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In vitro fermentation of xylooligosaccharides from wheat bran insoluble dietary fiber by *Bifidobacteria*

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ABSTRACT

The utilization of xylooligosaccharides (XOS) from wheat bran dietary fiber by four *Bifidobacterium* strains (*Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Bifidobacterium bifidum* and *Bifidobacterium breve*) was investigated. Among the tested strains, *B. adolescentis* displayed the highest growth on XOS, whereas *B. breve* showed no growth. Further, the *in vitro* kinetic analysis of fermentation of XOS by *B. adolescentis* was performed. The maximum value of biomass yield and the lowest pH value of the culture broth were arrived at 7.22 mg/mL and 5.1, respectively, after *in vitro* fermentation for 24 h. The consumption of XOS by *B. adolescentis* reached 70.4% after 24 h, which preferred XOS with a degree of polymerization (DP) of 3 with 88% of percentage consumption, followed by DP 2, and DP 4–7. The total production of short-chain fatty acids, predominately acetate, reached 7.47 mg/mL in the fermentation of XOS.

1. Introduction

Whole grains of cereals are a rich source of fermentable carbohydrates including dietary fiber, resistant starch and non-digestible oligosaccharides. Dietary fiber is concentrated in the outer layers of the grain, thus wheat bran can contain 45–50% of fiber (Hollmann & Lindhauer, 2005). Wheat bran as an important by-product of conventional milling is produced worldwide in enormous quantities and recognized as a good source of dietary fiber. Bran comprises the outer tissues of the wheat kernel and includes botanically distinct tissues of the pericarp (fruit coat), testa (seed coat), the hyaline layer and the aleurone layer, which is part of the endosperm (Evers & Millar, 2002). The pericarp testa is constituted of lignified cell walls, where cellulose microfibrils are dispersed in acidic arabinoxylans represent about half the dietary fibers component of wheat bran or wheat aleurone whereas cellulose and β-glucans contributed only smaller parts (Neyrinck et al., 2008).

The potential health benefits of cereal dietary fiber include reduced risk of coronary heart disease and colorectal and breast cancers. Many of the health effects are believed to be related to the microbial fermentation of dietary fiber in the large intestine (Karppinen, Liukkonen, Aura, Forssell, & Poutanen, 2000). The human intestinal microflora, a complex bacterial ecosystem, is composed of more than 400 bacterial species. This microflora metabolizes non-digested dietary carbohydrates, such as resistant starch, non-starch polysaccharides, non-digestible oligosaccharides, to a variety of products such as short-chain fatty acids (e.g., acetic acid, propionic acid, and butyric acid), other organic acids (e.g., lactic acid, succinic acid, and pyruvic acid), and gases (e.g., H_2, H_2S, CO_2, and CH_4). Bifidobacteria are a predominant group of the colonic microflora that can account for up to 25% of the total number of bacteria present (Pedreschi, Campos, Noratto, Chirinos, & Cisneros-zevallos, 2003). Due to their heterofermentative nature, bifidobacteria can produce short-chain fatty acid (SCFA) as the end product of oligosaccharide fermentation. The shift in intestinal pH induced by the acidic metabolites during carbohydrate fermentation inhibits the growth of undesirable, potentially pathogenic bacteria. Bifidobacteria can also confer several other benefits on their host, such as vitamin production, cholesterol lowering effects, immunostimulating effects, anticarcinogenic activity, a decrease of intestinal transit time, and protection against infections (Van der Meulen, Avonts, & Vuyst, 2004). Many attempts have been made to increase the number of bifidobacteria in the intestinal tract by supplying certain probiotics strains and prebiotics that stimulate the growth of bifidobacteria, which will positively affect human health.

Most of the currently known or suggested prebiotics in the functional food industry are non-digestible oligosaccharides such as lactulose, fructo-oligosaccharides, galacto-oligosaccharides, soybean oligosaccharides, lactosucrose, isomalto-oligosaccharides,

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gluco-oligosaccharides, xylooligosaccharides (XOS) or palatinose during the last few decades, and their industrial applications as functional food ingredients are continuously increasing (Mussatto & Mancilha, 2007). Recently, XOS released by enzymatically degrading wheat arabinoxylans have been proposed as alternative prebiotics (Hughes et al., 2007; Grootaert et al., 2007). XOS with different average degrees of polymerization and average degrees of substitution, which are fermented in different regions of gastrointestinal tract, may present different prebiotic properties, and long-term supplementation with these oligosaccharides increased the concentration of beneficial SCFA such as butyric and propionic acids in all colon vessels (Sanchez et al., 2009). Therefore, XOS substituted with arabinose can be fermented by intestinal bacteria in a way that brings about positive effects for human health, which can be produced with enzyme from cereal arabinoxylans (Rantanen et al., 2007). Wheat bran is hemicellulose-rich substrates, among which xylan represents 40% of dry matter (Thiago & Kellaway, 1982). Xylans consist of a linear backbone of β-(1 → 4) linked D-xylopyranosyl residues containing individual α-L-arabinofuranosyl residues attached through O-2 and/or O-3 (Izydorczyk & Biliaderis, 1993). The xylan backbone can be hydrolyzed randomly by endoxylanases (endo-1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8). Endoxylanases attack the xylan main chain internally in a random manner to release a mixture of various arabinoxylan-oligosaccharides. It has been previously reported that wheat bran insoluble dietary fiber was hydrolyzated by commercial xylanase preparation Sunzymes to release XOS with a degree of polymerization (DP) of 2–7 and the ratio of arabinose to xylose of 0.27 (Wang, Sun, Cao, Tian, & Wang, 2009).

To the best of our knowledge, XOS substituted with arabinose as novel oligosaccharides from wheat bran insoluble dietary fiber were rarely studied for the prebiotic effectiveness. The main objective of this study was to investigate the capability of XOS from wheat bran insoluble dietary fiber to support the *in vitro* growth of bifidobacteria. Four common strains, *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium breve*, and *Bifidobacterium bifidum* were used to perform fermentation with culture media containing the XOS mixtures. Their growth was tested by determining the maximum optical density and the lowest pH value. Further, the strain of *B. adolescentis* was grown on the XOS to estimate some kinetic parameters for growth and product formation.

2. Materials and methods

2.1. Materials

Wheat bran was obtained from Beijing Gongdeli Flour Factory (Beijing, The People's Republic of China). The bran was milled and passed through a 0.5 mm sieve. Sunzymes, which contains the glycoside hydrolase 10 family endo-1,4-β-xylanase from *Bacillus subtilis*, was obtained from Sunhy Biology Co., Ltd., Wuhan, China. Heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1 from *Bacillus licheniformis*, 120 KNU/g), protease Alcalase 2.4 L (EC 3.4.21.62, from *B. licheniformis*, 2.4 AU/g), and amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 AGU/g) were from Novo Nordisk (Bagsvaerd, Denmark). Amberlite XAD-2 was obtained from Rohm and Haas Company (Philadelphia, U.S.A.). Arabinose, D(+)-xylose (Sigma chemical Co.), 1,4-β-D-xylobiose, 1,4-β-D-xylotriose, 1,4-β-D-xylotetraose, 1,4-β-D-xylopentaose, 1,4-β-D-xylohexaose (Megazyme, Bray, Ireland) were used as carbohydrate standards. Acetic, butyric, lactic, and propionic acids were obtained from Sigma Chemical Co. All other chemicals and solvents in this study used were of analytical grade.

2.2. Preparation of XOS

The preparation of XOS from wheat bran insoluble dietary fiber was based on a procedure described previously (Wang et al., 2009). Briefly, wheat bran (100 g) was autoclaved to destroy the activities of endogenous cell wall-degrading enzymes (e.g., endogenous arabinoxylanase) and subsequently swollen in a 2 L beaker with 1 L water at 60 °C for 6 h. After that, 7.5 mL α-amylase was added to the suspension, and then was heated in a boiling water bath for 40 min with continuous stirring. After treatment with α-amylase, the pH of the suspension was adjusted to 7.5 with 275 mM NaOH, and 3.0 mL protease was then added to the samples. After incubation at 60 °C for 30 min, the pH of the suspension was acidified with 325 mM HCl to 4.5. Then, 3.5 mL amyloglucosidase was added and the mixture was incubated at 60 °C for 30 min with continuous agitation. The suspension was centrifuged at 10,000 × *g* for 10 min, and the residue was washed twice with hot water and cold water, respectively until no cloudiness was evident. The washed residue was dried at 40 °C overnight in a vacuum oven to obtain wheat bran insoluble dietary fiber (WBIDF). Ten grams of WBIDF were incubated in 200 mL of 0.5% (w/v) Sunzymes (in 50 mM acetate buffer at pH 5.0) at 50 °C in the dark for 16 h with constant stirring. After heat inactivation of the enzyme at 100 °C for 10 min, the hydrolysate was centrifuged at 10,000 × *g* for 20 min, and the supernatant solution was passed through a 0.45 μm filter. The filtrate was concentrated to 100 mL by rotary evaporation. The concentrated solution was applied to an open column (80 cm × 2.5 cm i.d.) packed with Amberlite XAD-2 (previously washed with 95% (v/v) ethanol and then water). Elution was successively carried out with four column volumes of distilled water. The eluted fraction was concentrated and lyophilized with a freeze dry system (AlPHA1-4, Christ, Germany) to get XOS.

2.3. Organism and culture conditions

B. adolescentis, *B. longum*, *B. bifidum* and *B. breve* used in this study were obtained from China Center of Industrial Culture Collection. The bacteria were maintained at −80 °C as glycerol stock solutions. The bacteria were thawed and revived by culturing in TPY broth (Scardovi, 1986) which contained, in gram per liter: trypticase, 10; phytone, 5; glucose, 5; Tween 80, 1; yeast extract, 2.5; cysteine, 0.5; dipotassium phosphate, 2; magnesium chloride hexahydrate, 0.5; zinc sulphate heptahydrate, 0.25; calcium chloride, 0.15; ferric chloride, trace, and the final pH of the medium was adjusted to 6.8 ± 0.1, using 4 M NaOH or HCl. The strains were incubated in the broth at 37 °C in an anaerobic atmosphere of 5% CO_2, 10% H_2 and 85% N_2 (YQX-1 type anaerobic incubator, Yuejing Medical Instrument Co., Ltd., Shanghai, China), and transferred weekly.

2.4. Growth experiments

The growth of *B. adolescentis*, *B. longum*, *B. bifidum* and *B. breve* on XOS was monitored by measuring the highest optical density (OD) at 600 nm and the lowest pH value throughout the fermentation. Cells were cultured anaerobically in 10 mL TPY for 48 h at 37 °C, then inoculated (5%, v/v) into each tube of TPY containing 5.0 mg/mL of XOS instead of glucose as the sole carbon source. Nutrient base medium with no carbohydrate solution was used as control. Each strain was anaerobically grown on XOS or the control in triplicate. The OD of the tubes was measured at 0, 2, 4, 6, 8, 24 and 48 h post-inoculation at 600 nm using a UV-1000 UV/VIS Recording Spectrophotometer (Rayleigh Analytical Instruments, Beijing, China).

2.5. In vitro kinetic analysis of fermentation of XOS by B. adolescentis

2.5.1. Analysis of growth characteristics

The strain was inoculated (5%, v/v) into the culture tube of TPY containing 5.0 mg/mL of XOS as the sole carbon source. Three replicates were prepared for each combination of strain/carbohydrate/fermentation time. Fermentations were anaerobically carried out at 37 °C under static conditions. Growth characteristics were monitored by measuring pH and the biomass dry weight (DW) of culture broth at the specific intervals. To determine the DW, the cells contained in 10 mL of fermentation broth were filtered onto preweighed cellulose nitrate membrane fibers, washed with distilled water, dried at 105 °C for constant weight. The pH of the samples was measured by potentiometric methods.

2.5.2. Determination of XOS and fermentation products in the cell-free supernatants

One milliliter samples taken from the culture tubes inoculated with *B. adolescentis* at specific intervals were centrifuged at 15,000 × *g* for 5 min to remove bacteria and particulate matter. XOS in the supernatant of samples were determined on a Water 600 system high performance liquid chromatography (HPLC) using a Sugar-Pak^{TM}1 column (300 mm × 6.5 mm i.d.). The column was maintained at 85 °C. The detector signal was electronically monitored with a Waters 2401 refractive index detector. A sample volume of 10 μL was run at a flow rate of 0.5 mL/min with water as mobile phase. Arabinose, xylose, xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose were used as standards for XOS analysis.

SCFA analysis was carried out using HPLC with an ion-exclusion Bio-Rad Aminex HPX-87H (7.8 mm × 300 mm). The supernatant (20 μL) was then injected onto a Water 600 system HPLC system equipped with a refractive index detector and automatic injector. The HPX-87H column was maintained at 50 °C. The eluent was 5 mM sulphuric acid in HPLC-grade water, and the flow rate was 0.6 mL/min. Quantification of the organic acids in the samples was carried out by using external calibration curves of acetic, propionic, butyric, and lactic acids, and the results expressed in mg/mL. The experiments were repeated in triplicate.

2.6. Statistical analysis

Where differences in the treatments were found, a two-sample Student's *t*-test was carried out between the samples. The differences were considered significant when the *P* value was <0.05.

3. Results and discussion

3.1. In vitro utilization of XOS by Bifidobacterium species

Bifidobacteria are gram-positive, saccharolytic anaerobes, which may comprise as much as 25% of the cultivable gut microflora, with *B. adolescentis* and *B. longum* predominating in adults and *B. bifidum* and *B. breve* in infants (Hopkins, Cummings, & Macfarlane, 1998; Ward, Niñonuevo, Mills, Lebrilla, & German, 2007). They obtain carbon and energy through fermentation of host and dietary carbohydrates. Bifidobacteria catabolize a variety of mono- and oligosaccharides released by glycosyl hydrolases acting on non-digestible plan polysaccharides or host-derived glycoproteins and glycoconjugates. XOS are reported to be selectively and preferentially fermented by bifidobacteria, which include non-substituted XOS and XOS substituted with arabinose or acetyl groups and/or 4-*O*-methyglucuronic acid, however, fermentability studies of XOS were mainly performed using linear and low molecular weight XOS with DP 2–4 (Kabel, Kortenoeven, Schols, & Voragen, 2002). In this paper, the ability to ferment XOS from wheat bran insoluble dietary fiber as sole carbon sources in the medium by *B. adolescentis*, *B. longum*, *B. bifidum* and *B. breve* was examined. In our previous study it was demonstrated that the DP of the tested XOS was 2–7 with a ratio of arabinose to xylose of 0.27 (Wang et al., 2009). The maximum optical densities and the lowest pH value obtained for the four strains on XOS are shown in Fig. 1. With the exception of *B. breve*, the other three tested strains displayed growth on XOS, and the amount of growth displayed was variable and depended on the strain. An increase in OD of the culture broth and decrease in the pH were observed for the tested strains grown on XOS. *B. adolescentis* displayed the highest growth on XOS with the maximum OD of 1.6827 ± 0.0478 and the lowest pH value of 5.1, followed by *B. longum* and *B. bifidum*, however, *B. breve* did not show any growth on XOS compared to the control. Crittenden et al. (2002) reported that the four tested strains were able to grow to high yields using XOS, which is specified to contain approximately 70% dry weight β-xyl-(1-4)-oligosaccharides ranging in size from DP 2–5, and *B. longum* was able to grow well using rye arabinoxylan as the sole carbon source, however, *B. adolescentis* displayed slight growth on the arabinoxylan. Pastel, Westermann, Meyer, Tuomainen, and Tenkanen (2009) reported that *B. adolescentis*, *B. longum*, and *B. breve* displayed clearly different growth characteristics when they were cultivated on a commercial XOS mixture and arabinoxylan hydrolysates. The commercial XOS mixture supported only the growth of *B. adolescenti*, whereas both *B. adolescentis* and *B. longum* showed low but clear growth on arabinoxylan hydrolysates, while *B. breve* was able to grow slowly on arabinoxylan hydrolysates. The growth of a microorganism on a particular oligosaccharide may be strain specific, which was due to the differences in the transport systems of oligosaccharides for different bifidobacteria (Holt, Miller-Fosmore, & Cô té, 2005). Van Craeyveld et al. (2008) reported that structurally different wheat-derived XOS have different prebiotic and fermentation properties in rats, a XOS preparation with an average degree of arabinose substitution of 0.27 exhibited the best combination of desirable effects on gut health characteristics.

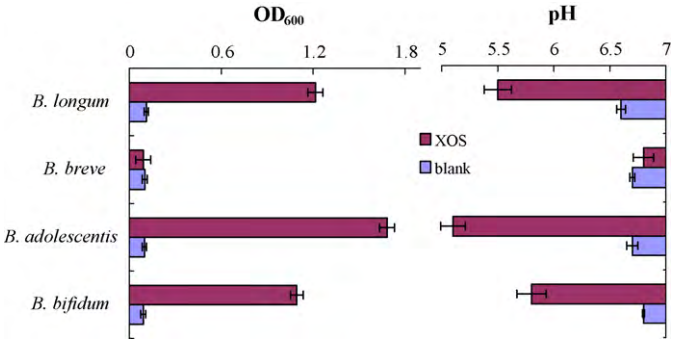

Fig. 1. The maximum OD_{600} and pH values were obtained by four *Bifidobacterium* strains grown on XOS for 48 h (data were the mean values of three replicate determinations).

3.2. In vitro kinetic analysis of fermentation of XOS by B. adolescentis

Due to the highest growth of *B. adolescentis* on XOS among the four tested bifidobacteria, which is a major bifidobacterial species in the adult intestinal microflora (Van Laere, Abee, Schols, Beldman, & Voragen, 2000), the *in vitro* kinetics of the strain growing on XOS from wheat bran insoluble dietary fiber were further performed in this paper. The kinetics of bacterial growth, carbohydrate consumption, and metabolite production were analyzed in detail. The time course of growth of *B. adolescentis* on XOS is shown in Fig. 2.

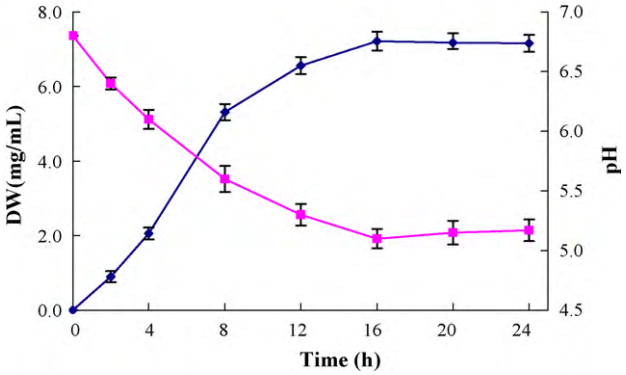

Fig. 2. The time course of growth of *B. adolescentis* on XOS for 24 h (data were the mean values of three replicate determinations). Symbols: (♦) biomass dry weight; (■) pH.

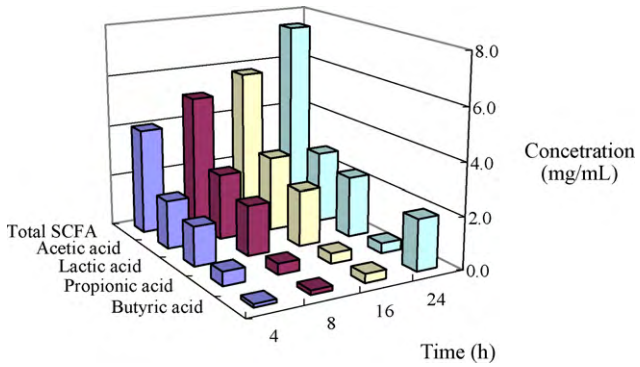

Fig. 4. The time course of the production of SCFA during *in vitro* fermentation of XOS by *B. adolescentis* for 24 h (data were the mean values of three replicate determinations).

A significant increase in biomass dry weight and a decrease in pH were observed throughout the fermentation. The biomass dry weight increased rapidly and reached 7.22 mg/mL at 16 h of post-inoculation, similarly, the pH value of the culture broth decreased rapidly and reached 5.1 from the beginning ($P < 0.05$). After 2 h of *in vitro* fermentation a significant increase of biomass dry weight with a small decrease of pH was observed ($P < 0.05$). After 16 h, the biomass dry weight and the pH value were shown no increase or decrease. The maximum specific growth rate of *B. adolescentis* (μ, determined by regression of the experimental data) during growth on XOS was $0.29 \pm 0.01\ h^{-1}$. It has been reported that *B. adolescentis* reached the stationary phase after approximately 7.5 and 12 h of fermentation, respectively when it was inoculated with the media containing XOS as sole carbon source, which was obtained from corn cobs and rice husks autohydrolysis (Gullón et al., 2008; Moura et al., 2007).

The time course of degradation of XOS during *in vitro* fermentation with *B. adolescentis* is shown in Fig. 3. The concentration profiles of various DP of XOS in the fermentation with *B. adolescentis* were demonstrated the carbohydrate consumption. The percentages of total XOS consumption were 13.0% after 4 h and 70.4% after 24 h. The consumption of XOS coincided with the increase of the biomass dry weight. A significant decrease of the concentration of XOS with DP 3, 2, 5 and 6 after 16 h of fermentation. The highest percentage of utilization corresponding to DP 3 (88.0%), followed by DP 2 (79.4%), DP 4 (75.0%), DP 5 (73.5%), DP 6 (59.5%), DP 7 (50.9%) at 24 h post-inoculation. However, free monosaccharide with an initial concentration of 0.25 mg/mL accumulated up to 0.33 mg/mL. Although the consumption of low and intermediate-DP oligosaccharides by bacteria and their generation from XOS of higher DP existed during the fermentation, it could be concluded that the utilization kinetics was faster for oligosaccharides with DP 2 and 3. The high consumption of XOS with DP 2 and 3 indicates that these oligosaccharides are the preferred substrates of *B. adolescentis*. Oligosaccharides with DP 4–7 were also suitable carbon sources. The results are in agreement with the findings of Gullón et al. (2008) in their investigation with rice husks-derived XOS.

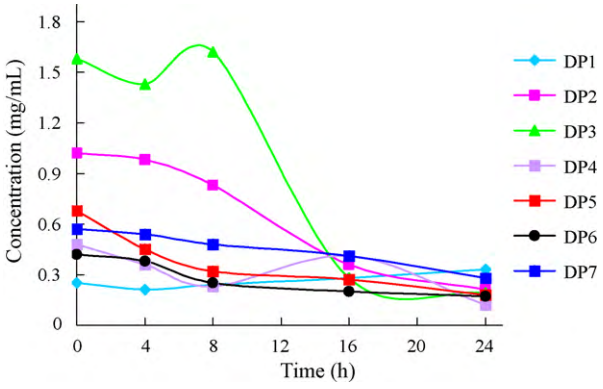

Fig. 3. The time course of the degradation of XOS during *in vitro* fermentation of XOS by *B. adolescentis* for 24 h (data were the mean values of three replicate determinations).

The time course of production of SCFA during in vitro fermentation of XOS by *B. adolescentis* is shown in Fig. 4. It was observed that the monitored metabolites in this study were found in the order acetic acid (2.76 mg/L) > lactic acid (2.34 mg/L) > butyric acid (1.98 mg/L) > propionic acid (0.39 mg/L) at 24 h post-inoculation, and the concentration of total SCFA reached 7.47 mg/mL in the fermentation of XOS. The production of SCFA results in the decrease of pH value of the culture broth. The concentration of total SCFA was 4.05 mg/mL after 4 h of fermentation while the pH value decreased from 6.8 to 6.1. During the first 16 h of the fermentation of XOS, acetic and lactic acids were formed rapidly, while the butyric acid was formed slowly, and the concentration of propionic acid remained constant or even decreased slightly, furthermore, the pH value of the culture broth decreased with the increase of the concentration of total SCFA. The production of SCFA coincided with the consumption of the XOS.

Bifidobacterium sp. has different preferences to ferment polysaccharides and/or oligosaccharides. In this study, *B. adolescentis*, *B. longum* and *B. fifidum* have the ability to ferment XOS from wheat bran dietary fiber, whereas *B. breve* is not able to ferment the oligosaccharides. The growth of *bifidobacteria* is linked to its ability to produce proteins, such as extra- and intracellular hydrolytic enzymes, as well as mono- and oligosaccharide transporters involved in the metabolism of non-digestive carbohydrates. For *B. adolescentis* a whole set of enzymes is described able to degrade the arabinoxylan-derived oligosaccharides completely into their monomeric sugars. Two different arabinofuranosidases (AXH-d3 and AXH-m23) were purified from a cell-extract of *B. adolescentis* DSM20083. Both enzymes were very specific in their mode of action. AXH-d3 hydrolyzed only C-3 linked arabinofuranosyl residues of doubly substituted xylopyranosyl residues of arabinoxylans and arabinoxylan-derived oligosaccharides. AXH-m23 released only arabinosyl units that were linked to the C-2 or C-3 position of single substituted xylose residues in arabinoxylan oligomers. Besides AXH-d3 and AXH-m23, a β-xylosidase activity towards linear XOS is produced by *B. adolescentis* DSM20083, enabling complete degradation of the branched oligosaccharides to monosaccharides (Van den Broek, Hinz, Beldman, Vincken, & Voragen, 2008). The *in vitro* kinetics *of B. adolescentis* growing on XOS from wheat bran insoluble dietary fiber also showed that

the strain preferred XOS with DP 2 and 3, and the concentration of the free monosaccharide displayed a slight increase. The preference of *B. adolescentis* for di- and oligosaccharides as compared with monosaccharides was also noted in previous studies (Crittenden et al., 2002; Palframan, Gibson, & Rastall, 2003). This special ability might be ascribed to the coexistence of two ways for oligosaccharide utilization. First, small oligosaccharides are transported into the interior of the cell where they were further hydrolyzed to monosaccharides by intracellular enzymes. Second, the oligosaccharides are hydrolyzed by extracellular enzymes and the monosaccharides formed are further transported into the cell (Pastel et al., 2009).

4. Conclusions

The *in vitro* studies carried out on *B. adolescentis*, *B. longum*, *B. bifidum* and *B. breve* suggest the prebiotic nature of XOS from wheat bran dietary fiber. Among the tested strains, *B. adolescentis* displayed the highest growth on XOS, whereas *B. breve* showed no growth. Further, the *in vitro* kinetic analysis of fermentation of XOS by *B. adolescentis* indicated that the consumption of XOS resulted in a significant increase of biomass dry weight and a significant decrease of pH in the culture broth due to the production of SCFA. *B. adolescentis* preferred XOS with DP 2 and 3, and the concentration of the free monosaccharide displayed a slight increase throughout fermentation. The *in vivo* stimulation of bifidobacteria by XOS will be further investigated.

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