-glucans by *B. infantis*, which has implications for potential use of these β-glucans as novel prebiotics in development of synbiotic application.

KEYWORDS: *β-glucan, Bifidobacterium longum subsp. infantis, proteome, fermentation*

INTRODUCTION

Prebiotics are nondigested food ingredients that affect the host by selectively stimulating growth or activity of one or a number of bacteria in the colon that can improve the host's health.[1] Among the emerging prebiotic candidates, β-glucans are receiving increasing attention due to their different origins and structural diversity as well as fermentation characteristics and human health benefits.[2,3] It was reported that laminarin, a linear β-1,3-glucan isolated from seaweed *Laminaria digitata*, could be highly fermented by human fecal inoculum in 24 h, and a total short-chain fatty acid (SCFA) concentration of 85 mM was produced with 60% acetic acid.[4] In another study, when Curdlan, a β-1,3-glucan from the bacteria *Alcaligenes faecalis*, was fed to rats for 4 weeks, there was a significant increase in bifidobacterial proliferation as well as production of SCFAs and lactate in the animal's fecal contents.[5] β-Glucan in cereals contains mixed β-1,3- and β-1,4-glycosidic linkages. Fermentation of β-glucans from barley and oat by the human faecal microbiota gave a unique SCFA profile that is high in propionic acid which might exert a hypocholesterolaemic effect.[2] β-Glucan from the mushroom sclerotia of *Pleurotus tuber-regium*, which is a highly branched β-1,3-linked polysaccharide, was reported to have antitumor and immunomodulating activities.[6] In an in vitro study, β-glucans from three mushroom sclerotia were fermented by human fecal homogenate to different extents due to their structural differences.[7] Mushroom β-glucans from *Poria cocos* and *Polyporous rhinocerus* stimulated growth of *Lactobacillus brevis* and *Bifidobacterium longum* but inhibited proliferation of *Clostridium celatum* in a 24 h in vitro fermentation study.[8]

Probiotics are living miroorganisms, which upon ingestion in sufficient amount can exert health benefits beyond inherent basic nutrition.[9] Bifidobacteria, accounting for nearly 95% of the gut microflora of breast-fed babies and up to 3% of the total colonic microflora in adults, are one of the most commonly used species in probiotic preparations.[10] There are 39 species that have been characterized in the *Bifidobacteirum* genus and 24 different strains that have been subjected to sequence analysis,[10] while the first complete genome sequence of *B. longum* subsp. *longum* NCC2705 was released in 2002.[11] Rapid growth in the genome study for bifidobacteria is a clear reflection of the growing scientific interest in this particular group aiming at the genetic basis for their health benefits, adaptation to colonization, and persistence in the human gastrointestinal tract (GIT).[11] More than 8% of the genes in a given bifidobacterial genome is thought to be involved in carbohydrate metabolism, and nearly one-half of these genes, that is about 5% of the total bifidobacterial genes, function in carbohydrate uptake. The ability of bifidobacteria to degrade

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complex carbohydrates in the colon has already been well established, and the presence of genes that encode various carbohydrate-modifying enzymes reflects their adaptation to the human gastrointestinal tract environment.[12]

In the present study, *B. infantis* was selected to be incubated with β-glucans from seaweed, mushroom, and barley and the differentially expressed proteins between different treatments in the obtained samples were compared by proteome analysis. Several genes were selected based on proteome results for transcriptional analysis, and both intracellular and extracellular hydrolyzing enzyme activity were tested. Combined with the released genome information of *B. infantis*[13] and the present results, a model on the catabolism of β-glucans by *B. infantis* was proposed. This study broadens the understanding on the metabolic pathway of the structurally different β-glucans in *B. infantis* and demonstrates their potential application as novel prebiotics and synbiotics.

MATERIALS AND METHODS

Sources of β-Glucans. Seaweed β-glucan (Laminarin) was obtained from *Laminaria digitata* (Sigma), and barley β-glucan was obtained from *Hordeum vulgare* (Megazyme). Mushroom β-glucan was prepared from the sclerotia of *Pleurotus tuber-regium* according to procedures reported previously.[3] Chemical structures of all β-glucans were characterized as previously described.[3]

In Vitro Fermentation of β-Glucans. In vitro fermentation of the above three different β-glucans with *Bifidobacterium infantis* (JCM 1222), obtained from the Japan Collection of Microorganism (JCM), was conducted by a batch fermentation system under strict anaerobic conditions described elsewhere.[3] Three biological replicates were performed for each growth condition. The fermentation system was monitored for pH, bacterial growth, and short-chain fatty acid (SCFA) production by protocols described previously.[3] Organic matter disappearance (OMD) was analyzed by the ashing method as previously described[8] to evaluate the utilization efficiency of the β-glucans in each system.

Two-Dimensional Electrophoresis (2-DE). The bifidobacterial cells for proteome analysis were collected at 24 h of fermentation and washed three times in low-salt buffer (3 mM KCl, 1.5 mM KH_2PO_4, 68 mM NaCl, and 9 mM NaH_2PO_4).[14] Cells were resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT) (GE Healthcare) and underwent ultrasonication for 10 min. Supernatant was collected by centrifugation at 12 000*g* for 10 min, and TCA/acetone (1:10 v/v) was added to the supernatant. The mixture was stored at −20 °C overnight for protein precipitation. The pellet obtained by centrifugation (10 000*g* for 10 min at 4 °C) was washed with ice-cold acetone before being air dried and resuspended in rehydration solution (8 M urea, 2% (w/v) CHAPS, 0.002% (w/v) bromophenol blue) (GE Healthcare). Protein concentration was determined using a PlusOne 2-D Quant Kit (GE Healthcare).

Samples of 300 μg of protein in rehydration stock solution and 0.5% (v/v) pH 4−7 Pharmalyte (GE Healthcare) made up to a volume of 250 μL and loaded to immobilized pH gradient strips (pH 4−7, linear, 13 cm, GE Healthcare), and isoelectric focusing (IEF) was performed on an Ettan IPGphor III isoelectric focusing system (GE Healthcare). The second dimension was performed with 12.5% polyacrylamide gel on the SE 600 Ruby Standard Dual Cooled Vertical Unit (GE Healthcare) overnight. Gels were stained using Coomassie Brilliant Blue R350 (GE healthcare) and scanned with ImageScanner (Amersham Biosciences). Image analysis was carried out by ImageMaster 2D Platinum software (Amersham Biosciences). Differentially expressed protein spots were compared among the gels from the cells incubated with β-glucans from seaweed, mushroom, and barley with the latter as reference. Spots with the volume ratio of at least 1.0-fold change and an analysis of variance statistical significance test ($P < 0.05$) were selected for mass spectrometric analysis.

In-Gel Digestion and Protein Identification by Mass Spectrometric Analysis. Individual protein spots selected form 2D-E were cut out and destained with 50 mM ammonium bicarbonate, dehydrated with ACN. Spots were rehydrated with 5 μL of Sequencing-grade modified trypsin (Promega) at 20 ng/μL in 50 mM ammonium bicarbonate and incubated at 37 °C overnight. Afterward, 3 μL of extraction buffer (75% ACN, 2.5% TFA) was added to the samples, followed by sonication for 10 min. The supernatant containing tryptic peptides (0.5 μL) was directly spotted onto a MALDI-TOF target plate along with an equal volume of α-cyano-4-hydroxycinnamic acid (4 mg/mL α-cyano-4- hydroxycinnamic acid in 35% ACN and 1% TFA).

Mass spectrometric analysis of the peptide solutions from spots was carried out on a MALDI-TOF/TOF tandem mass spectrometer ABI 4700 proteomics analyzer (Applied Biosystems, Foster City, CA). Mass data acquisitions were piloted by 4000 Series Explorer Software v3.0 with batched processing and automatic switching between MS and MS/MS modes. All MS survey scans were acquired over the mass range 800−3500 m/z in the reflectron positive-ion mode and accumulated from 2000 laser shots with an acceleration of 20 kV. MS spectra were internally calibrated using porcine trypsin autolytic products (m/z 842.509, m/z 1045.564, m/z 1940.935 and m/z 2211.104) resulting in mass errors of less than 30 ppm. The MS peaks (MH^+) were detected on a minimum S/N ratio ≥ 20 and cluster area S/N threshold ≥ 25 without smoothing and raw spectrum filtering. Peptide precursor ions corresponding to contaminants including keratins and the trypsin autolytic products were excluded in a mass tolerance of ±0.2 Da. Filtered precursor ions with a user-defined threshold (S/N ratio ≥ 50) were selected for the MS/MS scan. Fragmentation of precursor ions was performed using MS-MS 1 kV positive mode with CID on and argon as the collision gas. MS/MS spectra were accumulated from 3000 laser shots using default calibration with Glu-Fibrinopeptide B from 4700 Calibration Mixture (Applied Biosystems). MS/MS peaks were detected with a minimum S/N ratio ≥ 3 and cluster area S/N threshold ≥ 15 with smoothing.

MS and MS/MS data were loaded into the GPS Explorer software v3.5 (Applied Biosystems) and searched against Swiss-Prot database (released on Sept 18, 2006) by Mascot search engine v1.9.05 (Matrix science, London, U.K.) using combined MS (peptide-mass-fingerprint approach) with MS/MS (DeNovo sequencing approach) analysis for protein identification. The following search parameters were used: monoisotopic peptide mass (MH^+); 800−3500 Da; one missed cleavage per peptide; enzyme, trypsin; taxonomy, Bacteria; p*I*, 0−14; precursor-ion mass tolerance, 50 ppm; MS/MS fragment-ion mass tolerance, 0.1 Da; variable modifications, carbamidomethylation for cysteine and oxidation for methionine were allowed. Known contaminant ions corresponding to trypsin and keratins were excluded from the peak lists before database searching. The top 10 hits for each protein search were reported. Identified proteins were assigned to various functional classes with the help of UniProt (Universal Protein Resource, http://www.uniprot.org/).

Real-Time RT-PCR. Several genes encoding the proteins of interest were selected for analysis by real-time RT-PCR at the transcriptional level, and their primer sequences were designed by Beacon Designer 7 software listed in Table 1. Total RNA was extracted from *B. infantis* cells using a SV Total RNA Isolation System (Promega, Madison, WI) and quantified by spectrophotometry at 260 nm. Reverse transcription was carried out with an ImProm-II Reverse Transcription System (Promega) with 1 μg of total RNA input. PCR amplification was performed using the IQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA) on the Bio-Rad IQ5 Real-Time PCR Instrument. Thermal cycling was programmed as follows: 95 °C for 3 min; 40 cycles of 95 °C for 10 s, 55 °C for 30 s, 72 °C for 7 s; a melting curve was immediately run at the end of this thermal cycling. Results were analyzed using the $2^{-\Delta\Delta CT}$ method of relative quantification to represent relative expression values.[10] PCR runs were performed in triplicate for each of the three biological replicates, and the transcript from *B. infantis* 16 S rDNA (rDNA) (Bli008) was used as internal control since its expression is relatively constant in bacteria.[14]

Enzyme Activity Assay. Hydrolysis of different β-glucan substrates was quantified using the dinitrosalicyclic acid method[15] by measuring the reducing sugar released by glucanase. This glucanase

Table 1. Oligonucleotides Used for Gene Expression Analysis (Real-Time RT-PCR)

encoded protein name	nucleotide sequence
Bli 001 outer membrane porin protein	AGTTTGTTGTCAGAATCG;
	TAGAAGGTATCGGTGATG
Bli002 ABC transporter related protein	GATGTACTTCAAGATACGGGTGTA
	GTGAACCGTGCCAAGGAA
Bli003 phosphotransferase; PTS enzyme 1	CAATGGTGAGCAAGTCAT
	CATCCTCGATCTCAGCAG
Bli004 outer membrane protein	CCAGTTTGTTGTCAGAAT
	TAGAAGGTATCGGTGATG
Bli005 ATP binding protein of ABC transporter for sugars	AACAAGCTCACCACGATT
	CGGGTCGATCTTGATCTT
Bli006 glycogen phosphorylase	AATCACCGCACCGAAGTC
	TTTGAGTGTATTTCAGTTGTTCAC

assay was carried out in a pH 8.0 phosphate buffer with a substrate concentration of 0.25% in a reaction volume of 200 μL at 40 °C for 10 min. Under this condition, the amount of glucanase required for production of 1 μmol of reducing sugar per minute is defined as one unit of glucanase activity.[16] β-Glucosidase activity assay was carried out with *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as substrate in 50 mM phosphate buffer at 40 °C. After 10 min incubation, the reaction was stopped by adding saturated sodium tetraborate solution and the absorbance was read at 405 nm. One unit of β-glucosidase activity is defined as the amount of glucosidase required for production of 1 μmol of *p*NP per minute under this condition.[17,18]

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

RESULTS AND DISCUSSION

Comparison of Bifidobacterial Fermentation of β-Glucans from Seaweed, Barley, and Mushroom. In vitro fermentation was monitored for 24 h which should be sufficient to imitate the colonic microbial degradation of nondigestible carbohydrates in vivo and also corresponded to the late exponential growth phase of *B. infantis* cultures based on a previous study.[2] Results of pH, bacterial growth, utilization of substrate (represented by OMD), and production of metabolites (SCFAs) of the 24 h fermentation process are shown in Table 2. pH in all systems showed a significant decrease after 24 h of incubation, which might be due to production of SCFAs. The number of population of *B. infantis* during the 24 h fermentation monitored by viable count was increased by an average of 3 $\log_{10}$ CFU on the three β-glucans with the largest increase of 4 $\log_{10}$ CFU in laminarin, which was consistent with its highest bifidogenic effect found previously.[3] Laminarin had the highest OMD of nearly 37%, which was consistent with its largest increase of bifidobacterial population. SCFAs are the major organic acids from colonic fermentation of polysaccharides with acetic acid, propionic acid, and butyric acid as the principal components.[19] *B. infantis* produced a similar amount of total SCFAs when incubated with the three β-glucans with a ratio of acetic:propionic:butyric acid to be 8:1:1 (Table 2), which was consistent with a previous report.[3]

Comparative Proteome Analysis of Bifidobacteria Utilizing β-Glucans from Barley, Seaweed, and Mushroom. β-Glucan substrates from barley, seaweed, and mushroom are β-type glucans with different sugar linkages. Barley β-glucan has a mixed β-1,3 and β-1,4 in the main chain; laminarin possesses a linear β-1,3-linkage backbone with β-1,6-branches, and β-glucan from mushroom sclerotia has a complex chemical structure of a highly branched main chain with mixed β-1,3 and β-1,4 and β-1,6 linkages.[3] Representative 2-DE gel images of the *B. infantis* cultured with the β-glucan from seaweed, barley, and mushroom are shown in Figure 1A, 1B, and 1C, respectively. By comparing the proteome patterns of the bifidobacteria from fermentation of these substrates, 77 spots were found to be differentially expressed among these three substrates (Table 1 in the Supporting Information), and 17 of them are involved in carbohydrate metabolism (Table 3).

Spot 97 was one of the ATP binding proteins of ABC transporter for sugars. Bacterial ABC transporters are essential in many ways of bacterial life such as iron uptake, virulent defense, and so on. High expression of the ATP binding protein of ABC transporter for sugars (spots 97 and 164) in the laminarin and barley gels instead of in that of mushroom indicated that *B. infantis* could uptake the less complex β-glucan molecules from seaweed and barley probably through action of the ABC transporter system. Formate acetyltransferase (spot 99) is involved in formation of pyruvate through acetyl CoA. Enolase (spot 100) is an important enzyme in the bifid shunt, responsible for conversion from 2-phosphoglycerate to phosphoenolpyruvate (PEP). Ruiz et al. also reported that the membrane-associated enolase also played a role in bacterial colonization on the human gut.[20] Another two copies of enolase (spots 165 and 168) were also found. Glycogen phosphorylase (spot 101) belongs to a family of oligosaccharide phosphorylases and may be involved in glycogen metabolism.

Both spot 159 (chain A, Ompf porin mutant Y106f) and spot 160 (chain A, Ompf porin mutant D74a) might be constituent

Table 2. Fermentation of *B. infantis* in the Presence of β-Glucans from Different Sources[a]

β-glucan source	time (h)	pH	population of *B. infantis*[b]	OMD %	total SCFA production (mmol/L)	normalized molar percentage (%)		
						acetic acid	propionic acid	butyric acid
seaweed	0	6.5 ± 0.01	5.07 ± 0.16					
	24	6.02 ± 0.00a	9.25 ± 0.01b	36.9 ± 5.98	2.00 ± 0.03a	81.14 ± 1.77a	10.91 ± 0.79a	7.95 ± 0.98a
barley	0	6.52 ± 0.01	4.96 ± 0.26					
	24	6.04 ± 0.02a	8.63 ± 0.20ab	15.6 ± 4.51	1.97 ± 0.33a	79.20 ± 1.04a	11.73 ± 0.08a	9.08 ± 0.99a
mushroom	0	6.48 ± 0.01	5.07 ± 0.10					
	24	6.05 ± 0.05a	8.47 ± 0.12a	17.8 ± 8.47	2.24 ± 0.08a	78.70 ± 3.27a	11.78 ± 1.68a	9.52 ± 1.59a

[a]Values in the same column with different letters are significantly different (ANOVA Tukey's multiple comparison test; $P < 0.05$). [b]$\log_{10}$ CFU per mL culture medium

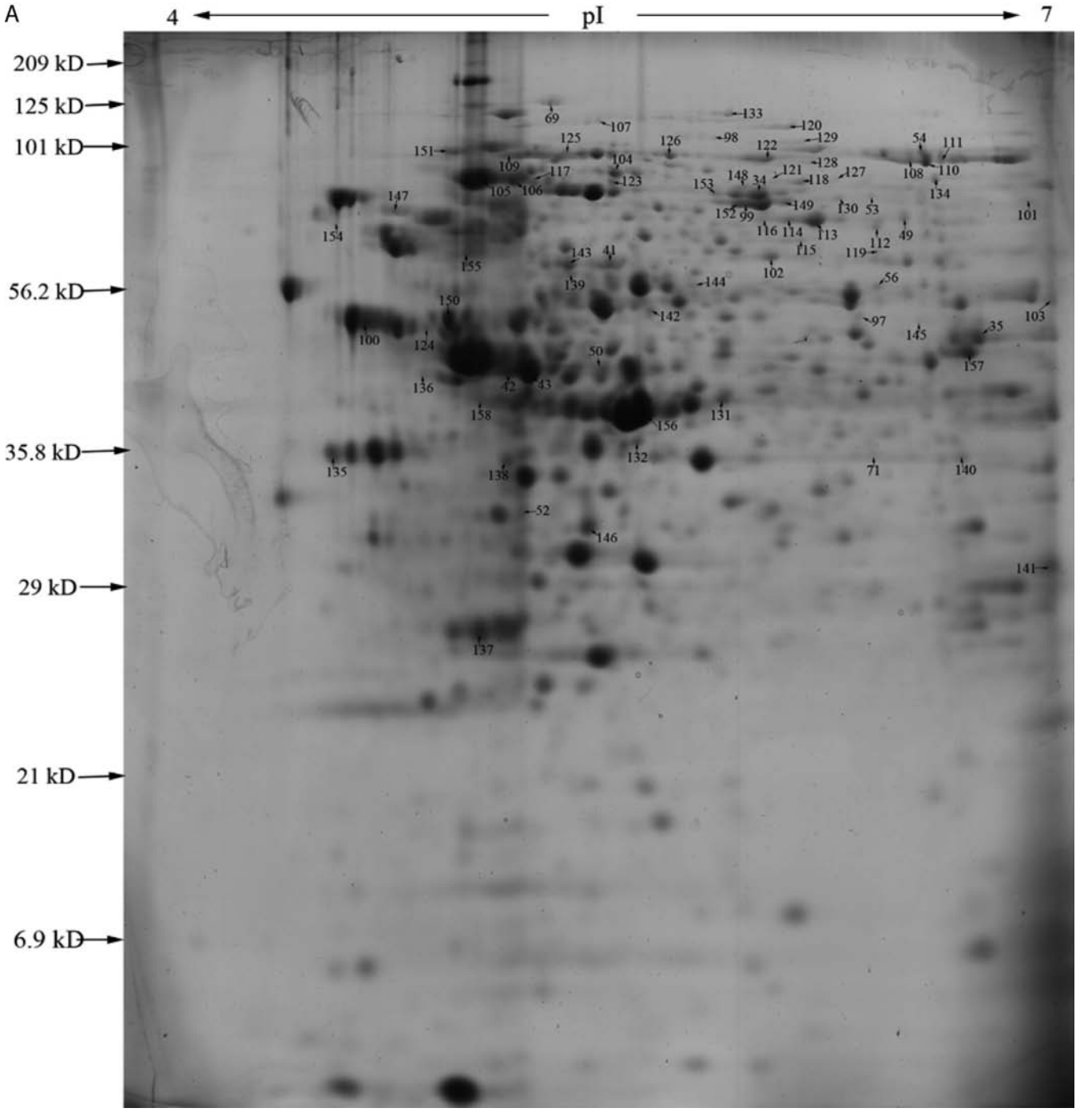

Figure 1. continued

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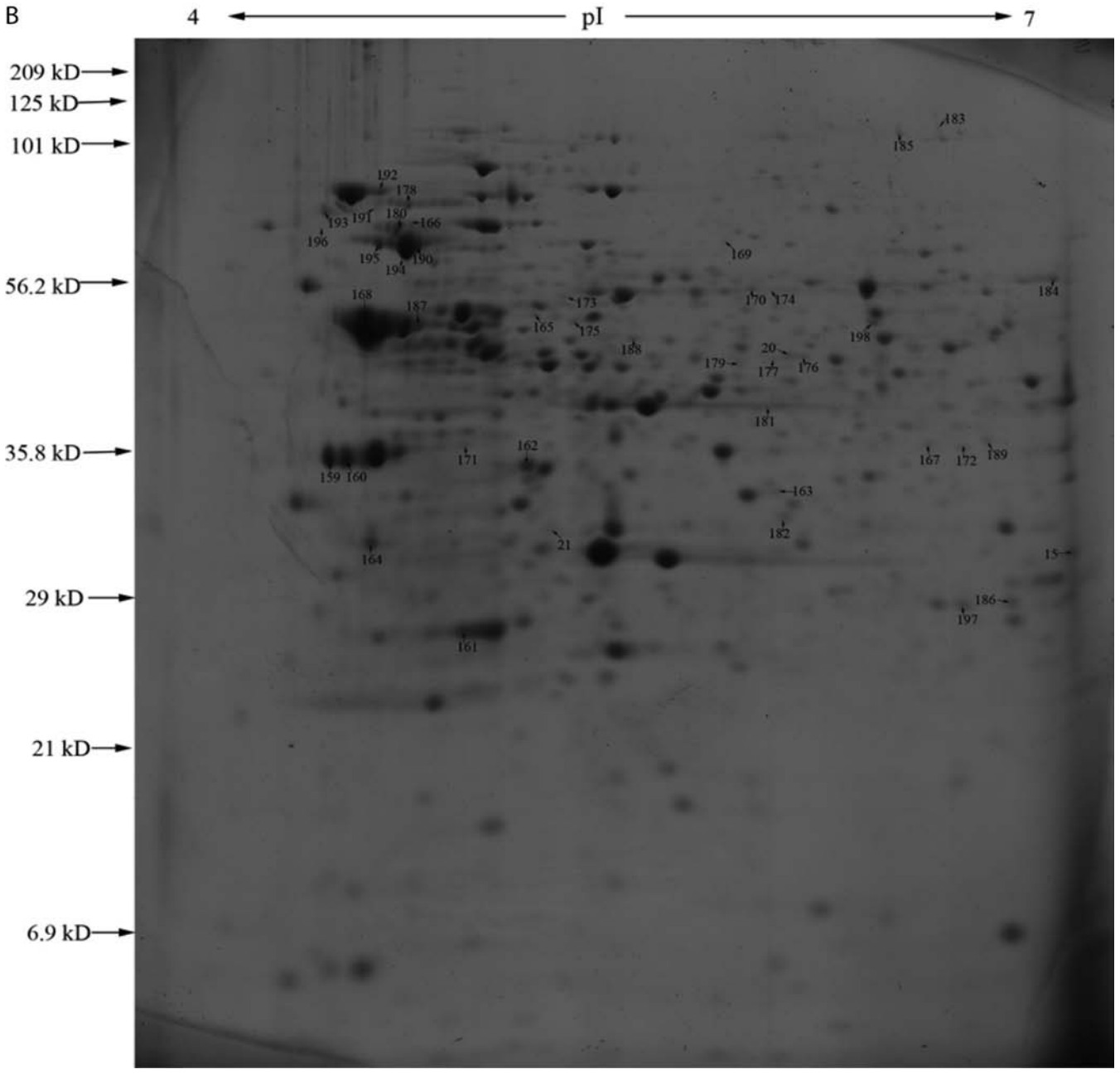

Figure 1. continued

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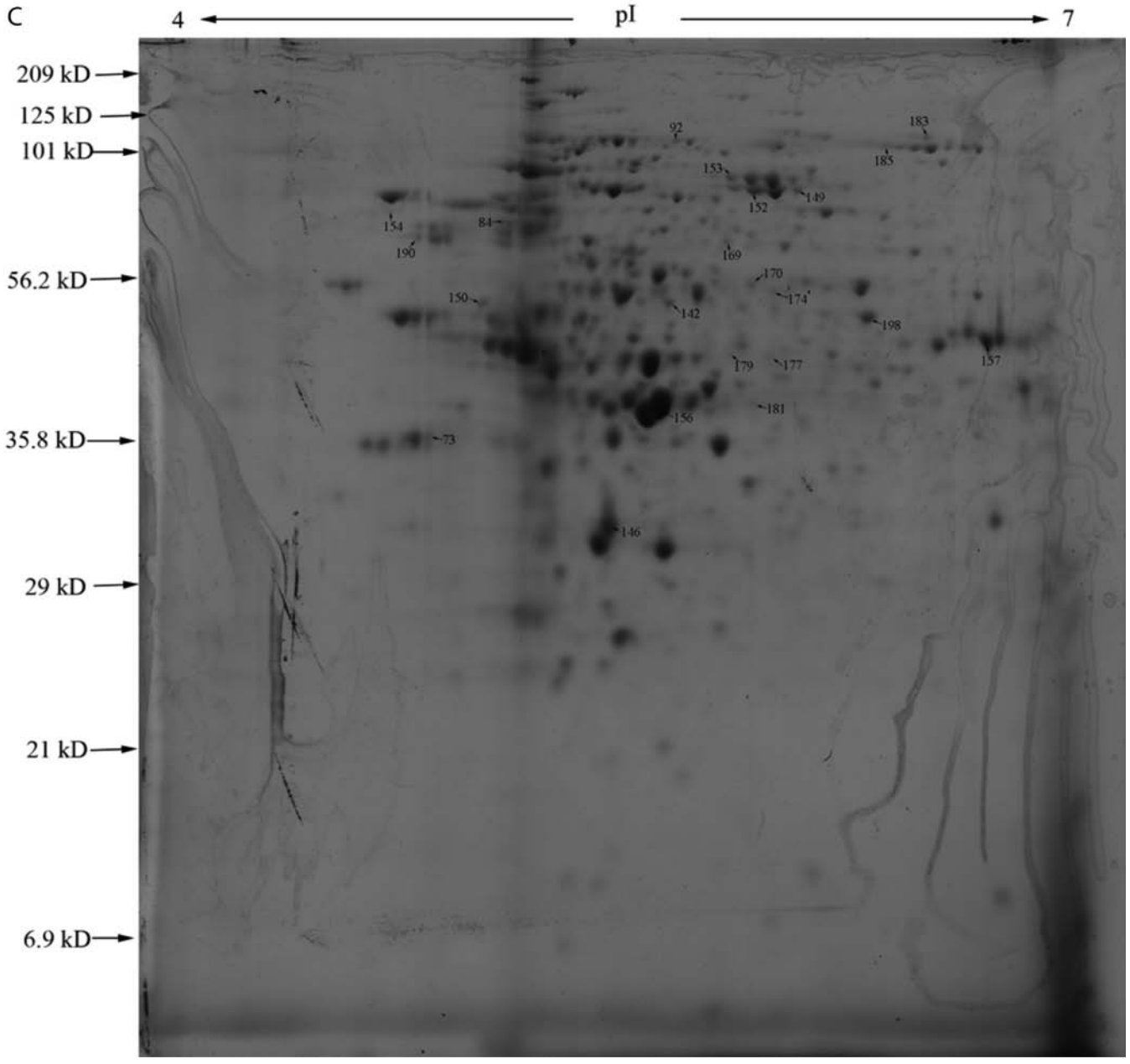

Figure 1. Representative 2-DE of total proteins from *B. infantis* treated with β-glucan from (A) seaweed, (B) barley, and (C) mushroom. Identified proteins are indicated by the arrow and number and listed as in Table 2.

proteins of cell membrane porins which might help transportation of molecules through the membrane. The larger amount of this kind of proteins in the presence of β-glucan from barley showed the more active response of *B. infantis* to it. Triose phosphate isomerase (spot 161) is an important glycolytic enzyme catalyzing interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate, which is an intermediate product in glucose fermentative pathway. Triose phosphate isomerase also links fucose metabolism and glucose metabolism through this conversion.[13]

Both spot 162 and spot 163 were identified to be fructose-1,6-bisphosphate aldolase. Phosphotransferase system (PTS) enzyme I (spot 166) belongs to the carbohydrate transport system 'phosphotransferase system PTS', which consists of enzyme II permease and HPr besides phosphotransferase enzyme I and is one of the several carbohydrate transport systems in *B. longum* NCC2705.[21,22] It was proposed that the PTS in *B. longum* was completely glucose specific, and *B. infantis* may possess respective PTS specific for glucose and *N*-acetylglucosamine.[13] By converting glucose to fructose-6-phosphate, the PTS incorporates glucose into the central fermentative pathway. UDP-galactose-4-epimerase (spot 167), also called UDP-glucose-4-epinerase, functions in the final step of the 'Leloir pathway' for galactose metabolism by producing UDP-glucose.[13] Oligo-1,6-glucosidase (spot 169) could break sucrose into fructose and glucose. The amount of oligo-1,6-glucosidase in the presence of mushroom β-glucan was more than 3 times higher than that of barley and twice that of

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Table 3. Proteins Involved in Carbohydrate Metabolism Differentially Expressed in *B. infantis* Cultured with β-Glucans from Different Sources

spot no.	protein description	organism	accession no.	theor. MW	theor. pI	fold change[a] barley	seaweed[b]	mushroom
97	ATP binding protein of ABC transporter for sugars	*B. longum*	gil23465255	40702.3	5.85	ND	1.00 ± 0.08	ND
98	pyruvate-flavodoxin oxidoreductase	*E. coli*	gil16129339	12 8743.2	5.52	ND	1.00 ± 0.01	ND
99	formate acetyltransferase	*H. influenzae*	gil16272145	86476.2	5.75	ND	1.00 ± 0.02	ND
100	enolase	*S. epidermidis*	gil27467479	47217.8	4.58	1.00 ± 0.74	0.33 ± 0.15	ND
101	glycogen/starch/α-glucan phosphorylase	*E. coli*	gil 213690992	90 329.7	7.12	1.00 ± 0.01	2.86 ± 0.02	ND
159	Ompf Porin Mutant Y106f	*E. coli*	gil14488510	37 045.8	4.64	1.00 ± 0.50	0.46 ± 0.06	ND
160	Ompf Porin Mutant D74a	*E. coli*	gil6729727	37 017.8	4.69	1.00 ± 0.54	0.63 ± 0.12	0.32 ± 0.16
161	triose phosphate isomerase	*B. cereus*	gil20146166	15 235.6	5	1.00 ± 0.14	1.71 ± 0.58	ND
162	fructose-1,6-bisphosphate aldolase	*G. stearothermophilus*	gil9297081	30 798.8	5.47	1.00 ± 0.08	0.56 ± 0.09	ND
163	fructose-1,6-bisphosphate aldolase	*G. stearothermophilus*	gil9297081	30 798.8	5.47	1.00 ± 0.01	ND	ND
164	ABC transporter ATP-binding protein	*B. halodurans*	gil15616033	29 163.5	4.65	1.00 ± 0.12	0.65 ± 0.02	ND
165	enolase	*S. epidermidis*	gil27467479	47 217.8	4.58	ND	1.00 ± 0.25	3.69 ± 0.41
166	PTS enzyme I	*L. plantarum*	gil50812224	63 039.3	4.78	1.00 ± 0.04	0.93 ± 0.03	ND
167	UDP-galactose-4-epimerase	*E. coli*	gil15800468	37 214.6	5.89	1.00 ± 0.01	0.97 ± 0.01	ND
168	enolase	*S. aureus*	gil15923766	47 087.8	4.55	1.00 ± 3.68	0.44 ± 0.86	ND
169	oligo-1,6-glucosidase	*E. coli*	gil146969	45 240.1	5.5	1.00 ± 0.01	1.59 ± 0.01	3.60 ± 0.01
170	glucose-6-phosphate 1-dehydrogenase	*S. enterica*	gil16760836	55 985.4	5.61	1.00 ± 0.02	2.28 ± 0.02	3.51 ± 0.01

[a]Fold-change up- or down-regulation values (mean v % ± SD) on β-glucans as compared to barley β-glucan (normalized) obtained using 2-DE analysis (for complete data on 2-DE analysis see Supporting Information). ND, proteins not detected. [b]Values of seaweed β-glucan were normalized for comparison when the value of barley β-glucan was not detected.

laminarin (Table 3). Although no extra sucrose as carbon source was added into the fermentation medium, oligo-1,6-glucosidase (spot 169) may help degradation of the barley and mushroom β-glucans in the medium with unclear explanation so far. Glucose-6-phosphate 1-dehydrogenase (spot 170) acts in the pentose phosphate pathway, transforming D-glucose 6-phosphate to D-glucono-1,5-lactone 6-phosphate. In the final steps of this pentose phosphate pathway,[23] D-glucono-1,5-lactone 6-phosphate is finally converted to ribose-5-phosphate or xylulose-5-phosphate, which could be inserted into the central fermentative pathway. This pentose phosphate pathway may act as an alternative to glycolysis for regulation of the intermediate compounds.

There were 28 spots, representing 17 proteins, selected to be differentially expressed among the different β-glucan substrates, which may play a role in energy production and conversion. Changes of proteins that work on translation or transcription were also observed, and 10 spots were identified (Table 1 in the Supporting Information). This might indicate a modulation of protein synthesis and energy utilization in the presence of different β-glucan substrates. Stress proteins were also expressed in response to different substrates. Hsp60 (spots 190 and 194) together with chaperonin GroEL (spot 195), which was found overproduced in the bile salt response of *B. longum*,[24] were detected in the presence of the three β-glucan substrates with the highest amount in the presence of barley β-glucan. Chaperone protein dnaK (spots 191, 192, and 193) are important for bacterial stress response,[25] which might exert their functions in response to heat, acid, bile salt, or other kinds of stress. In this case, in the absence of simple sugars ready for use, these proteins might help the bacteria utilizing the structurally complicated β-glucans. Some proteins involved in amino acid metabolism, nucleotide metabolism, fatty acid metabolism, and other unknown functions were also identified at different levels in the presence of the different β-glucan substrates (Table 1 in the Supporting Information).

Real-Time RT-PCR Analysis. A total of 6 genes (Table 1) from 6 spots were selected in real-time RT-PCR. Genes coding for those proteins that were expected to play an important role in carbohydrate transport and metabolism were tested for their changes at the transcription level in fermentation of three β-glucans, and results are shown in Table 4.

Bli 001 is the gene coding for an outer-membrane porin protein, which may help the carbohydrate molecules pass through bacterial cell membrane.[26] Its protein abundance in the barley β-glucan gel was 3.1-fold higher than that in the mushroom β-glucan gel and 1.5-fold higher than that in the

Table 4. Changes of Identified Proteins in *B. infantis* Probably Involved in β-Glucan Catabolism at the Transcription Level by Real-Time RT-PCR

spot no.	protein description	fold of change real-time RT-PCR seaweed[a]	mushroom[a]	barley[a]
160	Bli 001 outer membrane porin	2.9[c]	1.4	1.0[b]
164	Bli002 ABC transporter related	0.93	2.6[c]	1.0
166	Bli003 PTS system enzyme 1	0.90	13	1.0
159	Bli004 outer membrane protein	2.1	nil	1.0
97	Bli005 ABC transporter ATP-binding protein	0.51[c]	0.44[c]	1.0
101	Bli006 glycogen phosphorylase	1.6	1.4	1.0

[a]Source of origin of β-glucans. [b]Normalized value for comparison with other values in the same row. [c]Significant difference compared with the normalized value of Barley β-glucan (ANOVA Tukey's multiple comparison test; $P < 0.05$).

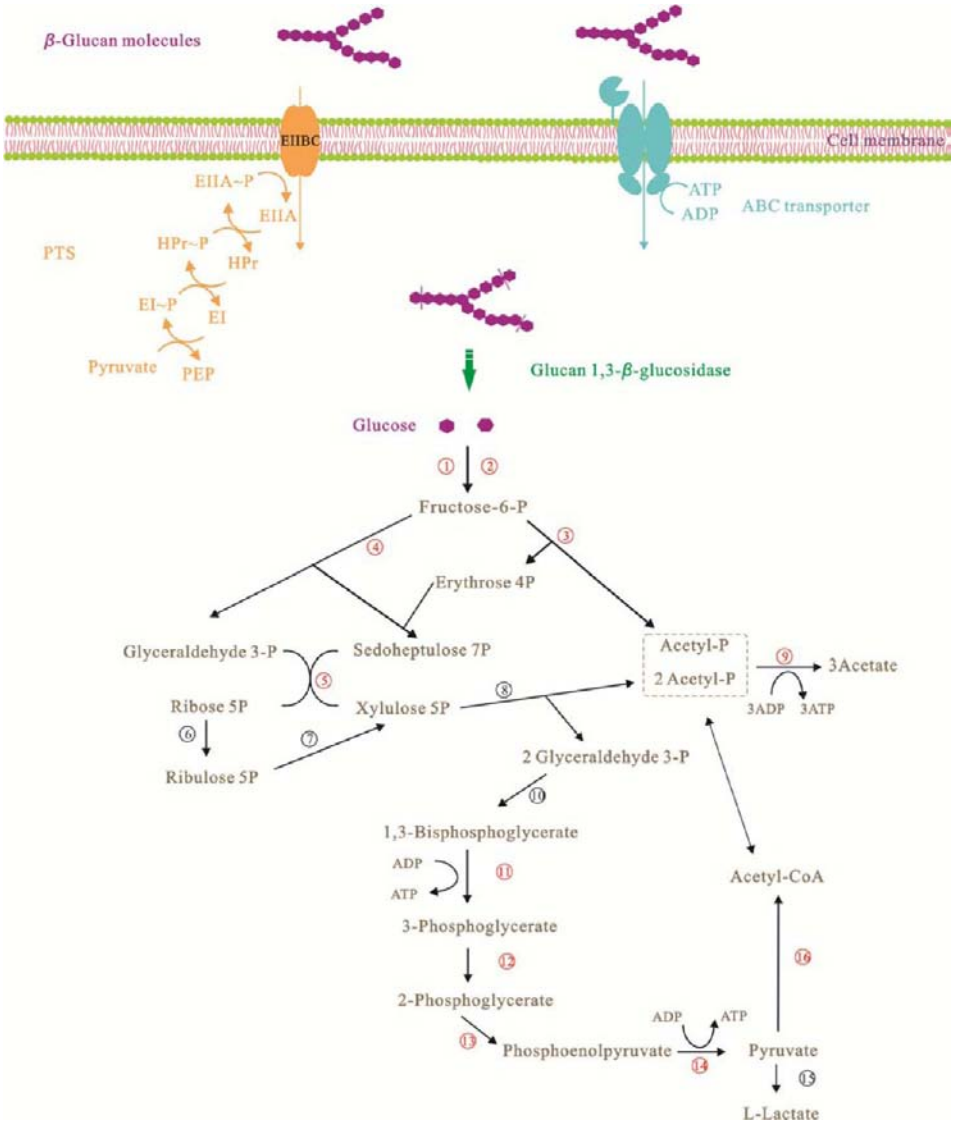

Figure 2. Schematic representation of the proposed catabolic pathway of β-glucans by *B. infantis*. Enzymes involved in the carbohydrate catabolism are denoted by numbers: 1, glucokinase; 2, glucose-6-phosphate isomerase; 3, fructose-6-phosphate phosphoketolase; 4, transaldolase; 5, transketolase; 6, ribose 5-phosphate isomerase; 7, ribulose 5-phosphate epimerase; 8, xylulose-5-phosphate phosphoketolase; 9, acetate kinase; 10, glyceraldehyde-3-phosphate dehydrogenase; 11, phosphoglycerate kinase; 12, phosphoglycerate mutase; 13, enolase; 14, pyruvate kinase; 15, lactate dehydrogenase, 16, pyruvate formate lyase. Numbers circled in red denote the proteins identified in this study with different abundances among treatments. Adapted from ref 13.

seaweed β-glucan gel (Table 3). At the transcriptional level, Bli 001 was upregulated 2.9-fold in the seaweed β-glucan gel with only 1.4- and 1.0-fold in the mushroom and barley β-glucan gels, respectively (Table 4). Bli004 is the gene for another membrane protein, which may also play a role in passage of carbohydrate molecules through the membrane. Protein was not detected on the gels of mushroom β-glucan (Table 3), which is in accordance with the fact that its transcription was not detected in mushroom β-glucan treatment (Table 4), and laminarin gave rise to 2.1-fold change compared to that by barley β-glucan at the transcription level (Table 4).

Bli 002 is the gene for ABC transporter-related protein. At the protein level, it was expressed in the presence of barley and seaweed β-glucans while not detected for mushroom β-glucan (Table 3). At the transcription level, it was up-regulated to 2.6-fold in mushroom β-glucan culture while only 0.93-fold was found in both the seaweed and the barley β-glucan cultures. Although at the protein level the ATP binding protein of ABC transporter could not be identified in all β-glucan systems due to the inherent limitations of the 2D-DIGE technique, the real-time RT-PCR study verified that *B. infantis* could use the ABC transporter systems for uptake of all three kinds of β-glucans, with different fold of change in its expression levels (Table 4). Bli005 is coding for the ATP binding protein of another ABC transporter, and it was down-regulated in seaweed and mushroom β-glucans. Expression of different ABC transporters in all treatments indicated that *B. infantis* probably can use diverse ABC transporters to different extents for one particular substrate. *B. infantis* genome contains many ABC transporter-related genes, which may facilitate incorporating different kinds of substrates/nutrients into the bacterial cells.

Bli003 is the gene coding for PTS enzyme I, phospho-transferase. PTS is one characterized transport system in bifidobacteria,[14] and phosphotransferase is one of its

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components. The PTS system enzyme I protein showed a similar level of abundance in the presence of laminarin and barley β-glucans (Table 3). This was consistent with the trend at the transcription level that seaweed β-glucan gave a similar induction of 0.90-fold of that by barley β-glucan (Table 4). However, in the presence of mushroom β-glucan, gene expression varied greatly among replicates with an average of 13-fold (Table 4), which might imply that *B. infantis* adopted preferentially the PTS system for carbohydrate transport compared with the other β-glucan treatments due to the more complex structure of the mushroom β-glucan.

Bli006 encodes for glycogen phosphorylase, which is an important enzyme responsible for sugar phosphorylation. This enzyme was highly expressed in treatment of seaweed β-glucan with the spot density 2.9-fold up-regulated than that by barley β-glucan. Transcription levels for this enzyme were similar in all three β-glucan cultures with laminarin 1.6, mushroom β-glucan 1.4, and barley β-glucan 1.0. This might indicate that this phosphorylase assists *B. infantis* in the integral metabolism of different β-glucans to some extent.

Hydrolytic Enzyme Activity for β-Glucans during Bifidobacterial Fermentation. All cell-free fermentation broths and intracellular extracts from bifidobacterial cells collected after 24 h fermentation were tested for glucanase activity and β-glucosidase activity. All cell-free fermentation broths from different β-glucan systems displayed neither glucanase activity nor β-glucosidase activity (data not shown), which might indicate that *B. infantis* did not secrete these hydrolases into the culture medium. As for the intracellular activity, *B. infantis* exhibited 7.1 and 4.1 μU/mL fermentation broth of glucanase activity in the presence of the mushroom and seaweed β-glucans, respectively, but no activity was detected for the barley β-glucan. These might be due to the different enzyme-inducing ability of these β-glucans on the bifidobacteria. No β-glucosidase activity was observed intracellularly in all treatments (data not shown).

There have been documented studies on the β-glucosidase activity assay of *Bifidobacterium* species. It was reported that three strains from *Lactobacillus* and *B. animalis* showed β-glucosidase activity which gave them potential for production of the biologically active aglycones in soymilk.[27] It was also reported that *B. longum* CRL849 produced β-glucosidase in the presence of raffinose, which was used as the principal energy source.[28] Although β-glucosidase was detectable in general in the genus *Bifidobacterium*,[29] *B. infantis* did not exhibit any activity when encountering different sugars, including glucose, raffinose, and lactose, which was consistent with the present results.

Proposed Mechanistic Model for Utilization of β-Glucans by *B. infantis*. On the basis of the identified proteins that were differentially expressed in different β-glucan substrates, changes of the proteins at the transcription level, and enzyme activity assay, a proposed model for metabolism of β-glucans from different sources by *B. infantis* is shown in Figure 2.

Uptake and degradation of β-glucan molecules by *B. infantis* proceeded in a multistep manner. First, it is the binding of the β-glucan molecules on the cell surface, followed by internalization of the β-glucan molecules through the cell membrane by ABC-type carbohydrate transporters or PTS proteins, and then breakdown of β-glucan polymer into glucose or oligosaccharides by the action of β-glucan hydrolases. Lastly, it involves incorporation of glucose into the central fermentative pathway—the bifid shunt.

ATP-binding cassette transporters (ABC transporters) are a family of proteins which are universally present from prokaryotes to humans. In bacteria, ABC transporters could help in mediating the import of nutrients into the cells, including ions, amino acids, peptides, sugars, etc. In Gram-positive bacteria, the ABC transporters for sugars are assembled by several protein components: a substrate binding protein anchored in the cell membrane exposed to outside the cell specific for target carbohydrate molecules; two integral membrane proteins responsible for formation of the transport channel; the ATP binding protein as ATP hydrolyzing component (ATPase) for the energy source of the translocation.[30,31] All three types of β-glucans (seaweed, barley, and mushroom) could be recognized and uptake through the ABC transporter systems by *B. infantis*, although the transport efficiency might be different due to their large difference in molecular size (MW 6×10^3, 590×10^3, and 96×10^3 Daltons for seaweed, barley, and mushroom β-glucan, respectively).[3] The captured β-glucan molecules are translocated through the channel of the ABC assembly energized by ATP hydrolysis. Utilization of the milk oligosaccharides through the ABC transporter complex in *B. infantis* has also been reported, which underscores the importance of this family of transporters in colonization of the infant GIT.[13]

In *B. infantis*, β-glucan molecules were not only transported by ABC transporters but also channeled across the cell membrane through the phosphotransferase system (PTS). The PTS is involved in the transport and phosphorylation of a large variety of carbohydrates, both in Gram-negative and in Gram-positive bacteria. The PTS is composed of the non-specific soluble proteins: enzyme I (E I) and histidine protein (HPr), and the carbohydrate-specific component enzyme II (E II).[32] The PTS catalyzes the carbohydrate phosphorylation coupled with its translocation through the cell membrane energized by the glycolytic intermediate phosphoenolpyruvate (PEP).[32] One complete glucose-specific PTS in the genome of *B. longum* NCC2705 has been experimentally verified.[22] According to the genome information, *B. infantis* possesses the PTS specific for glucose and *N*-acetylglucosamine,[13] although the specificity needs further experimental verification. In this study, proteomic and real-time RT-PCR analysis revealed that the PTS could be used for the β-glucan substrate comprised solely of glucose units.

The real-time RT-PCR analysis also showed a remarkable high transcription of PTS enzyme I gene by induction of mushroom β-glucan (Table 4). As proposed by Postma et al.,[32] accumulation of carbohydrates by the non-PTS systems requires more than one ATP equivalent for both transport and phosphorylation on one monosaccharide unit while only one ATP is required by PTS system. This makes the PTS system a more favorable system for carbohydrate metabolism by anaerobic bacteria. The relatively lower solubility of mushroom β-glucan in water makes it more difficult to be used for production of energy by *B. infantis* than seaweed and barley β-glucans. Therefore, it was reasonable that the PTS might be adopted to a much larger extent having an average change fold of 13 in RT-PCR analysis (Table 4) by *B. infantis* when incubated with mushroom β-glucan in face of the limited availability of carbon source.

The β-glucan molecules transported inside the cell were predicted to be processed by intracellular carbohydrate

hydrolases. On the basis of the glycoside hydrolase classification system available in the CAZY database (www.cazy.org) and previous study,[13] *B. infantis* has one glucan 1,3-β-glucosidase (EC 3.2.1.58), which is capable of successive hydrolysis of β-D-glucose units from the nonreducing ends of β-(1→3)-glucans, releasing individual glucose units (http://www.enzyme-database.org/query.php?ec=3.2.1.58). Enzyme activity results (Table 4) confirmed expression of the active glucanase located intracellularly in the presence of laminarin and mushroom β-glucans. The documented study on β-(1→3)-glucan exohydrolase from *Euglena gracilis* demonstrated that this type of enzyme had a high specificity toward β-1,3-glucosidic linkages and its action was not prevented by substitution at the C-6 position in the β-1,3-linked backbone.[33] In their substrate specificity test, seaweed β-glucan was efficiently hydrolyzed and led to production of glucose and laminaribiose,[33] which is consistent with the result in this study that seaweed β-glucan strongly promoted growth of *B. infantis*. On the other hand, it had been proposed that the low degree of hydrolysis of lichenin (mixed linkage of β-1,3 and β-1,4) with production of glucose alone was ascribed to breakdown of the terminal β-1,3 linkages in the mixed-linked backbone.[33] This phenomenon is also similar with the case of mushroom β-glucan with mixed β-1,3 and β-1,4 linkages, which was utilized by *B. infantis* at a relatively slower rate (Table 2). The released glucose molecules could be readily tunneled into the bifid shunt, and the oligoresidues, if any remained, might be further degraded or exported as metabolites by the bifidobacterial cells. Further analysis for the detailed fate of the hydrolyzed residues is required.

Similarly, those β-glucan molecules phosphorylated and imported by the PTS may also be broken down by glucan 1,3-β-glucosidase to yield simple sugars which are incorporated into the main fermentative pathway accordingly. It was also documented that a predicted β-1,3-exoglucanase in *B. longum* NCC2705 belonging to the same group (EC 3.2.1.58) shared more than 50% similarity to the characterized enzymes in yeast.[11] The absence of glucanase activity in those bifidobacterial cells treated with barley β-glucan might be due to the high proportion of β-1,4 linkages (nearly 75%, data not shown) in the backbone of this β-glucan, which might not efficiently induce production of the glucanase. The detailed substrate specificity along with the enzymatic mechanism of the glucan 1,3-β-glucosidase in *B. infantis* needs further experimental studies. The absence of β-glucosidase in *B. infantis* could be one of the reasons for the slow rate of degradation of all β-glucans substrates in the fermentation (Table 2).

The final step of the model involves integration of simple sugars from the previous steps into the central fermentative pathway (Figure 2). The glucose molecules released from the former steps were phosphorylated by glucokinase into glucose-6-phosphate. The probable glucose-phosphate from degradation of those β-glucans imported through the PTS could be transformed to glucose-6-phosphate by phosphoglucomutase. The glucose-6-phosphate from the above two sources could be isomerized by the action of glucose 6-phosphate isomerase to β-D-fructose-6-phosphate, which is a key intermediate in the carbohydrate metabolism pathway—the bifid shunt in bifidobaceria.[10,13]

A model for xylo-oligosaccharides (XOS) catabolism in *B. aninalis* subsp. *lactis* BB-12 has been proposed previously, which is more or less similar to this model and comprised of substrate transport across the cell membrane by the ABC system and degradation inside the cell.[34] On the other hand, an alternative model for XOS catabolism in *B. longum* subsp. *longum* has recently been proposed.[35] *B. longum* possesses 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 and degrade XOS substrate, with the hydrolyzed residues being transported into the cells. In fact, these two types of strategies for nutrient uptake adopted by *Bifidobacterium* species have been postulated previously.[36]

The present study indicates the capability of *B. infantis* to ferment β-glucans with diverse chemical structures from different sources as primary carbon sources. It could be inferred from the above results that *B. infantis* is able to alter its metabolic patterns in face of the availability of different substrates.[10] Such flexibility in *B. infantis* provides a more favorable outcome for its own benefits under nutrient-limited conditions for competitive colonization in human colon. β-Glucans from mushroom, notable for their ability to modulate the immune system, are gaining more and more attention as potential prebiotics. The proposed model for β-glucan catabolism by *B. infantis* is anticipated to broaden our understanding of how this type of nondigestible carbohydrates could be utilized by bifidobacteria to function as novel prebiotics. It is anticipated that β-glucans combined with bifidobacteria such as *B. infantis* and other species can be used as synbiotics for the functional food industry.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Full list of differentially expressed proteins in *B. infantis* in the comparative proteome study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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