Note

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Effects of prebiotic oligosaccharides consumption on the growth and expression profile of cell surface-associated proteins of a potential probiotic *Lactobacillus rhamnosus* FSMM15

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To investigate carbohydrate preference of a potential probiotic, *Lactobacillus rhamnosus* FSMM15, six prebiotics, including two milk-derived prebiotics, galactooligosaccharides and lacto-*N*-biose I, and four plant-origin prebiotics, beet oligosaccharide syrup, difructose anhydride III, fructooligosaccharides, and raffinose, were examined. The strain utilized the milk-derived prebiotics at similar levels to glucose but did not utilize the plant-origin ones in the same manner, reflecting their genetic background, which allows them to adapt to dairy ecological niches. These prebiotics had little influence on the expression pattern of cell surface-associated proteins in the strain; however, an ATP-binding cassette transporter substrate-binding protein and a glyceraldehyde-3-phosphate dehydrogenase were suggested to be upregulated in response to carbon starvation stress.

Key words: ABC transporter substrate-binding protein, glyceraldehyde-3-phosphate dehydrogenase, lacto-*N*-biose I, starvation-stress response

Lactobacillus rhamnosus strain FSMM15 (FSMM15) was recently isolated as a potential probiotic from a fermented mare milk, which was traditionally produced by domestic farmers living on Sumbawa Island in Indonesia [1]. FSMM15 was tolerant to low pH and bile salts at a level comparable to a commercial probiotic, *L. rhamnosus* GG ATCC53103 (LGG) [1]. FSMM15 showed the ability to adhere to a porcine gastric mucin at a level similar to LGG, whereas it was significantly less adhesive to a mouse derived glycoprotein, laminin, which constitute the extracellular matrix [1]. Prebiotics are nutritional substances that promote growth of probiotics preferentially, but known prebiotics are not necessarily utilized by all the probiotics reported. Furthermore, little is known about effects of prebiotics consumption on the cell surface proteins in lactobacilli and Bifidobacteria, although Schar-Zammaretti et al. [2] reported that the concentration of cell surface-associated proteins of *Lactobacillus acidophilus* increased slightly, with the physicochemical properties and structure of the cell wall remaining almost unchanged, when the carbohydrate content in the culture medium was largely reduced. In terms of cytosolic proteins, van Zanten et al. [3] demonstrated that a potential prebiotic, cellobiose, upregulated two β-glycoside hydrolases in *L. acidophilus* NCFM. It is important for desirable use of aimed probiotics in the food industries to clarify which prebiotics are effectively utilized by them. We evaluated the growth of FSMM15 for this purpose using six prebiotics, beet oligosaccharide syrup (BOS), difructose anhydride III (DFA III), fructooligosaccharides (FOSs), galactooligosaccharides (GOSs), lacto-*N*-biose I (LNB I), and raffinose (RAF), as sole carbon sources added to de Man, Rogosa and Sharpe (MRS) broth in this study. Furthermore, since cell surface proteins, such as S-layer proteins [4] and LPXTG-anchored proteins [5], are directly related to host-bacteria interactions, effects of prebiotics supplementation on expression profiles of cell surface-associated proteins extracted by a combination of lysozyme and lithium chloride were investigated.

FSMM15 was obtained from our library of lactic

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Table 1. Prebiotics used in this study

Name	Description
Beet oligosaccharide syrup (BOS)	Beet Oligo (Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan), made from beet molasses, containing 11% of oligosaccharides and 67% other carbohydrates
Difructose anhydride III (DFA III)	A cyclic disaccharide, di-D-fructofuranose-1,2':2,3'-dianhydride, enzymatically produced from inulin (Nippon Beet Sugar Manufacturing Co., Ltd.)
Fructooligosaccharides (FOSs)	Meioligo P (Meiji Seika Kaisha Ltd., Tokyo, Japan), a mixture of 42% 1-kestose, 46% nystose, and 9% 1F-β-fructofuranosylnystose
Galactooligosaccharides (GOSs)	Oligomate-55NP (Yakult Honsha Co., Ltd., Tokyo, Japan), containing not less than 55% galactooligosaccharides and not more than 45% simple sugar and lactose
Lacto-*N*-biose I (LNB I)	A disaccharide, 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-D-glucopyranose, enzymatically synthesized by Dr. Nishimoto and Dr. Kitaoka at the National Food Research Institute (Tsukuba, Japan)
Raffinose (RAF)	A trisaccharide, β-D-fructofuranosyl-*O*-α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside; purity>98% (Nippon Beet Sugar Manufacturing)

acid bacteria at Obihiro University of Agriculture and Veterinary Medicine. A single colony of FSMM15 formed on an MRS agar plate was inoculated into 5 ml of MRS broth supplemented with 2% glucose as a carbon source and incubated statically at 37°C for 24 hr under anaerobic conditions (a CO_2 concentration of approximately 20%) using an AnaeroPack Kenki system (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan). A 20-µl aliquot of this culture broth was inoculated into 5 ml of a fresh MRS broth under sterile conditions and incubated as described above. After the 24-hr incubation, cells were harvested, washed twice with phosphate-buffered saline (PBS), and inoculated into 50 ml of a modified MRS broth supplemented with 2% each of the six 0.2-µm-filter-sterilized prebiotics as a sole carbon source (Tables 1 and 2), resulting in an optical density (OD) of 0.3 at a wavelength of 600 nm. Modified MRS broths supplemented with or without glucose were used as positive (termed GLC) and negative (termed BM) controls, respectively. All these broths were then incubated at 37°C for 132 hr under the anaerobic conditions. One-ml aliquots of the culture broths were sampled under sterile conditions in every 12 hr. OD_{600nm} values of the samples were measured spectrophotometrically, and their colony forming units (CFUs) were determined by counting colonies formed by streaking diluted culture broth with PBS on MRS agar plates under sterile conditions. All the experiments were done in triplicate. Generation time (Tg) was calculated with the following equation:

$$Tg = \mathrm{t}\log 2/\log(N_{24hr}/N_{0hr}),$$

where t is the appropriate time interval during the logarithmic phase, N_{0hr} indicates the viable cell count at the starting time point (0 hr) of the interval, and N_{24hr} indicates the viable cell count at the end time point (24 hr) of the interval.

The major structural difference between the milk-derived (GOSs and LNB I) and plant-origin (DFA III, FOSs, and RAF) prebiotics is the presence of galactose and fructose residues, respectively, in the nonreducing end. To utilize such carbohydrates, bacteria should possess specific sugar transporters and enzymes that enable metabolism of them. As shown in Fig. 1, DFA III (Tg = 204 min), FOSs (Tg = 195 min), and RAF (Tg = 129 min) exhibited almost the same growth as BM (Tg = 227 min), indicating these three prebiotics were apparently unutilized by FSMM15. Hence, FSMM15 may lack either enzymes metabolizing these prebiotics, such as fructosidase [6] and DFA IIIase [7], or functional transporter machinery, such as the phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS) and sugar-specific permease, which are required for uptake of them. These plant-origin prebiotics are simply not the primary carbohydrates for FSMM15 to adapt to the ecological niche where they survive. On the other hand, as expected, the best prebiotic for the growth of FSMM15 was GOSs (Tg = 198 min), which resulted in an $OD_{600\,nm}$ value of approximately 3.7 and a similar cell population, about 8.5 logCFU/ml, compared with GLC (Tg = 89 min) in the stationary phase. FSMM15 grew on BOS (Tg = 136 min) in a manner similar to its growth on GOSs; however, this was not due to its major oligosaccharide content, RAF, but was due to other carbohydrates in BOS (Table 1). It is well documented that lactose and GOSs are utilized by lactobacilli via the common metabolic pathways, PEP-PTS/phospho-β-galactosidases and permease/β-galactosidase [reviewed in 8]. Moreover, FSMM15 was capable of utilizing LNB I (also known as β-D-galactosyl-1,3-*N*-acetyl-D-glucosamine; Tg =

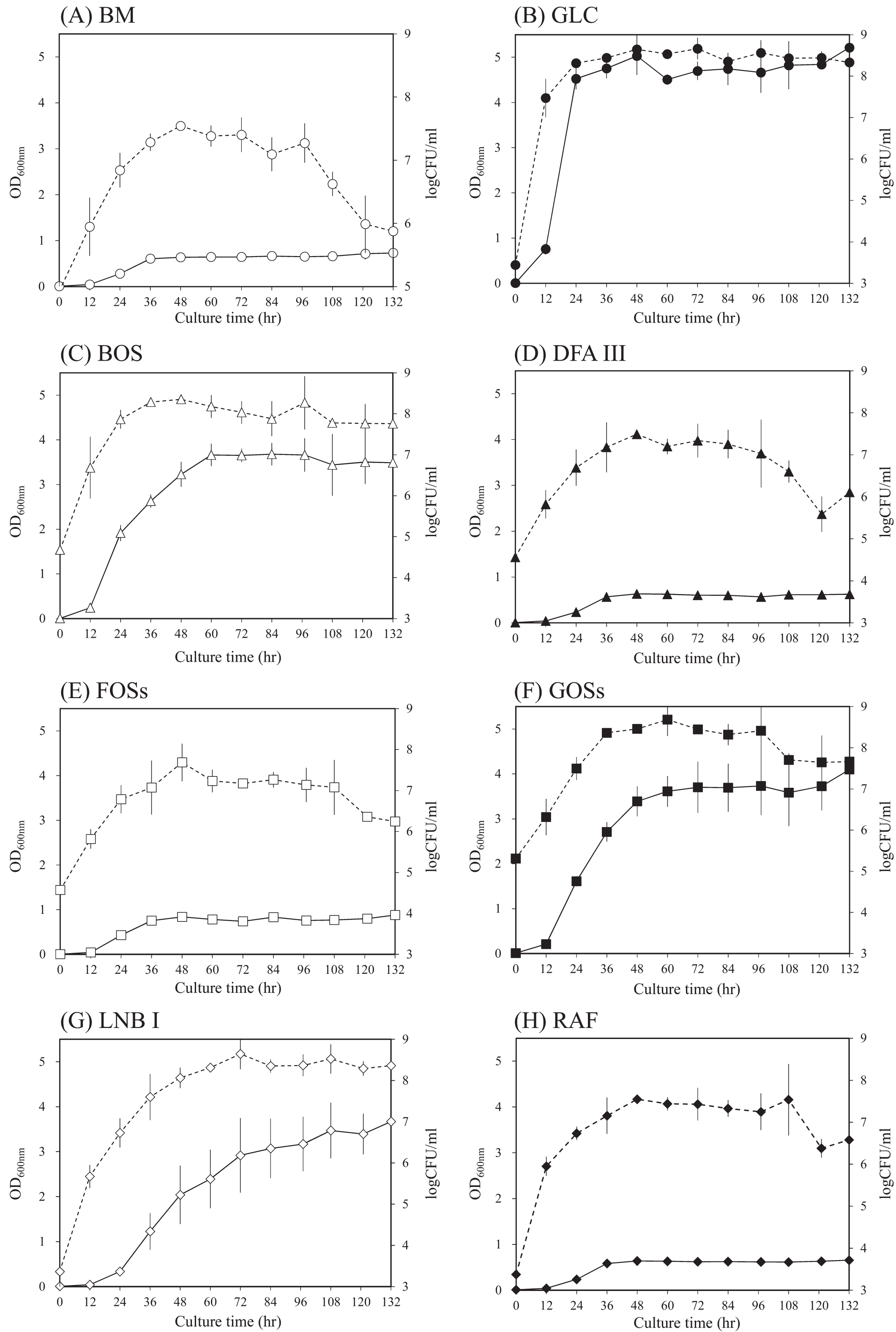

Fig. 1. Growth curves of *L. rhamnosus* FSMM15 cultivated in the modified MRS broth supplemented with various prebiotics as solo carbon sources.

Individual growth curves for BM (A), GLC (B), BOS (C), DFA III (D), FOSs (E), GOSs (F), LNB I (G), and RAF (H) are shown. Values of $OD_{600\ nm}$ and logCFU are indicated by solid and broken lines, respectively. These experiments were performed in triplicate.

129 min), which is found in nature as a partial structure of human milk oligosaccharides [9], in a manner unlikely to *Bifidobacterium longum* strains that have a gene cluster for the utilization of LNB I, including a lacto-*N*-biose phosphorylase gene [10]. Although the *Tg* with LNB I supplementation (129 min) was shorter than that of GOSs (198 min) for the first 24 hr of cultivation, it decreased afterward, leading to slower growth of FSMM15 with LNB I than GOSs. Despite the fact that the maximum $OD_{600\ nm}$ value obtained for LNB I was around 3.5 in the stationary phase, the cell population was similar to those with GLC and GOSs supplementation. In fact, utilization of LNB I by FSMM15 was not surprising, because it was previously reported that strains belonging to the *Lactobacillus casei/paracasei/rhamnosus* subgroup have a unique gene cluster, *gnbREFGBCDA*, enabling utilization of galacto-*N*-biose, which is found in mucin, and LNB I via the action of phospho-β-galactosidase [11]. To elucidate the presence of a similar gene cluster in FSMM15, further studies are needed.

Next, to investigate effects of prebiotic supplementation on expression profiles of cell surface-associated proteins of FSMM15, cell surface-associated proteins of FSMM15 were extracted from cells harvested at the end of the logarithmic growth phase by centrifugation (3,000 × *g*, 15 min, 4°C) from 50 ml of the modified MRS broth (Table 2). The harvested cells were washed twice with cold PBS, suspended in an adequate volume (1 ml/0.1 g wet weight of the harvested cells) of 2 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1 M Tris-HCl (pH 8.0) containing 15 mM NaCl and 50 mM $MgCl_2$, and then incubated at 37°C for 1 hr. After that, the supernatant was collected by centrifugation (12,000 × *g*, 15 min, 4°C). The cell pellet was suspended consecutively in an adequate volume (1 ml/0.1 g wet weight of the harvested cells) of 1 M LiCl (Sigma-Aldrich) solution and then incubated at 20°C for 20 hr according to the method of a previous report [12]. The supernatant was collected by centrifugation (8,000 × *g*, 30 min, 4°C). Each of the collected supernatants was filtered with a nitrocellulose membrane (0.2 µm of pore size, Merck Millipore, Billerica, MA, USA). Their protein concentrations were estimated by measuring the absorbance at a wavelength of 280 nm, assuming the molar absorption coefficient, $E^{1\%}_{1cm}$, to be 10. After subjecting the filtrates to a desalting procedure using a PD-10 column (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions, they were lyophilized and kept as extracted protein fractions in a desiccator until used.

The cell viability of FSMM15 after the extraction treatments is summarized in Table 3, in which representative values obtained by two independent experiments are shown. The wet weights of the harvested cells grown in the modified MRS broth supplemented with the prebiotics, except for LNB I, showed good agreement with their viable cell counts in Fig. 1. The low yield of cells from the modified MRS supplemented with LNB I seems simply to be an experimental error, as large variations were observed in the viable cell counts of FSMM15 grew on LNB I, especially at the end of the logarithmic growth phase (Fig. 1). In general, cell viability was drastically decreased, to less than 20%, by treatment with lysozyme and LiCl, indicating possible leakage of cytoplasmic proteins into the extracts. Interestingly, damage to the cells by the extraction procedure was strongly prevented, by more than 80%, when FSMM15 grew on DFA III and FOSs and moderately prevented (63%) when FSMM15 grew on LNB I (Table 3), but the mechanism is unclear. In contrast to cell growth, the yields of extracted cell surface-associated proteins per cell were notably high in BM, DFA III, and RAF. In general, nutrient starvation leads to growth arrest, and hence lactobacilli have developed individual strategies to survive starvation, e.g., modification of cell morphology and cell division at entry into the stationary phase, resulting in diminished cell size [13]. Cytosolic proteins differentially expressed in response to starvation in fact varied among lactobacilli. For example, Hussain et al. [14] reported that *L. casei* upregulates 16 proteins that are primarily responsible for sugar metabolism in cytosol during the stationary growth phase. The same author also

Table 2. Composition of the modified MRS broth[a)]

Component[b)]	Concentration (g/l)
Ammonium citrate tribasic	2
Carbon source[c)]	20
Dipotassium hydrogen phosphate	2
Lab-Lemco powder[d)]	8
Magnesium sulfate heptahydrate	0.2
Manganese (II) sulfate tetrahydrate	0.05
Peptone[e)]	10
Sodium acetate trihydrate	5
Tween 80	1
Yeast extract[e)]	4

[a)]All components except for the carbon source were dissolved in distilled water, followed by pH adjustment to 6.2–6.6 and sterilization at 121°C for 15 min. A filter-sterilized carbon source was added under sterile conditions to the mixture afterward. [b)]All chemicals were analytical grade unless otherwise stated. [c)]Modified MRS media supplemented with or without glucose was prepared as a positive control (GLC) and a negative control (BM), respectively. [d)]From Oxoid Ltd. (Basingstoke, UK). [e)]From BD Biosciences (Sparks, MD, USA).

Table 3. The cell viability of *L. rhamnosus* FSMM15 after the lysozyme-LiCl extraction treatments

Carbon source	Harvest time (hr)	Wet weight of the harvested cells (g)	Viable cell counts (CFU/ml)		Cell viability[c)] (%)
			Before extraction[a)]	After extraction[b)]	
BM	48	0.10	6.75×10^6	7.50×10^5	11.11
GLC	36	0.62	1.57×10^9	1.68×10^8	10.66
BOS	36	0.69	1.45×10^8	2.54×10^7	17.53
DFA III	48	0.24	7.59×10^6	6.70×10^6	88.3
FOSs	48	0.35	2.75×10^8	2.29×10^8	83.36
GOSs	48	0.69	8.50×10^7	1.48×10^7	17.35
LNB I	72	0.29	4.75×10^7	3.00×10^7	63.16
RAF	48	0.17	6.88×10^6	6.25×10^5	9.09

c=(b/a)*100

reported that 12 out of 19 upregulated proteins in starved cells were associated with amino acid metabolism, lipids biosynthesis, or energy metabolisms other than glycolysis in *L. rhamnosus*, suggesting that starved cells began to hunt for alternative energy sources, i.e., amino acids, lipids, or pentose sugars, when they starved for lactose [15]. It was unclear why FOSs produced such a low protein yield per cell, although they exhibited low cell growth similar to BM, DFA III, and RAF.

Expression profiles of the cell surface-associated proteins extracted with LiCl were then monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% acrylamide gel and Tris-glycine buffer system (0.025 M Tris-HCl (pH 8.3), 0.192 M glycine, 0.1% SDS). Each of the lyophilized protein extracts was dissolved in 100 µl of loading buffer (0.06 M Tris-HCl (pH 6.8), 24.8% glycerol, 1.9% SDS, 0.0095% bromophenol blue, 5% 2-mercaptoethanol), and then a 30-µl aliquot of each solution was heat denatured at 95°C for 5 min, cooled down on ice, and loaded onto the polyacrylamide gel. Proteins were migrated at 200 V for approximately 40 min at room temperature using a Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, Hercules, CA, USA) and a PowerPac Basic Power Supply (Bio-Rad Laboratories) and then were visualized with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories). Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories) were used as protein size markers. For peptide-PAGE to monitor the expression profiles of lysozyme extracts, a 16.5% polyacrylamide gel and Tris-tricine buffer system (0.01 M Tris-HCl (pH8.3), 0.01 M tricine, 0.01% SDS) was used. The lyophilized protein extracts were dissolved in 100 µl of loading buffer (100 mM Tris-HCl (pH 6.8), 1% SDS, 20% glycerol, 0.02% Coomassie Brilliant Blue R-250) and further treated as described above. Proteins were migrated at 100 V for approximately 90 min at room temperature using the same equipment as above. Kaleidoscope Polypeptide Standards (Bio-Rad Laboratories) were used as protein size markers.

Protein bands that appeared on the SDS-PAGE gel were excised and washed thoroughly with 50% acetonitrile (ACN) in 10 mM NH_4HCO_3. After removing the solvent, gel pieces were dehydrated with 100% ACN and then dried using a SpeedVac (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Next, the sulfhydryl group of cysteine residues was protected by carbamidomethylation. In brief, the dehydrated gels were individually incubated with gently shaking in 100 µl of 10 mM DL-dithiothreitol (Sigma-Aldrich) dissolved in 10 mM NH_4HCO_3 at room temperature for 10 min. Then, the solvent was replaced by 100 µl of 55 mM iodoacetamide (Sigma-Aldrich) dissolved in 10 mM NH_4HCO_3 and incubated with gently shaking at room temperature for 10 min in the dark. The excised gels were then washed twice with 50% ACN in 10 mM NH_4HCO_3 and then dried as described above. Each 3 µl of 100 ng/µl proteomic grade trypsin (Sigma-Aldrich), dissolved in 8.1% ACN in 36 mM NH_4HCO_3 containing 0.1 mM HCl, was added to the dried gels and incubated on ice for 5 min. To this mixture, 30 µl of 10 mM NH_4HCO_3 was added and incubated at 37°C overnight. The tryptic digests were extracted twice from the gel pieces with 50 µl of a mixture of 0.1% trifluoroacetic acid (TFA):100% ACN (1:1, v/v) by sonication for 15 min. Furthermore, the remaining tryptic digests were extracted with 50 µl of a mixture of 0.1% TFA:100% ACN (1:2, v/v) by sonication for 15 min. Solvents were totally removed from the extracted peptide solutions using the SpeedVac. The dried peptides were dissolved in 50 µl of 0.1% TFA by sonication for 15 min, and then desalted using ZipTip C18 pipette tips (Merck Millipore), according to the manufacturer's instructions. One microliter of the desalted peptide solution was mixed with an equal volume of saturated α-cyano-4-

hydroxycinnamic acid (Bruker Daltonik GmbH, Bremen, Germany) dissolved in a mixture of 0.1% TFA:100% ACN (2:1, v/v), and then a 1-µl aliquot was mounted on an MTP 384 target plate ground steel T F (Bruker Daltonik GmbH). After the spots were dried, the target plate was loaded into a mass spectrometer (autoflex II TOF/TOF, Bruker Daltonik GmbH). Peptide mass fingerprinting (PMF) was carried out using the pre-installed method, RP_1–3kDa (a reflector positive ion mode focusing on the mass range of 1–3 kDa), flexAnalysis 2.0 software (Bruker Daltonik GmbH), and BioTools 3.0 interface (Bruker Daltonik GmbH) connected to the Mascot search engine [16]. Tandem mass (MS/MS) analysis was performed by LIFT mode with the autoflex II TOF/TOF. Peptide Calibration Standard II (Bruker Daltonik GmbH) was used for external calibration of the mass spectrometer.

Proteomic analyses using one-dimensional PAGEs were performed independently on the proteins extracted with lysozyme and consecutively with LiCl by peptide-PAGE and SDS-PAGE, respectively. No significant difference was observed in the protein expression profiles of cell surface-associated proteins extracted with lysozyme (Fig. 2A). The major band that appeared around 16 kDa was identified as lysozyme by PMF (data not shown). On the other hand, there was variation in the protein band profiles among the extracted proteins from the modified MRS broth supplemented with the prebiotics (Fig. 2B). Although sufficient amounts of proteins from GLC (35 µg) and LNB I (17 µg) were loaded onto the SDS-PAGE gel, the results showed no visible bands. This is likely because the major parts of these fractions were contaminated by other biological compounds such as nucleic acids and small peptides, which resulted in UV absorbance at 280 nm. On the other hand, 4 protein bands at most were seen in the expression profiles for BM, BOS, DFA III, FOSs, GOSs, and RAF. These bands were very faint in the expression profiles for BOS and GOS, implying that the expression levels of these proteins were enhanced under starved conditions in BM, DFA III, RAF, and FOSs. Among the four protein bands, two were successfully identified by PMF (Fig. 3). Band 2, indicated by an arrowhead in Fig. 2B, was identified as an ATP-binding cassette transporter substrate-binding protein (ABC-SBP) (gi:636738117). Also, band 3 turned out to be glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (gi:511645614). As a result of MS/MS analysis, band 4 was suspected to be a partial fragment of ABC-SBP (gi:229312913) with a probability-based MOWSE score of 38, which was lower than the threshold value for significance of 42 (data not shown). ABC-SBPs associate with ABC transporters, which are membrane proteins that translocate a wide variety of molecules, including sugars, amino acids, lipids, metabolites, and drugs, across the cellular membrane [17]. ABC-SBPs bind ligands with high affinity and deliver them to the transmembrane domain of ABC transporters. According to the annotation of ABC-SBP (gi:636738117) in the database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), it contains a periplasmic component of the ABC-type sugar transport system, strongly suggesting that this cell surface-associated protein is upregulated in response to carbohydrate starvation. Watanabe et al. [18] reported that Lam29, which is an adhesion-like protein of *Lactobacillus mucosae* ME-340 capable of binding to human blood group A and B antigens, showed high similarity to an ABC-SBP from *Lactobacillus fermentum*

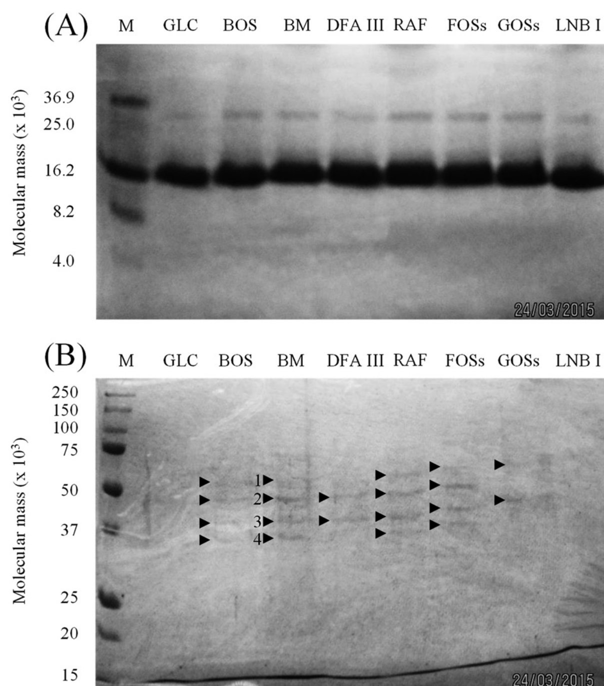

Fig. 2. Expression profiles of cell surface-associated proteins extracted with lysozyme (A) and LiCl (B) from *L. rhamnosus* FSMM15 grown in the modified MRS broth supplemented with various prebiotics.

M indicates molecular size markers. The extracted proteins loaded onto the gels were as follows: GLC (312 µg), BOS (431 µg), BM (391 µg), DFA III (440 µg), RAF (456 µg), FOSs (456 µg), GOSs (452 µg), and LNB I (452 µg) in panel A and GLC (35 µg), BOS (30 µg), BM (50 µg), DFA III (43 µg), RAF (38 µg), FOSs (41 µg), GOSs (30 µg), and LNB I (17 µg) in panel B. Visible protein bands in panel B are indicated by arrowheads with numbers on the left side of the bands.

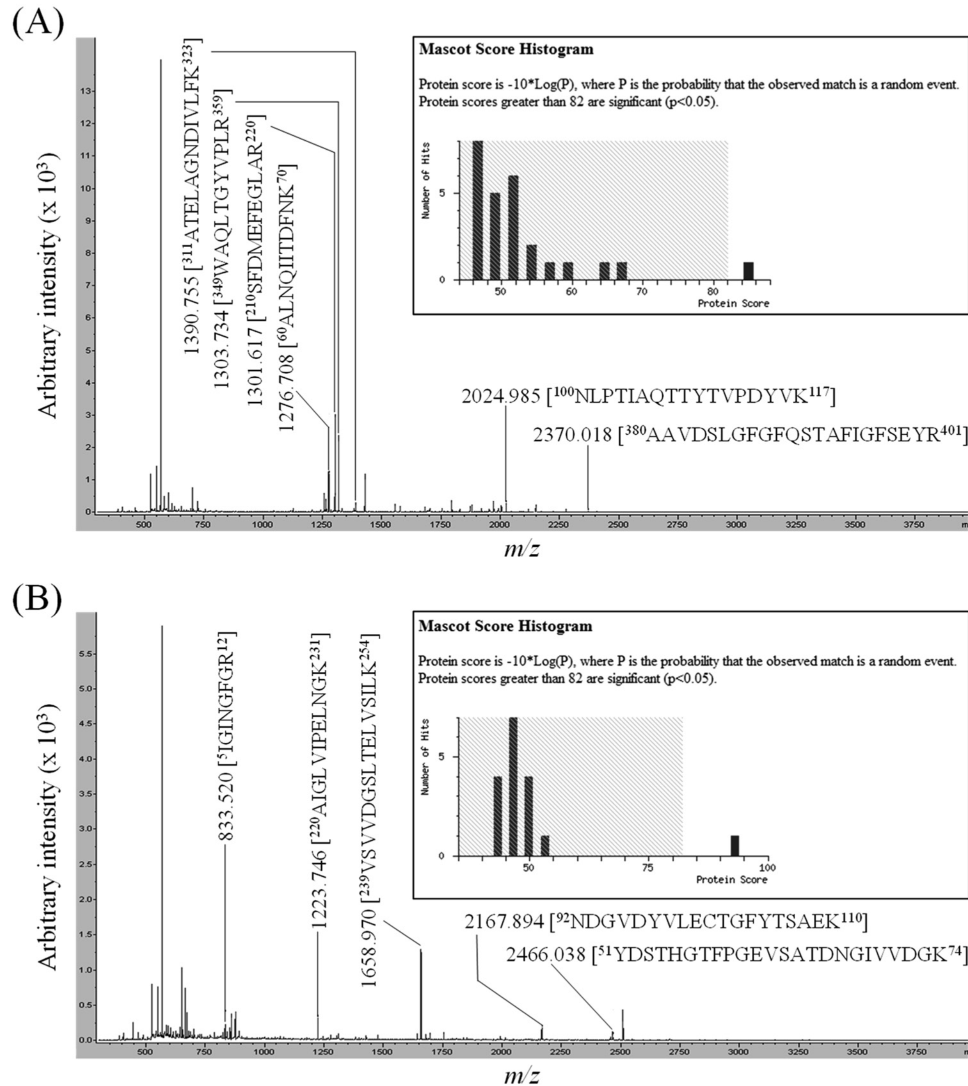

Fig. 3. PMF analyses of cell surface-associated proteins extracted with LiCl from *L. rhamnosus* FSMM15

MS spectra obtained for bands 2 and 3, indicated by arrowheads in Fig. 2B, are shown in panels (A) and (B), respectively. MASCOT search results are indicated as insets in the figure. Band 2 was identified as ATP-binding cassette transporter substrate-binding protein (gi:636738117) with a probability-based MOWSE score of 85. Band 3 was identified as glyceraldehyde-3-phosphate dehydrogenase (gi:511645614) with a probability-based MOWSE score of 93. The threshold value for significance was 82 ($p<0.05$). One-letter representations of assigned amino acid sequences and their monoisotopic masses are shown in the figure. Amino acid residue numbers from the starting methionine are indicated as superscripts in the sequences.

IFO 3956. In this context, the ABC-SBP of FSMM15 may function as an adhesin. Several proteins show multiple biological functions when they are expressed in different cellular locations, and these proteins are referred to as “moonlighting proteins” [19]. GAPDH is an enzyme involved in the glycolytic pathway in cytoplasm; however, it is also known to moonlight as an adhesin with respect to carbohydrates when it is present on or attached to the cell surface of many bacteria, including different strains of *Lactobacillus* [20], *Staphylococcus* [21], *Candida* [22], *Streptococcus* [23–25], and *Listeria* [26]. Therefore, GAPDH is also likely to be upregulated in response to carbohydrate starvation. In *L. casei*, enolase and GAPDH identified on the bacterial cell surface correlated with acid stress conditions [27], while in *L. rhamnosus*, these proteins were identified on the bacterial cell surface in response to both heat and acid stress conditions [28]. Furthermore,

Nezhad et al. [27] demonstrated that lactobacilli strains rapidly modified their surface properties by upregulation of these glycolytic enzymes in response to changes in pH. However, the molecular mechanism of transport of moonlighting proteins onto the cell surface is still unclear. Cell viability of FSMM15 significantly decreased after lysozyme treatment under our experimental conditions, and hence it requires further experiments to clarify the original localization of the extracted GAPDH.

In conclusion, the results of this study unraveled favorable prebiotics for the potential probiotic *L. rhamnosus* FSMM15. *L. rhamnosus* FSMM15 preferred milk-related prebiotics, GOSs and LNB I, rather than plant-origin prebiotics, DFA III, FOSs, and RAF. Prebiotic supplementation had little effect on the variation of cell surface-associated proteins of FSMM15, whereas energetically starved conditions enhanced the expression levels of ABC-SBP and GAPDH, which were most likely expressed on the cell-surface to promote the chance to capture carbohydrates in the surrounding area. Therefore, GOSs or LNB I consumption is unlikely to have an influence on the interaction between FSMM15 and its host via cell surface-associated proteins.

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REFERENCES

1. Shi T, Nishiyama K, Nakamata K, Aryantini NP, Mikumo D, Oda Y, Yamamoto Y, Mukai T, Sujaya IN, Urashima T, Fukuda K. 2012. Isolation of potential probiotic *Lactobacillus rhamnosus* strains from traditional fermented mare milk produced in Sumbawa Island of Indonesia. Biosci Biotechnol Biochem 76: 1897–1903. [Medline] [CrossRef]
2. Schär-Zammaretti P, Dillmann ML, D'Amico N, Affolter M, Ubbink J. 2005. Influence of fermentation medium composition on physicochemical surface properties of *Lactobacillus acidophilus*. Appl Environ Microbiol 71: 8165–8173. [Medline] [CrossRef]
3. van Zanten GC, Sparding N, Majumder A, Lahtinen SJ, Svensson B, Jacobsen S. 2015. The differential proteome of the probiotic *Lactobacillus acidophilus* NCFM grown on the potential prebiotic cellobiose shows upregulation of two β-glycoside hydrolases. Biomed Res Int 2015: 347216. [Medline] [CrossRef]
4. Buck BL, Altermann E, Svingerud T, Klaenhammer TR. 2005. Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol 71: 8344–8351. [Medline] [CrossRef]
5. Roos S, Jonsson H. 2002. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. Microbiology 148: 433–442. [Medline] [CrossRef]
6. Barrangou R, Altermann E, Hutkins R, Cano R, Klaenhammer TR. 2003. Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by *Lactobacillus acidophilus*. Proc Natl Acad Sci USA 100: 8957–8962. [Medline] [CrossRef]
7. Saito K, Sumita Y, Nagasaka Y, Tomita F, Yokota A. 2003. Molecular cloning of the gene encoding the di-D-Fructofuranose 1,2′:2,3′ dianhydride hydrolysis enzyme (DFA IIIase) from *Arthrobacter* sp. H65-7. J Biosci Bioeng 95: 538–540. [Medline] [CrossRef]
8. Titgemeyer F, Hillen W. 2002. Global control of sugar metabolism: a gram-positive solution. Antonie van Leeuwenhoek 82: 59–71. [Medline] [CrossRef]
9. Thurl S, Munzert M, Henker J, Boehm G, Müller-Werner B, Jelinek J, Stahl B. 2010. Variation of human milk oligosaccharides in relation to milk groups and lactational periods. Br J Nutr 104: 1261–1271. [Medline] [CrossRef]
10. Kitaoka M, Tian J, Nishimoto M. 2005. Novel putative galactose operon involving lacto-*N*-biose phosphorylase in *Bifidobacterium longum*. Appl Environ Microbiol 71: 3158–3162. [Medline] [CrossRef]
11. Bidart GN, Rodríguez-Díaz J, Monedero V, Yebra MJ. 2014. A unique gene cluster for the utilization of the mucosal and human milk-associated glycans galacto-*N*-biose and lacto-*N*-biose in *Lactobacillus casei*. Mol Microbiol 93: 521–538. [Medline] [CrossRef]
12. Wang B, Wei H, Yuan J, Li Q, Li Y, Li N, Li J. 2008. Identification of surface protein from *Lactobacillus reuteri* JCM 1081 that adheres to porcine gastric mucin and human enterocyte-like HT-29 cells. Curr Microbiol 57: 33–38. [Medline] [CrossRef]
13. van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD, Maguin E. 2002. Stress responses in lactic acid bacteria. Antonie van Leeuwenhoek 82: 187–216. [Medline] [CrossRef]
14. Hussain MA, Knight MI, Britz ML. 2009. Proteomic analysis of lactose-starved *Lactobacillus casei* during stationary growth phase. J Appl Microbiol 106: 764–773. [Medline] [CrossRef]
15. Hussain MA, Natt KN. 2014. Comparison of changes in the cytosolic proteomes of lactose starved and acid stressed *Lactobacillus rhamnosus* cells. Am J Microbiol Biotecnol 1: 56–63.
16. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. 1999.

Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20: 3551–3567. [Medline] [CrossRef]

17. Konings WN, Lolkema JS, Bolhuis H, van Veen HW, Poolman B, Driessen AJ. 1997. The role of transport processes in survival of lactic acid bacteria. Energy transduction and multidrug resistance. Antonie van Leeuwenhoek 71: 117–128. [Medline] [CrossRef]
18. Watanabe M, Kinoshita H, Nitta M, Yukishita R, Kawai Y, Kimura K, Taketomo N, Yamazaki Y, Tateno Y, Miura K, Horii A, Kitazawa H, Saito T. 2010. Identification of a new adhesin-like protein from *Lactobacillus mucosae* ME-340 with specific affinity to the human blood group A and B antigens. J Appl Microbiol 109: 927–935. [Medline] [CrossRef]
19. Jeffery CJ. 1999. Moonlighting proteins. Trends Biochem Sci 24: 8–11. [Medline] [CrossRef]
20. Kinoshita H, Uchida H, Kawai Y, Kawasaki T, Wakahara N, Matsuo H, Watanabe M, Kitazawa H, Ohnuma S, Miura K, Horii A, Saito T. 2008. Cell surface *Lactobacillus plantarum* LA 318 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) adheres to human colonic mucin. J Appl Microbiol 104: 1667–1674. [Medline] [CrossRef]
21. Terrasse R, Amoroso A, Vernet T, Di Guilmi AM. 2015. *Streptococcus pneumoniae* GAPDH is released by cell lysis and interacts with peptidoglycan. PLoS ONE 10: e0125377. [Medline] [CrossRef]
22. Gozalbo D, Gil-Navarro I, Azorín I, Renau-Piqueras J, Martínez JP, Gil ML. 1998. The cell wall-associated glyceraldehyde-3-phosphate dehydrogenase of *Candida albicans* is also a fibronectin and laminin binding protein. Infect Immun 66: 2052–2059. [Medline]
23. Brassard J, Gottschalk M, Quessy S. 2004. Cloning and purification of the *Streptococcus suis* serotype 2 glyceraldehyde-3-phosphate dehydrogenase and its involvement as an adhesin. Vet Microbiol 102: 87–94. [Medline] [CrossRef]
24. Maeda K, Nagata H, Yamamoto Y, Tanaka M, Tanaka J, Minamino N, Shizukuishi S. 2004. Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus oralis* functions as a coadhesin for *Porphyromonas gingivalis* major fimbriae. Infect Immun 72: 1341–1348. [Medline] [CrossRef]
25. Fu Q, Wei Z, Liu X, Xiao P, Lu Z, Chen Y. 2013. Glyceraldehyde-3-phosphate dehydrogenase, an immunogenic *Streptococcus equi* ssp. *zooepidemicus* adhesion protein and protective antigen. J Microbiol Biotechnol 23: 579–585. [Medline] [CrossRef]
26. Schaumburg J, Diekmann O, Hagendorff P, Bergmann S, Rohde M, Hammerschmidt S, Jänsch L, Wehland J, Kärst U. 2004. The cell wall subproteome of *Listeria monocytogenes*. Proteomics 4: 2991–3006. [Medline] [CrossRef]
27. Nezhad M, Knight M, Britz ML. 2012. Evidence of changes in cell surface proteins during growth of *Lactobacillus casei* under acidic conditions. Food Sci Biotechnol 21: 253–260. [CrossRef]
28. Deepika G, Karunakaran E, Hurley CR, Biggs CA, Charalampopoulos D. 2012. Influence of fermentation conditions on the surface properties and adhesion of *Lactobacillus rhamnosus* GG. Microb Cell Fact 11: 116. [Medline] [CrossRef]