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Galactoglucomannan Extracted from Spruce (*Picea abies*) as a Carbohydrate Source for Probiotic Bacteria

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ABSTRACT: A prebiotic is a nonviable food component that confers a health benefit on the host associated with modulation of the microbiota. Hemicelluloses are the second most common group of polysaccharides in nature and they occur in plant cell walls. The predominant hemicellulose in softwood species is galactoglucomannan, and based on its chemical structure and information available about similar saccharides, galactoglucomannan may be postulated to have prebiotic properties. In this study we demonstrated that *Bifidobacterium* species are able to ferment hemicellulose-derived saccharides. Significant stimulatory effects on the growth rates of bifidobacteria were found when galactoglucomannan or its hydrolysis products were present. *Bifidobacterium animalis* subsp. *lactis* strain Bb12, a commonly used probiotic, was able to adapt to the galactoglucomannan leading to more efficient utilization of hemicellulose-derived saccharides. Our study demonstrates prebiotic properties for galactoglucomannan and warrants the next step, that is, characterization of the effects of galactoglucomannan in food.

KEYWORDS: *Galactoglucomannan, hemicellulose, bifidobacteria, prebiotic, softwood, adaptation, polysaccharide, Bifidobacterium lactis Bb12*

INTRODUCTION

Hemicelluloses constitute a complex group of heterogeneous polysaccharides and represent one of the major sources of renewable organic matter in nature.[1] Wood-based hemicelluloses have not been characterized and utilized as extensively as the other major components of wood, that is, cellulose and lignin. Recently, utilization of hemicellulose biomass as an alternative source for fossil fuels, precursor for microbe-fermented ethanol or other molecules have been studied,[2,3] but also some health-related more value-added purposes have been suggested.[4] It has been reported that a galactoglucomannan-containing diet given to a dog increased the concentration of fecal short-chain fatty acids and the total amount of fecal bifidobacteria, and also fecal pH was lowered similarly.[5] Recent reports suggest that in vivo produced acetic acid can reduce the amount of pathogens in the colon.[6] A recent study suggests that galactoglucomannan supplemented diet increases the total concentration of fecal *Bifidobacterium* spp.[5] Also galactoglucomannan has been recently suggested to prevent selenium-induced liver damage and fibrosis in rats when taken intragastrically.[7] Spruce-derived galactoglucomannan extract was shown to stimulate the proliferation of thymocytes in vitro suggesting potential immunomodulating properties. In addition, galactoglucomannan extract was also shown to possess a low antioxidative activity.[8]

The most common natural complex polysaccharides in food include cellulose, glycogen, pectins, and hemicelluloses. In general, plant hemicellulose polysaccharides are important natural food ingredients that are present in the cell walls of all edible plants. However, specific information about nutritional value and metabolic pathways of hemicelluloses is lacking. Slowly digestible carbohydrates are considered important for the health of the human gastrointestinal tract, through modulation of its microbiota.[9] In addition to health of gastrointestinal tract, complex poly- and oligosaccharides may affect other functions, such as energy metabolism and also modulate lipid metabolism and endocrine signaling.[10]

In softwoods like spruce (*Picea abies*) and pine (*Pinus sylvestris*) hemicelluloses consist mainly of galactoglucomannan, whereas in hardwoods xylan polysaccharides are the main type of hemicellulose present. These polysaccharides can be separated from paper mill process waters, or be extracted from wood or pulp with pressurized hot water, where they are partly hydrolyzed to oligo- and monosaccharides.[2,11] These hemicelluloses are still largely an untapped and wasted material, but they are a potential resource with health-promoting properties. Even though there has recently been a growing interest in the structure and utilization of softwood-based hemicelluloses,[12] bioactive properties of softwood-derived galactoglucomannans have not been evaluated for food applications.

Some wood-derived specialty sugars, such as xylitol and D-mannose, are already used as functional food ingredients and specific health claims have been approved, for example some xylitol products in the European Union. Diet-derived Konjac glucomannan, which has structural similarities to galactoglucomannan, was recently shown to increase the fecal concentration of both bifidobacteria and lactobacilli.[13] Based on the beneficial effects of mannose-containing saccharides from traditional food components,[14] also galactoglucomannan may have health effects when consumed as a part of the recommended daily

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diet. These components could be described as potential prebiotics. The prebiotic properties usually include an increase of beneficial modification of gut microbiota by promotion of beneficial bacteria such as bifidobacteria and displacement of pathogens.[15] Bifidogenic effects are not sufficient without demonstrated physiological health benefits. The European Food Safety Authority requires human studies demonstrating for instance antagonism against specific pathogens and/or reduction in the number of pathogens in specific populations.[16]

In this study, potential prebiotic properties of hemicellulose-derived saccharides; galactoglucomannan polysaccharide, and manno- and xylooligosaccharides, were assessed by measuring the ability of specific *Bifidobacterium*-species and *Lactobacillus rhamnosus* to utilize and ferment these saccharides as their main carbohydrate source. For the first time, we assess the impact of galactoglucomannan and galactoglucomannan derived mannan oligosaccharide on the growth of three extensively used probiotic microbes: *Bifidobacterium animalis* subsp. *lactis* Bb12 (DSM 15954), *Bifidobacterium longum* (JCM1217), and *Lactobacillus rhamnosus* GG (ATCC 53103).

MATERIALS AND METHODS

Reagents and Microbe Strains. All reagents were provided by Sigma (Sigma-Aldrich Finland, Helsinki, Finland), expect Gifu Anaerobic Medium (GAM) Broth (Nissui Pharmaceutical Co., Tokyo, Japan). *Bifidobacterium animalis* subsp. *lactis* Bb12 (DSM 15954) was originally obtained from Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). *B. longum* (JCM 1217) was originally obtained from Japan Collection of Microorganisms (JCM RIKEN BioResource Center, Saitama, Japan).

Galactoglucomannan Preparation. Galactoglucomannan polysaccharide was extracted from ground fresh spruce sapwood by pressurized hot water at 170 °C for 20 min using an ASE-300 accelerated solvent extractor (Dionex, Sunnyvale, CA). These conditions are optimal for extracting high-molar-mass galactoglucomannan in native form and in high yield.[17] The water extract was centrifuged and brown sediment removed (containing mainly lignin and lignin-related substances). High-molar-mass hemicelluloses were precipitated by adding the water extract to ethanol (99.5% purity) to a water:ethanol volume ratio of 15:85. After standing overnight the suspension was centrifuged and the supernatant decanted. The precipitate was filtered and washed with ethanol, acetone and methyl *tert*-butyl ether, and finally vacuum-dried, thus providing a white amorphous galactoglucomannan-rich preparation in a yield of 7% on original wood basis.

The molar mass determined by size-exclusion chromatography with multiangle laser light scattering technique[17] was mainly in the range of 4000 to 20 000 g/mol. The ethanol-precipitated galactoglucomannan contained also other sugars than galactose, glucose, and mannose. The sum of the three galactoglucomannan sugars analyzed by acid methanolysis followed by gas chromatography amounted to 81.6% of the total sugar units. Other polysaccharides in the fraction were arabinoglucuronoxylans (arabinose + xylose +4-O-methyl glucuronic acid, 11.9%) and pectins (rhamnose + galacturonic acid, 3.8%). The acetyl group content as the molar ratio of acetyl:mannose units was 0.50. The galactoglucomannan preparation contained also about 1% of lignin, probably mainly in the form of lignocellulose complexes. No other wood extractives were precipitated with ethanol but remained in the ethanol solution. Carbon-13 NMR verified that the preparation was very similar in composition to one extracted from spruce wood at milder conditions, i.e. 90 °C for 60 min.[11]

Mannan, Xylan and Fructan Oligosaccharides. Xylooligosaccharide was obtained from Shandong Longlive Biotechnology (Qingdao, Shandong, China) (Figure 1). Galactoglucomannan-derived mannooligosaccharide was prepared by controlled cleavage of the galactoglucomannan preparation with commercial *β-endo*-mannanase (Megazyme, Wicklow, Ireland) (Figure 1). The fermentation was done at 37 °C and pH 6.5. Fructooligosaccharide Orafti P95 was obtained from Beneo Orafti (Tienen, Belgium). It included 95% di- to octasaccharides and 5% monosaccharides.

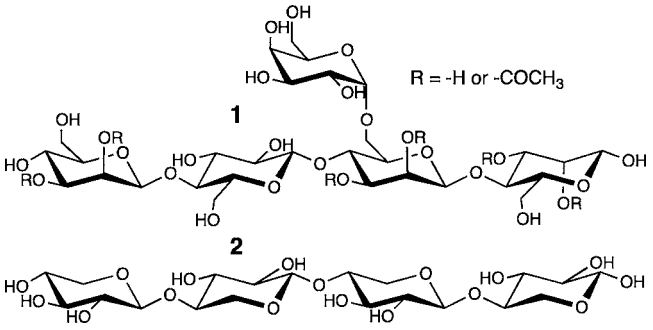

Figure 1. Representative structures of hemicellulose-derived oligosaccharides utilized in this study. Typical oligosaccharides present in mannooligosccharide (**1**) and xylooligosaccharide (**2**) preparations.

Hemicellulose-derived manno- and xylooligosaccharide were analyzed by size-exclusion chromatography (SEC) (Figure 2) and GC-MS. For SEC the preparations were acetylated with acetic acid anhydride in pyridine. A Shimadzu SEC instrument with an evaporative light scattering detector (Sedere Sedex 85 LF) was used, with two Jordi Gel DVB 550 Å columns (300 × 7.8 mm i.d.) in series in an oven at 40 °C. The eluent was tetrahydrofuran with 1% acetic acid and the flow 0.8 mL/min. Calibration was done by analysis of glucose, mannobiose, cellobiose, mannotetraose, mannopentaose, and mannohexaose for mannan oligosaccharides, and xylose and xylobiose for xylooligosaccharide. SEC showed that mannooligosaccharide contained oligomers with 2−8 sugar units, with predominance of dimers, trimers, tetramers and pentamers, whereas xylooligosaccharide contained mainly dimers and trimers (Figure 2). Both manno- and xylooligosaccharide contained less than 1% of monosaccharides. GC-MS analysis of the collected dimer fraction of mannooligosaccharide showed that it contained several individual compounds, however with mannobiose as main component.

Stock solutions of all saccharides in Milli-Q-water were prepared at a concentration of 6% (w/v). Solutions were sterilized by filtration through a 0.22 μm filter.

Carbohydrate Free Medium. Carbohydrate-free basal medium (CFBM) previously presented by Ruas-Madiedo et al.,[18] was used as basal medium for testing wood-derived galactoglucomannans as a possible carbon sources for selected bifidobacteria. The CFBM broth includes only a small amount (0.2% (w/v)) of glucose and no long-chained saccharides, but still all other nutrients needed to grow bifidobacteria. CFBM solution was supplemented with each hemicellulose-derived polysaccharide at 1% (w/v), or with 1% (w/v) glucose as a positive control, or with a similar volume of filtered Milli-Q water as a vehicle control.

Growth Assay. Single colonies of *B. lactis* Bb12, *B. longum* (JCM1217), and *L. rhamnosus* GG (ATCC 53103) were cultured for 2 days in anaerobic conditions (Anaerobic Workstation Concept 400) (Ruskinn Technology Limited, Leeds, United Kingdom) in GAM broth medium at 37 °C using 1% inoculum. The cells were concentrated by centrifugation of the broth at 4800 RCF for 10 min. After centrifuging pellet was suspended in phosphate buffered saline to remove possible sugars left from GAM broth and resuspended using 50 μL inoculum in 5 mL of CFBM at pH 6.5 and temperature 37 °C supplemented with saccharides. Samples in CFBM were incubated in an anaerobic atmosphere (10% H_2, 80% N_2, and 10% CO_2) for 3−4 days. Absorbance (λ = 600 nm) was measured daily on new samples. Results are presented as a mean value of optical densities of parallel samples (n = 6). No samples were incubated further after the absorbance reading due to contamination. Assays with *Bifidobacteria* spp. and *L. rhamnosus* were measured three times, variation being negligible.

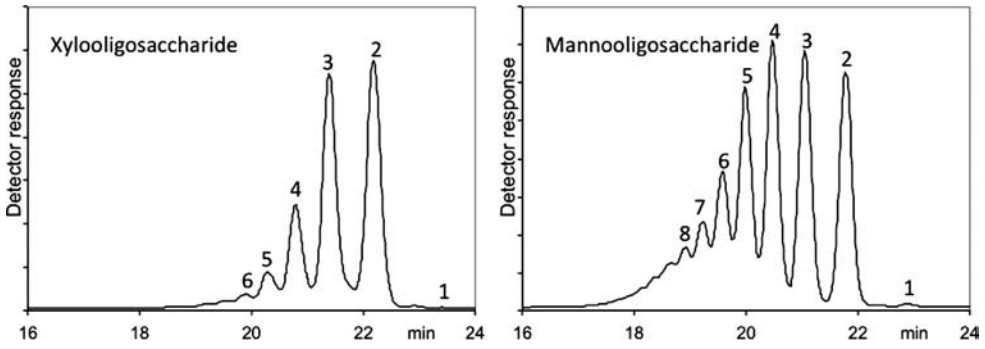

Figure 2. Size-exclusion chromatographic analysis of acetylated xylooligosaccharide and mannooligosaccharide preparations. 1: monosaccharides, 2: disaccharides, etc.

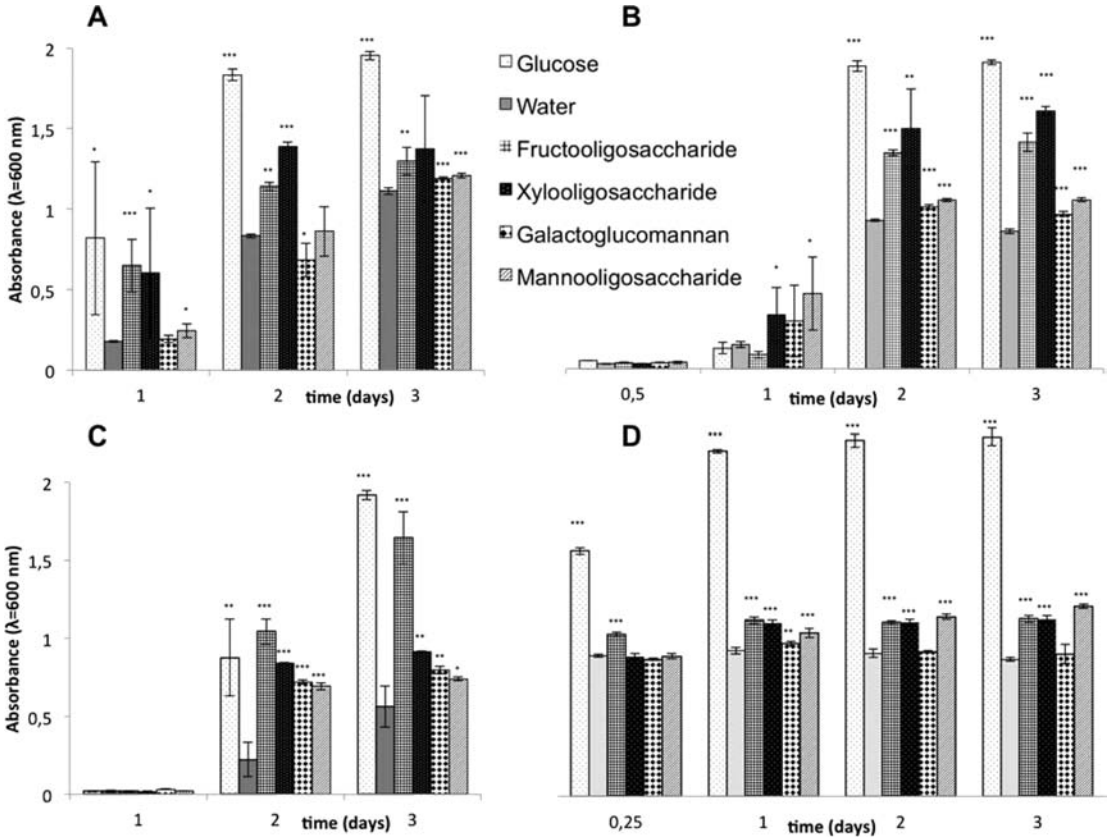

Figure 3. Growth of bacteria in carbohydrate-free basal medium with different substrates as carbohydrate sources after 0.25−3 day incubations. A. Growth of *Bifidobacteria lactis* Bb12. B. Growth of *B. lactis* Bb12 that has been adapted two weeks to galactoglucomannan-rich medium before assay. C. Growth of *B. longum* (JCM1217). D. Growth of *L. rhamnosus* GG. Bars represent the average of six replicate samples (mean ± SD) based on OD_{600} values, blank value subtracted. Glucose and four oligo- and polysaccharides are compared to negative control (water). * $P < 0.05$ vs negative control, ** $P < 0.01$ vs negative control, *** $P < 0.001$ vs negative control.

Optical density represents the relative amount of bacteria in broth medium, while plate counts were used as a control method to measure the total amount of viable bacteria present. Samples were plated on GAM agar and incubated at least for 24 h under anaerobic conditions at 37 °C. Results are presented as a mean value of plated agar dishes with 15−300 colonies on each.

In the galactoglucomannan-adaptation assay, bifidobacteria were grown 2 weeks in CFBM with 1% (w/v) galactoglucomannan preparation as the only carbohydrate source. Medium was changed every second day. After the adaptation period the growth of adapted strain with different hemicellulose-based oligosaccharides was analyzed as described earlier.

Statistical Analyses. Data are represented as mean values and standard deviations of parallel samples ($n \geq 4$). Student's *t*-test using two-tailed distribution was used to compare the growth results of bacteria with different saccharides to control values. Differences among means are considered significant at $P < 0.05$. Statistical analysis was conducted using Microsoft Excel for Mac 2011 Version 14.1 (Microsoft Corporation, Redmond, WA).

RESULTS AND DISCUSSION

The ability of gut microbes to degrade and ferment different oligo- and polysaccharides varies greatly among different species of *Bifidobacterium* genera and even among strains of the same species.[19] Hemicellulose-derived saccharides are cost-effective and natural compounds with prebiotic potential.[5,20,21] Our aim was to assess the ability of three commercially used

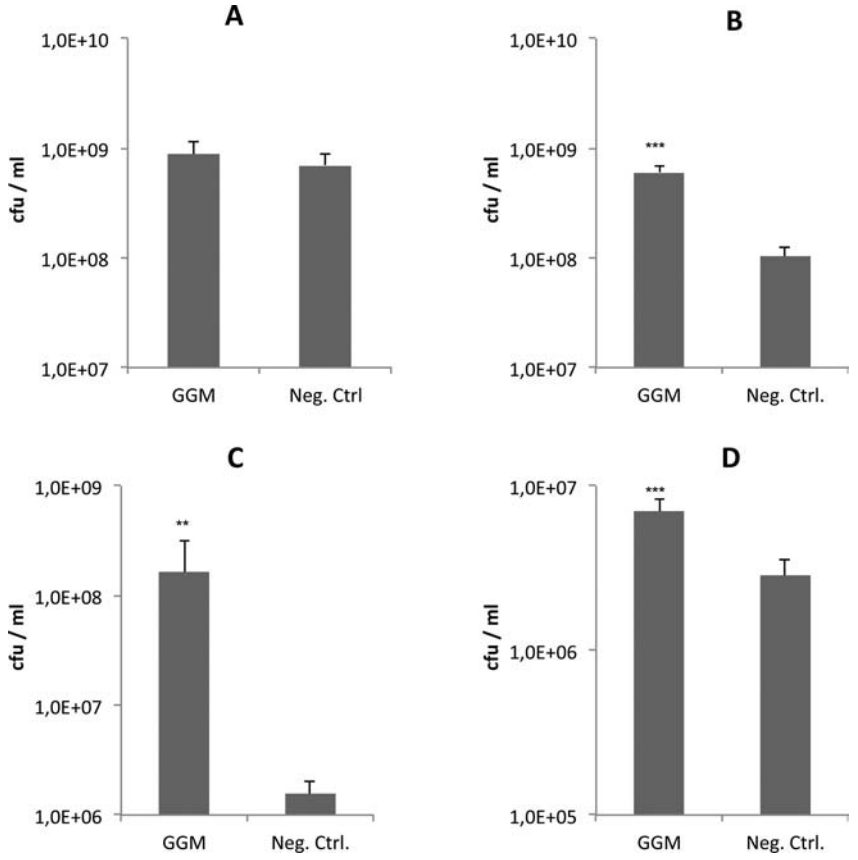

Figure 4. Concentrations of viable bacteria after three days anaerobic incubation with 1% galactoglucomannan polysaccharide (GGM) or carbohydrate-free control (Neg. Ctrl.): A. Concentration of *Bifidobacteria lactis* Bb12. B. Concentration of *B. lactis* Bb12 that has been adapted two weeks to GGM-rich medium before assay. C. Concentration of *B. longum* (JCM1217). D. Concentration of *L. rhamnosus* GG. The numbers of bacteria were measured by plate count method with 48 h anaerobic incubation on GAM agar. * $P < 0.05$ vs negative control, ** $P < 0.01$ vs negative control, *** $P < 0.001$ vs negative control.

probiotic bacteria to utilize these saccharides as their main carbohydrate source.

In this study we demonstrated that galactoglucomannan had a statistically significant positive effect on the concentration of viable *B. longum*, *B. lactis*, and *L. rhamnosus* bacteria in broth medium. The fermentation of different poly- and oligosaccharides as prebiotic candidates were studied in anaerobic in vitro growth assays, and according to daily optical density assays, galactoglucomannan polysaccharide, its derivative mannooligosaccharide, and fructo- and xylooligosaccharides affected growth rate and concentration of bacteria in anaerobic conditions (Figure 3).

Galactoglucomannan Increases Viability of Probiotic Bacteria. Our results clearly demonstrated that the addition of galactoglucomannan to growth medium increased the concentration of bifidobacteria, although there was an initial lag before the bifidobacteria caught up with enhanced growth (Figure 3A and C). The viability of the bacteria needed to be also assayed because the optical density of growth medium does not decrease notably when the amount of viable bacteria starts to decrease. And during 4-day assay it is likely that some bacteria will finally become dormant or die due to lack or depletion of substrate. Plate counts from the growth assay of *B. longum* clearly showed that while there were moderate changes in absorbance between growth days 2 and 3, the amount of viable bacteria was nearly 100 times higher in samples with galactoglucomannan preparation compared to the control (Figures 3C and 4C). This shows that bifidobacteria not only started to proliferate earlier and reached higher concentration, but they also remained viable for a longer period when there was galactoglucomannan added in medium. Also, the concentration of viable *L. rhamnosus* was significantly higher with galactoglucomannan substrate (Figure 4D). Any adverse effects of galactoglucomannan on bacterial growth were not observed.

***Bifidobacterium lactis* is Able to Adapt Galactoglucomannan Contain Medium.** Many bacterial species are known to be able to adapt to changes in the environment.[22] According to our results, the commonly used probiotic *B. lactis* Bb12 was fully adapted to galactoglucomannan-containing medium after culturing them for two weeks with galactoglucomannan as the major carbohydrate source of CFBM. Change in growth rates after the adaptation process was notable, especially in the early growth phase (Figure 3B). While adaptation to galactoglucomannan proceeded, the galactoglucomannan-adapted strain of *B. lactis* seemed to be able to benefit from mannooligosaccharide present in medium as well. The adapted bacteria strain grew, with galactoglucomannan preparation or mannooligosaccharide as a carbohydrate source, faster than with glucose added to medium. The concentration of viable *B. lactis* was also five times higher in galactoglucomannan containing medium compared to the control after the adaptation (Figures 3B). In accordance with these results, a concentration of nonadapted *B. lactis* was only slightly higher after three days incubation in

galactoglucomannan containing medium compared to control (Figure 4A). In addition, the growth rate of *B. lactis* with other oligosaccharides was also changed during galactoglucomannan-adaptation process (Figures 3A and B). Concentrations after one-day incubation were significantly different: nonadapted *B. lactis* benefit from fructo- and xylooligosaccharide and glucose, whereas the galactoglucomannan-adapted strain favored galactoglucomannan, and manno- and xylooligosaccharides. However, after three days incubation the growth results were mostly normalized, whereas utilization xylo- and fructooligosaccharide were also slightly enhanced (Figures 3A and B).

One of the possible mechanisms of galactoglucomannan-adaptation may be an increase in activity and/or concentration of enzymes needed to metabolize and break down galactoglucomannan.[23] It is notable that in case of each test compound the concentration of bacteria at late stationary phase was the same before and after adaptation (Figure 3A and B). This result supports the hypothesis that instead of selection, only enzymatic activity is changed during the adaptation process, while bacteria could again "normalize" their enzymatic activity during a few days assays.

Oligosaccharides Stimulate the Growth of *L. rhamnosus*. In contrast to *Bifidobacterium*, *Lactobacillus rhamnosus* GG started to grow quickly in CFBM with all substrates tested. Based on optical density, the concentration of *L. rhamnosus* differed significantly from the control when manno-, fructo-, or xylooligosaccharide was present in the growth medium (Figure 3D). In addition, the total concentration of viable *L. rhamnosus* was statistically higher in the stationary phase of the growth compared to the control when galactoglucomannan were present in the medium (Figure 4D). Previous results about *L. rhamnosus* strains being able to partially ferment fructooligosaccharide are compatible with our findings.[24]

CFBM medium reduced the growth rate of *Bifidobacteria* when compared to results with other tested commercial mediums like Gifu anaerobic medium broth. The beginning of the logarithmic growth phase was delayed due to potential adaptation problems of the custom-made CFBM or lack of monosaccharides. However, similar delays in beginning of exponential growth phase of *Bifidobacterium* spp. have been reported in earlier studies in carbohydrate-free medium.[18,25] Commercial growth media were not suitable, because they include different sugar monomers, especially glucose, which if present would probably prevent prebiotic effects of hemicellulose-based carbohydrate components being assayed.

Comparison of Different Saccharides. There were no major differences between any of the growth results of *Bifidobacteria* obtained for galactoglucomannan preparation and mannooligosaccharide containing medium. In all cases the growth curves were close to each other. Our results suggest galactoglucomannan preparation being a slightly more growth-inducing saccharide to *B. longum* (Figure 3C), whereas *B. lactis* seems to benefit more from oligosaccharide (Figure 3A and B). Still there was a systematic statistically significant difference between those results. *L. rhamnosus* GG benefits equally from all three oligosaccharides tested, galactoglucomannan preparation being weaker substrate than manno-, fructo- and xylooligosaccharide (Figure 3D).

Lyophilized galactoglucomannan preparation included a less than 5% (m/m) fraction that was not soluble in the CFBM broth or water and it was removed by filtration (0.22 μm filter). Also galactoglucomannan dilutions had a slightly yellowish color solution still being clear and with a mild odor caused possibly by vanillin esters.[12] All oligosaccharides were easily soluble and the dilutions were clear and odorless. This finding was, however, of no surprise since galactoglucomannan preparation is a natural extract and contains mostly polysaccharides, which have been previously reported to have a limited solubility.[26] Fructooligosaccharide and xylooligosaccharide utilized here are commercial, purified products, and mannooligosaccharide is chemically processed after actual extraction, which reduce the amount of odor causing and nonsoluble compounds. However, when comparing galactoglucomannan preparation and its derivative mannooligosaccharide, especially if large-scale commercial utilization is being considered, it has to be noted that the galactoglucomannan preparation is considerably more economical to produce compared to mannooligosaccharide. Galactoglucomannan is extracted with pressurized hot water from wood chips followed by precipitation and lyophilization; and the raw material is abundantly available. Our mannaooligosaccharide is prepared from galactoglucomannan extract and the process requires enzymatic cleavage of galactoglucomannan polysaccharides and purification of final product.

In most assays, bacteria finally reached higher concentrations in xylo- or fructooligosaccharide containing media when compared to one containing galactoglucomannan (Figure 3). One possible explanation is that remaining impurities of extracted galactoglucomannan, most notably lignin in the form of lignocellulose complexes, reduced the growth of bacteria. Also size difference between poly- and oligosaccharides could have an effect on transportation and fermentation of the compounds.[23] Fructooligosaccharide also included 5% sugar monomers and in xylooligosaccharide there were more dimers and trimers present compared to MOS (Figure 2).

The Effect of Saccharide Fermentation on Acidity. The acidic side products of fermentation of galactoglucomannan may affect pH values of in vitro assays and thereby prevent the growth of specific bacteria. This phenomenon may only be present in isolated systems such as test tubes where acid concentration may reach inhibitory levels.[25] However, in in vivo conditions this should not prevent growth. We monitored the acidity of media during the growth assays with galactoglucomannan -adapted *B. lactis* Bb12. While pH of pure CFBM broth was 6.5; addition of galactoglucomannan preparation and bacteria inoculum did not have an effect on that. After three days anaerobic incubation with Bb12, acidity of medium were increased to pH 5.4 (galactoglucomannan preparation), 5.2 (mannooligosaccharide), 4.8 (fructooligosaccharide), 4.2 (xylooligosaccharide), 4.1 (glucose) and 5.6 (water). These results support the data from our absorbance assays, whereas those samples having the highest absorbance reading also had the lowest pH value. This suggests metabolic activity being related to the amount of bacteria as expected.[23] These results also suggest that decreased acidity in in vitro conditions was not a factor preventing growth in our test system.

Prebiotic Potential of Galactoglucomannan. Our results clearly demonstrate that *Bifidobacterium* spp. are able to directly utilize galactoglucomannan saccharides present in their growth mediums. The growth of the bacteria was faster and also the maximum concentration of viable microbes present in medium was higher when galactoglucomannan was present. Our results are in accordance with previous data about number of bifidobacteria being increased in vivo with a diet derived galactoglucomannan substrate and in fecal medium in

vitro.[5,27] These results suggest that galactoglucomannan has potential as a prebiotic product or a component for synbiotics.

The growth results with galactoglucomannan are comparable to those measured when already recognized prebiotics, such as fructooligosaccharide, were present in the growth medium. An assessment of the components as potential ingredients in foods needs to be completed. For the first assessment of safety, information on the history of use of galactoglucomannan and similar components is needed.

Taken together, the results suggest a significant stimulatory effect on the growth rate and concentration of *B. lactis* Bb12, *B. longum*, and *L. rhamnosus* GG when galactoglucomannans are present in growth mediums. These bacteria, which are among the most common used probiotics, started to grow faster, reached higher concentrations and also stayed viable for longer periods. Hemicellulose is an untapped natural resource, which can be produced economically in large quantities. Therefore, the study of probiotics and hemicellulose together could also have potential for synbiotic formulations.

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Notes

The authors declare no competing financial interest.

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