

# Expression of four $\beta$ -galactosidases from *Bifidobacterium bifidum* NCIMB41171 and their contribution on the hydrolysis and synthesis of galactooligosaccharides

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**Abstract** This paper deals with two aspects tightly related to the enzymatic characteristics and expression of four  $\beta$ -galactosidases (BbgI, BbgII, BbgIII and BbgIV) from *Bifidobacterium bifidum* NCIMB41171. The growth patterns of this strain indicated a preference towards complex (i.e. lactose, galactooligosaccharides (GOSs)) rather than simple carbohydrates (i.e. glucose and galactose) and a collaborative action and synergistic relation of more than one  $\beta$ -galactosidase isoenzyme for either lactose or GOS hydrolysis and subsequent assimilation. Native polyacrylamide gel electrophoresis analysis of protein extracts from cells growing on different carbohydrates (i.e. glucose, lactose or GOS) indicated that two lactose hydrolysing enzymes (BbgI and BbgIII) and one GOS hydrolysing enzyme (BbgII) were constitutively expressed, whereas a fourth lactose hydrolysing enzyme (BbgIV) was induced in the presence of lactose or different GOS fractions. Furthermore, the  $\beta$ -galactosidase expression profiles of *B. bifidum* cells and the transgalactosylating properties of each individual isoenzyme, with lactose as substrate, clearly indicated that mainly three isoenzymes (BbgI, BbgIII and BbgIV) are implicated in GOS synthesis when whole *B. bifidum* cells are utilised. Two of the isoenzymes (BbgI and BbgIV) proved to have better transgalactosylating properties giving yields ranging from 42% to 47% whereas the rest (BbgI and BbgIII) showed lower yields (15% and 29%, respectively).

**Keywords** *Bifidobacterium bifidum* ·  $\beta$ -Galactosidase · Prebiotic

## Introduction

The gastrointestinal tract is a natural habitat for a large and diverse microbial ecosystem (Tannock 2002). Many species of bacteria have evolved and adapted to live in the human intestine and constitute a factor in both human health and disease (Guarner and Malagelada 2003). Bifidobacteria are an important inhabitant of this ecosystem (Biavati and Mattarelli 2001), playing a beneficial role in human health either by providing metabolic products useful to the host or as a crucial line of defence against colonisation by potentially harmful microorganisms (Leahy et al. 2005).

Even from the early steps of research, bifidobacteria have been identified as saccharolytic organisms (Bezkorovainy and Miller-Catchpole 1989). Today, complete or still ongoing genome sequencing projects of some bifidobacteria species (Schell et al. 2002; Suzuki et al. 2006) and the isolation and characterisation of many glycosidases from them (Hung et al. 2001; Katayama et al. 2004; Kitaoka et al. 2005; Fujita et al. 2005) clearly show their saccharolytic capabilities and metabolic preferences. Galactose-based carbohydrates are a major group of sugars present in many food products (milk) and/or additives (prebiotics) that can reach the colon and enhance bifidobacteria over other microorganisms (Macfarlane et al. 2008). The extensive variety of  $\beta$ -galactosidases identified in bifidobacteria justifies the importance of these enzymes for survival of bifidobacteria in the colonic environment. The normal action of  $\beta$ -galactosidases is to liberate galactose moieties from galactosides and fuel metabolic pathways with the necessary energy and carbon source. Therefore, character-

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isation of these enzymes with respect to their substrate preferences is of great interest in order to understand bifidobacterial physiology.

Moreover, characterisation of the synthetic capabilities of bifidobacterial  $\beta$ -galactosidases is of interest for prebiotic applications. It is now established that the modulation of the gut microecology towards a potentially healthier gastrointestinal tract can be achieved by different dietary approaches such as ingestion of probiotics, prebiotics or synbiotics (Collins and Gibson 1999). Amongst them, prebiotics have gained increased interest due to their technological properties and the selective stimulation that they exert on indigenous flora (Gibson and Roberfroid 1995). Although chemical synthesis of prebiotics is possible, the enzymatic approach is more attractive due to the need for less reaction steps (Crout and Vic 1998).  $\beta$ -Galactosidases are a group of enzymes currently utilised for galactooligosaccharide synthesis. They act by cleaving the  $\beta$ -glycosidic bond of  $\beta$ -galactosides to form a galactosyl–enzyme complex with simultaneous liberation of glucose or other simpler moieties depending on the carbohydrate used as substrate. This galactosyl–enzyme complex reacts with various nucleophiles (acceptors). Water is the dominating nucleophile in dilute aqueous solutions but carbohydrates in the reaction mixture can also act as nucleophiles resulting in a transferase reaction and hence oligosaccharides formation (Prenosil et al. 1987; Hansson and Adlercreutz 2001).

In previous studies, we demonstrated the existence of four different  $\beta$ -galactosidase isoenzymes from a GOS producing *Bifidobacterium bifidum* NCIMB41171, which were cloned, isolated and characterised with respect to their biochemical properties and substrate preferences (Tzortzis et al. 2005; Goulas et al. 2007b, 2009). The present work extends these studies to reveal the participation and synergistic relation of those isoenzymes during GOS assimilation in growth cultures of *B. bifidum* or GOS synthesis from lactose when the  $\beta$ -galactosidase activity of whole *B. bifidum* cells are utilised.

## Materials and methods

**Chemicals and growth media** All chemicals were purchased from Sigma (Dorset, UK) and were of the highest purity available unless otherwise stated. Bacteriological nutrient media were purchased from Oxoid (Basingstoke, UK).

**GOS fractionation** The GOS mixture (produced as described before Tzortzis et al. 2005; Goulas et al. 2007a) was separated in different fractions by gel filtration chromatography on a Biogel P2 column (1×5 cm; Bio-Rad, UK) maintained at room temperature and using water as

mobile phase at 2 ml min<sup>-1</sup> flow rate. Different fractions were collected with degree of polymerisation (DP) of 2, 3 and 4. The carbohydrate content was analysed by high performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD).

**Bacterial strains, culture conditions and carbohydrate fermentation** Modified tryptone peptone yeast extract (TPY) broth (Scardovi 1986; tryptone 10 g l<sup>-1</sup>; soya peptone 0.5 g l<sup>-1</sup>; yeast extract 2.5 g l<sup>-1</sup>; L-cysteine 0.5 g l<sup>-1</sup>; Tween 80 1 g l<sup>-1</sup>; NaCl 0.5 g l<sup>-1</sup>; K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O 2.6 g l<sup>-1</sup>; MgCl<sub>2</sub>·6H<sub>2</sub>O 0.5 g l<sup>-1</sup>; ZnSO<sub>4</sub>·7H<sub>2</sub>O 0.25 g l<sup>-1</sup>; FeCl<sub>3</sub> 0.01 g l<sup>-1</sup>; CaCl<sub>2</sub>·2H<sub>2</sub>O 0.2 g l<sup>-1</sup>; pH 6.0 or 6.8 was adjusted with HCl) was used for all the fermentation experiments. TPY broth after autoclaving was supplemented with 0.5% (w/v) of filter-sterilised carbohydrate solution (glucose, galactose, lactose or different GOS fractions). *B. bifidum* NCIMB41171 cells were grown anaerobically (10:10:80; H<sub>2</sub>/CO<sub>2</sub>/N<sub>2</sub>) in 10 ml Hungate tubes containing TPY broth at 37°C for 14 h with shaking at 150 rpm min<sup>-1</sup>. In separate experiments, *B. bifidum* cells were inoculated into 100 ml of TPY broth and grown in the presence of glucose, lactose or different GOS fractions, under the above-described conditions. When the cells reached an optical density of ≈0.3 (beginning to middle log phase), they were harvested by centrifugation (5,000×g for 20 min), resuspended in 20 ml buffer (50 mM sodium phosphate, pH 6.4) and lysed by sonication at 4°C (three times for 1 min at 21 amplitude microns) with a Soniprep 150 (SANYO Gallenkamp PLS, UK). Cell debris was removed by filtration through 0.2 μm filters. The enzyme extracts were then used for activity determinations and native polyacrylamide gel electrophoresis (native-PAGE).

**Enzymatic assays** Purification of the recombinant  $\beta$ -galactosidase isoenzymes (BbgI, BbgII, BbgIII and BbgIV) was carried out as described before (Goulas et al. 2009).  $\beta$ -Galactosidase activity against *o*-nitrophenyl- $\beta$ -D-galactopyranoside (*o*NPG) was measured and defined as described before (Goulas et al. 2009).

Activity determinations towards 0.1% (w/v) of lactose or different GOS fractions was performed in buffered solutions (50 mM sodium phosphate, 1 mM MgCl<sub>2</sub>) at the optimum pH for each enzyme preparation (pH 6.4 for BbgI, pH 5.8 for BbgII, pH 6.8 for BbgIII and pH 6.4 for BbgIV; Goulas et al. 2009). All the reactions were performed for 10 min at 37°C and with ≈0.1 U ml<sup>-1</sup> of reaction (in respect to lactose) with each enzyme preparation and with all the tested substrates except for the hydrolysis of GOS with DP=3 and DP=4 from BbgII where ≈0.0011 U ml<sup>-1</sup> of reaction were used. The reactions were stopped with heat inactivation at 90°C for 10 min. Activity towards different GOS fractions was calculated based on the amount of

galactose liberated per minute and compared to the rate of lactose hydrolysis under the same conditions. Galactose concentration was determined by HPAEC-PAD analysis. All protein concentrations were measured using the Bradford method (Bradford 1976) with bovine serum albumin as a standard.

**Determination of expression profiles of  $\beta$ -galactosidase isoenzymes** The  $\beta$ -galactosidase activity of freshly prepared crude protein extracts from *B. bifidum* was determined by the released galactose analysed as described above using 2.77 mM lactose (Sigma, UK) or  $\beta$ -D-(1  $\rightarrow$  6) galactobiose (Dextra Laboratories Ltd, UK) as substrate in buffered solution (100 mM sodium phosphate, 1 mM  $\text{MgCl}_2$ ) at pH 6.4. The results were calculated as percentage of relative activity towards  $\beta$ -D-(1  $\rightarrow$  6) galactobiose in comparison to lactose. Native-PAGE was carried out using 7.5% polyacrylamide gel in an ATTO mini PAGE system (ATTO Corporation, Japan). Pure recombinant enzyme preparations (BbgI, BbgII, BbgIII and BbgIV) and freshly prepared crude protein extracts ( $\approx 6 \mu\text{g}$ ) from *B. bifidum* cells growing on different substrates were loaded and electrophoresed on the nondenaturing PAGE system. The  $\beta$ -galactosidase activities were detected by incubating the gels in 1 mg  $\text{ml}^{-1}$  4-methylumbelliferyl- $\beta$ -D-galactopyranoside solution (MUG) in 100 mM sodium phosphate buffer, 1 mM  $\text{MgCl}_2$  at pH 6.4. Fluorescent bands were visualised and photographed under UV light after incubation at 37°C for 10–20 min.

**Synthesis of GOS** GOS synthesis was performed in 100 mM sodium phosphate buffered solutions at pH 6.4 and at 40°C in a shaking water bath (150 rpm  $\text{min}^{-1}$ ). Substrates used for the synthesis reactions were 30% to 40% (w/v) lactose and 30% (w/v) lactose with 10% (w/v) galactose. For the synthesis reactions, approximately 1.28 U  $\text{ml}^{-1}$  of enzyme activity (determined using oNPG as substrate) of either BbgI, BbgIII, BbgIV or whole *B. bifidum* cells and 172 U  $\text{ml}^{-1}$  of BbgII were used. All synthesis reactions were performed in duplicate. The reactions were followed over a 24-h period time and the enzymes heat inactivated at 90°C for 10 min before analysis of their carbohydrate content. Samples were analysed by high performance liquid chromatography (HPLC), HPAEC-PAD and selected samples by gas chromatography–mass spectrometry (GC–MS).

**Sample analysis of their carbohydrate content** Samples were analysed by HPLC using an Aminex HPX-87C  $\text{Ca}^{++}$  resin-based column (300 $\times$ 7.7 mm; Bio-Rad Laboratories Ltd, UK) and an HPLC analyser coupled to a refractive index detector. The column was maintained at 85°C and HPLC grade water was used as mobile phase at a flow rate 0.6  $\text{ml min}^{-1}$ . Quantitative determination of the oligosac-

charides (DP $\geq$ 3), disaccharides and monosaccharides was performed by using standard calibration curves of maltotriose, lactose, glucose and galactose, respectively.

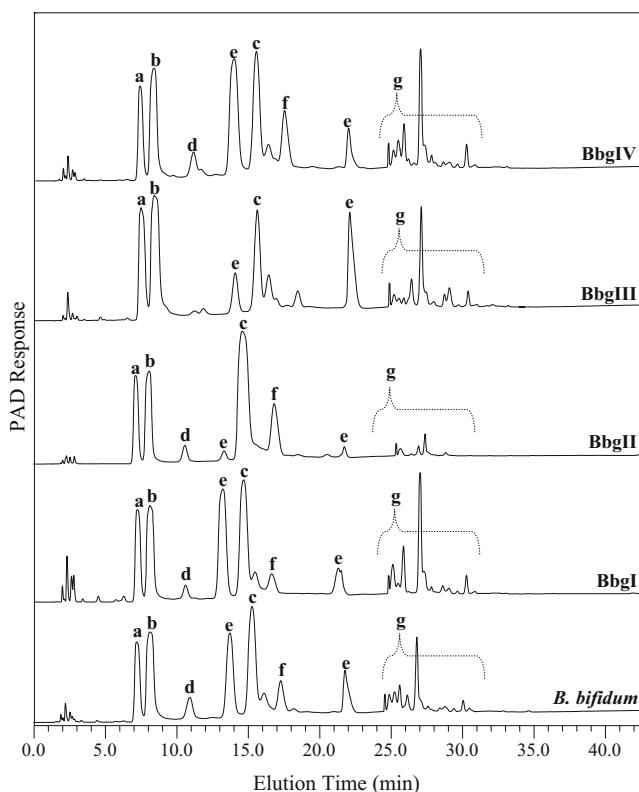
Samples were also analysed by HPAEC-PAD using a pellicular anion-exchange resin-based column CarboPac PA-1 (Dionex Chromatography, UK). Carbohydrates were eluted at 1  $\text{ml min}^{-1}$  flow rate using gradient mobile phase concentrations of sodium hydroxide and sodium acetate solutions at 20°C. Quantification of the carbohydrate concentration of samples (glucose, galactose and lactose) was carried out as described in Dionex Corporation (2001) (Technical Note 155; Sunnyvale, CA, USA). Identification of the different carbohydrates was done based on galactose, glucose, lactose and  $\beta$ -D-(1  $\rightarrow$  6) galactobiose standards, whereas identification of the degree of polymerisation of the rest carbohydrates present in GOS was done based on the different fractions isolated from the GOS fractionation (described above).

Selected samples were further analysed by GC–MS after derivatisation to sugar oximes using hydroxylamine chloride in pyridine and persilylation using hexamethyldisilazane and trifluoroacetic acid (Sanz et al. 2004). The column used during the analysis was the DB-17MS (length 30 m, ID 0.25 mm, Film 0.25  $\mu\text{m}$ ; J&W Scientific, USA).

## Results

**GOS composition and fractionation** The GOS mixture synthesised from lactose with the  $\beta$ -galactosidase activity of whole *B. bifidum* cells consisted of different fractions of mono-, di- and oligosaccharides. In order to purify the GOS mixture from monosaccharides and to obtain fractions of different degree of polymerisation, the mixture was subjected to size exclusion chromatography. The pooled fractions were analysed in HPAEC-PAD and the different components are shown in Fig. 1. Fractions with DP=2 and DP $\geq$ 2 contained amounts of lactose, whereas fractions with DP=3 and DP=4 were lactose free. The purified GOS consisted mainly from  $\beta$ -D-(1  $\rightarrow$  3) and to a lesser extent  $\beta$ -D-(1  $\rightarrow$  4) and  $\beta$ -D-(1  $\rightarrow$  6) glycosidic linkages, as described by Depeint et al. (2008).

**GOS assimilation from *B. bifidum*** *B. bifidum* grown in TPY broth containing glucose (0.5% w/v) was used to inoculate fresh TPY broth containing 0.5% (w/v) of either glucose, galactose, lactose, GOS (DP=2), GOS (DP $\geq$ 2) or GOS (DP $\geq$ 3). For the incubation periods tested, the results obtained from the growth of the microorganism indicated a clear preference for complex carbohydrates rather than their simpler moieties (i.e. glucose and galactose; Fig. 2). No growth was observed in TPY with galactose (data not shown), whereas minor growth was observed in the

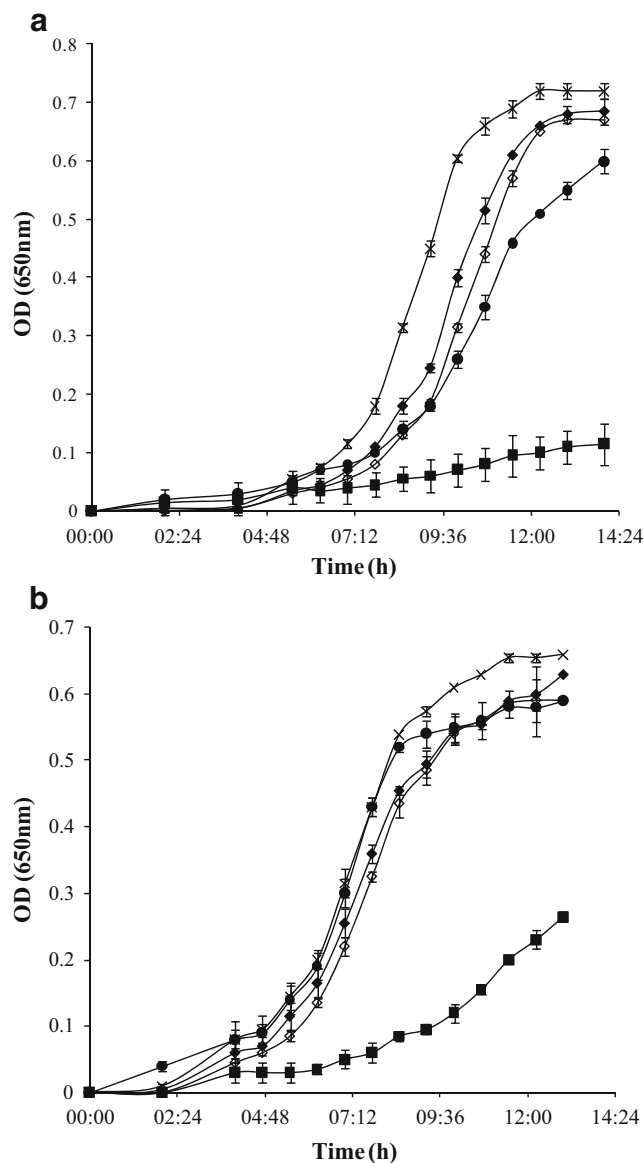


**Fig. 1** HPAEC-PAD analysis of the GOS mixtures synthesised by *B. bifidum* whole cells and the purified recombinant enzymes BbgI, BbgII, BbgIII and BbgIV, separately. *a* Galactose, *b* glucose, *c* lactose, *d*  $\beta$ -D-(1  $\rightarrow$  6) galactobiose, *e* DP=2, *f* DP=3, *g* DP $\geq$ 3. The identity of each peak has been confirmed with the respective carbohydrate standard and/or size exclusion chromatography

presence of glucose when compared to the other carbohydrates. More complex carbohydrates, such as the different GOS fractions or lactose, supported efficiently the microbial growth in both starting pH tested. The ability of the *B. bifidum* to grow on these carbohydrates indicated the existence of  $\beta$ -galactosidases able to efficiently hydrolyse them into their simpler moieties for further assimilation.

*Activity of  $\beta$ -galactosidases towards different GOS fractions* Subsequently, the enzymatic activity of the recombinant enzymes was determined against different fractions of GOS and compared to that against lactose (Table 1). The BbgII isoenzyme was shown to hydrolyse GOS preferentially compared to lactose. Degradation of GOS resulted to liberation of monosaccharides but also smaller oligosaccharides (Fig. 3). The appearance of smaller oligosaccharides into the medium indicated that they were not hydrolysed as efficiently as the initial GOS fractions. Contrary to BbgII, the other isoenzymes showed a clear preference for lactose hydrolysis over the different GOS fractions (Table 1). However, simpler oligosaccharides from the hydrolysis of GOS were appearing as in the case of BbgII isoenzyme (data not shown).

*Expression profiles of the  $\beta$ -galactosidase isoenzymes* In order to identify which isoenzyme is expressed, protein extracts from cells grown either on glucose, lactose, GOS (DP $\geq$ 2) or GOS (DP $\geq$ 3) were subjected to native-PAGE electrophoresis and the  $\beta$ -galactosidase expression profiles visualised after activity staining with MUG as substrate (Fig. 4). Purified recombinant  $\beta$ -galactosidases were used as standards. As indicated from the gels, BbgII and BbgIV standards were migrating at the same rate. Comparison of the enzyme profiles of cultures grown in different carbohydrates showed that BbgI and BbgIII were consistently



**Fig. 2** Growth curves of *B. bifidum* in TPY broth containing 0.5% (w/v) of carbohydrates. **a** Growth curves with starting pH 6.8; **b** growth curves with starting pH 6.0. Filled square glucose, unfilled diamond lactose, filled diamond GOS (DP=2), multiplication symbol GOS (DP $\geq$ 2), filled circle GOS (DP $\geq$ 3). Values are presented as means and standard deviation of the means ( $n=3$ )

**Table 1** Percentage of relative activity of the *B. bifidum* recombinant  $\beta$ -galactosidases (BbgI, BbgII, BbgIII and BbgIV) against different GOS fractions compared to the activity against lactose

GOS fraction	Enzyme			
	BbgI	BbgII	BbgIII	BbgIV
DP=2	+++	–	+++	+++
DP=3	++	+++++	++	+
DP=4	++	++++	–	+

DP degree of polymerisation, – not determined, + <10%, ++ 10–50%, +++ 50–100%, ++++ 100–500%, +++++ >500%

expressed. A protein corresponding to either BbgII or BbgIV was observed to be induced in the presence of either lactose or different GOS fractions in comparison to glucose growing cells.

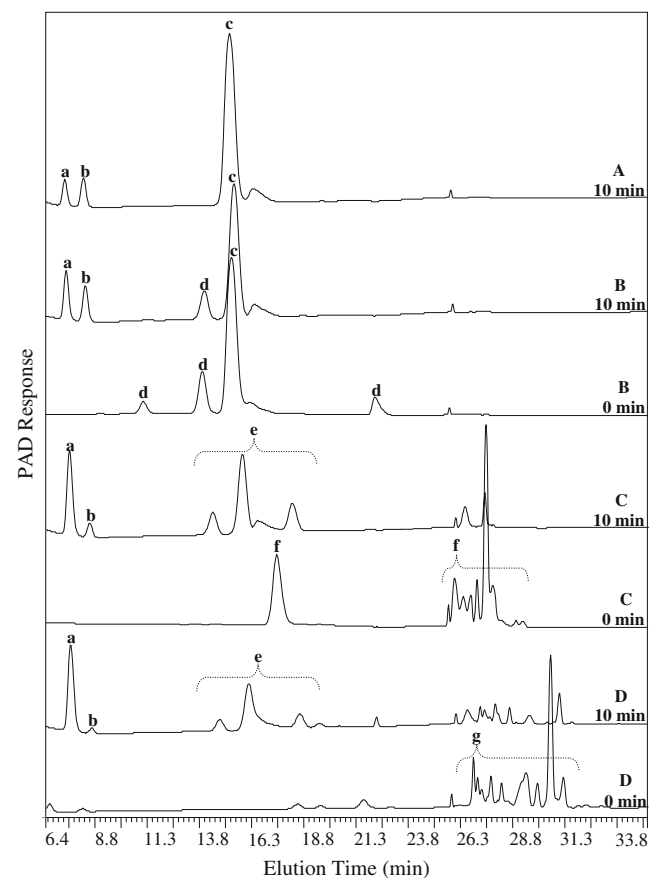
As was proven before (Goulas et al. 2009), pure protein preparations of BbgI, BbgIV and BbgIII showed minor activity towards  $\beta$ -D-(1  $\rightarrow$  6) galactobiose (<4.7%) in comparison to lactose, whereas BbgII had a more than 22-fold preference for that substrate. Subsequently, bearing in mind the above observation, *B. bifidum* protein extracts were used for activity determinations towards lactose or  $\beta$ -D-(1  $\rightarrow$  6) galactobiose (Table 2). A 93% relative activity towards  $\beta$ -D-(1  $\rightarrow$  6) galactobiose in comparison to lactose was observed when protein extracts from glucose-growing cells were used indicating that the third band appearing in the native-PAGE gel (from the top of the gel; Fig. 4) corresponded to BbgII  $\beta$ -galactosidase. An approximate three- to four-fold increase of the lactose hydrolysing activity and a relatively constant  $\beta$ -D-(1  $\rightarrow$  6) galactobiase activity were observed in cells growing either on lactose or different GOS fractions in comparison to glucose growing cells. This resulted in a decrease of the relative activity towards  $\beta$ -D-(1  $\rightarrow$  6) galactobiose (23–29%) in comparison to lactose and indicated that the induced protein corresponded to BbgIV  $\beta$ -galactosidase.

A fourth band has been observed to appear in cells growing either in lactose or the different GOS fractions. The presence of it has been attributed to the existence of a different BbgIV isoform since it has been observed to appear also with the recombinant BbgIV standard used on this study.

**GOS synthesis** Transferase activity was studied for each pure recombinant enzyme preparation and whole *B. bifidum* cells, under the same enzymatic conditions. Synthesis of oligosaccharides was observed with all the enzyme–substrate combinations. Under increased lactose concentration (40% w/v), the formation of oligosaccharides was favourable, especially towards GOS with DP $\geq$ 3 (data not shown). Figure 5 shows a typical time course during the production of GOS observed in all synthesis reactions

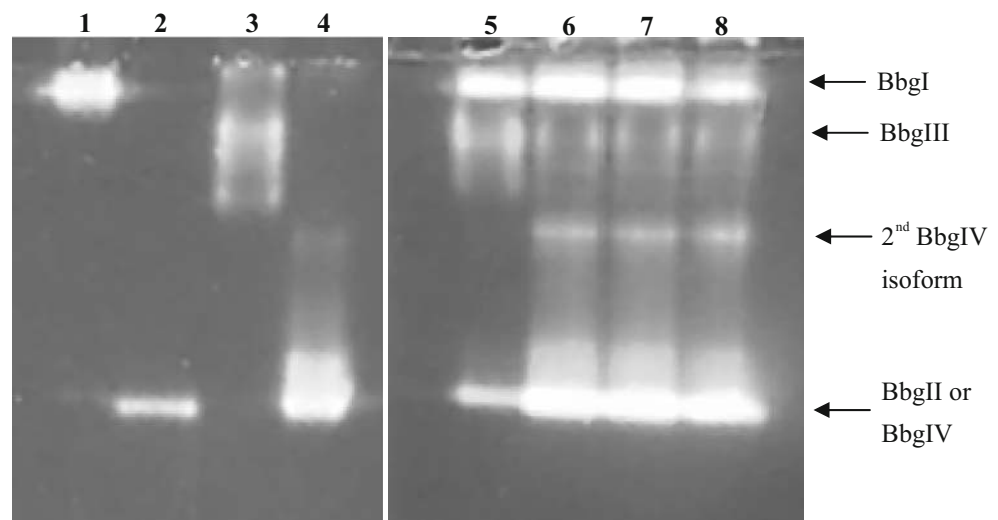
performed. Oligosaccharide concentration increased initially to a maximum and subsequently decreased when transgalactosylation activity became less pronounced than the hydrolytic activity (Boon et al. 2000).

Analysis of the carbohydrate compositions (Table 3) at optimum synthesis (with 40% w/v lactose) showed that the highest oligosaccharide concentrations were observed at 76.6% to 86.2% lactose conversion with all the enzyme preparations, except for BbgII  $\beta$ -galactosidase (50.7%). Yields of oligosaccharides were similar amongst BbgI, BbgIV and *B. bifidum* whole cells, with BbgIV being more efficient (47.0%) in the conversion of lactose into GOS. BbgIII proved to be less effective converting only 28.7% of the lactose whereas BbgII was poorly synthesising GOS with a yield of 15.2%. Another characteristic difference amongst the enzymes was that BbgIII preferred to synthesise equal amounts of DP=2 and DP $\geq$ 3 oligosaccharides, whilst BbgI, BbgIV and *B. bifidum* whole cells preferentially synthesised higher amounts of oligosaccharides with



**Fig. 3** HPAEC-PAD analysis of BbgII activity against different carbohydrates. 0.1% of either lactose (A), GOS (DP=2; B), GOS (DP=3; C) and GOS (DP=4; D) have been hydrolysed under the same assay conditions (i.e. enzyme units, temperature and pH). a Galactose, b glucose, c lactose, d GOS with DP=2, e products of hydrolysis, f GOS with DP=3, g GOS with DP=4

**Fig. 4**  $\beta$ -Galactosidase activity staining on native-PAGE. Protein extracts of *B. bifidum* cells grown either on glucose (lane 5), lactose (lane 6), GOS (DP $\geq$ 2; lane 7) or GOS (DP $\geq$ 3; lane 8) were compared with the profiles of pure recombinant  $\beta$ -galactosidase isoenzymes from *B. bifidum* (BbgI, lane 1; BbgII, lane 2; BbgIII, lane 3; BbgIV, lane 4)



DP=2 than DP $\geq$ 3. The opposite was observed for the BbgII enzyme.

In Fig. 1, representative HPAEC-PAD chromatograms are shown with the profiles of the oligosaccharides produced from each enzyme preparation separately. The profiles of the synthesis products from BbgI, BbgIV and *B. bifidum* whole cells are similar whereas BbgIII does not produce a disaccharide that elutes at the same retention time as the  $\beta$ -D-(1  $\rightarrow$  6) galactobiose standard (results confirmed by gas chromatography–mass spectrometry after derivatisation to the sugar oximes).

## Discussion

Over the time, a number of studies have demonstrated the existence of more than one  $\beta$ -galactosidase isoenzymes in bifidobacteria (Tochikura et al. 1986; van Laere et al. 2000; Møller et al. 2001; Hung et al. 2001; Goulas et al. 2007a). However, the number of reports showing the contribution of these isoenzymes in the bacterial physiology and the existence of possible synergistic relations amongst them for more efficient carbohydrate assimilation

is limited (van Laere et al. 2000). Moreover, no reports seem to exist showing the participation of different  $\beta$ -galactosidase isoenzymes in the synthesis of transgalactooligosaccharides when whole bifidobacterial cells are utilised. Here, a *B. bifidum* strain (NCIMB41171) was studied in an attempt to reveal some more properties of its  $\beta$ -galactosidase isoenzymes in order to better understand their contribution to the bacterial physiology and their participation in the synthesis of a GOS mixture that has been shown to enhance the growth of the specific strain (Depeint et al. 2008).

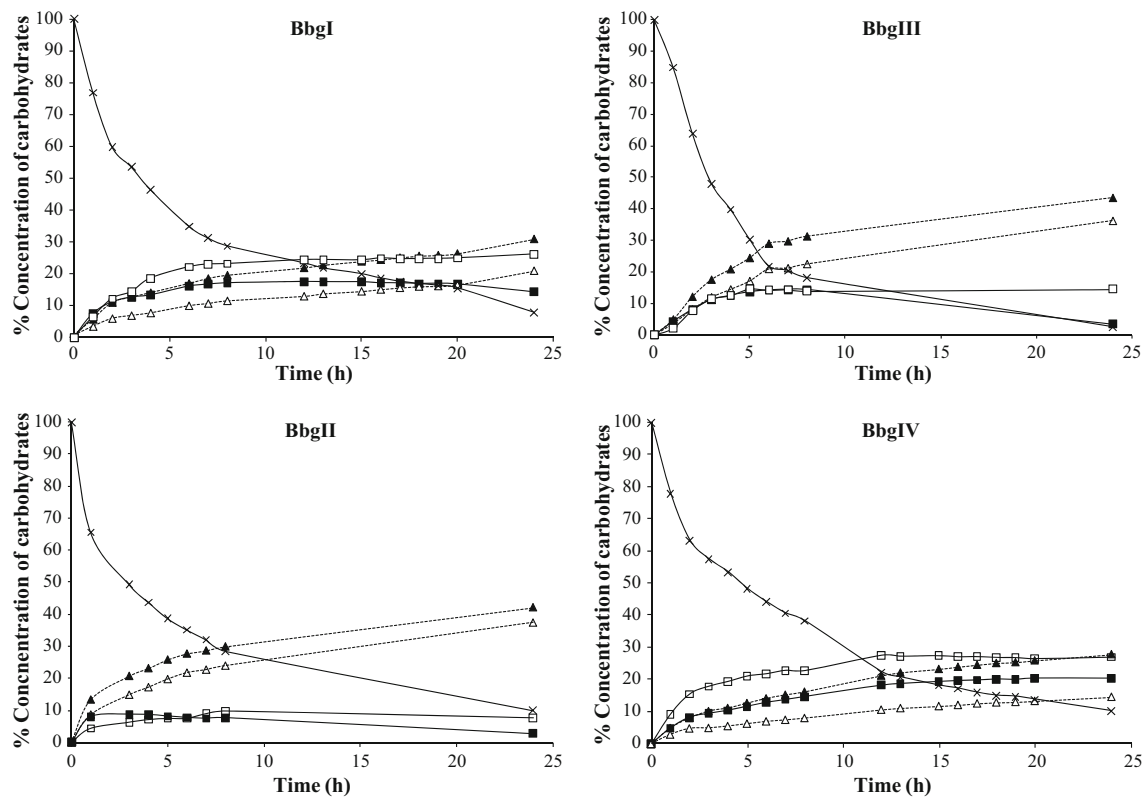
The fermentative behaviour of *B. bifidum*, described herein, indicates that this strain relies on the existence of complex carbohydrates rather than on their simpler moieties. *B. bifidum* showed poor growth on glucose and especially galactose when these carbohydrates were used as single carbon sources. More complex carbohydrates such as lactose or different GOS fractions proved to be more favourable for cell growth. It seems, therefore, that *B. bifidum* incorporates more efficient transport systems for those carbohydrates than for glucose and galactose. Krzewinski et al. (1996, 1997) proved the existence of glucose, galactose and an inducible lactose transport system

**Table 2** Percentage of relative and specific activity in unit per milligram (in brackets) of *B. bifidum* protein extracts towards lactose or  $\beta$ -D-(1  $\rightarrow$  6) galactobiose

Substrate	Protein extracts from <i>B. bifidum</i> grown on			
	Glc	Lac	GOS (DP $\geq$ 2)	GOS (DP $\geq$ 3)
Lactose	100% (0.262 $\pm$ 0.021 U mg $^{-1}$ )	100% (0.809 $\pm$ 0.064 U mg $^{-1}$ )	100% (0.661 $\pm$ 0.04 U mg $^{-1}$ )	100% (0.967 $\pm$ 0.015 U mg $^{-1}$ )
$\beta$ -D-(1 $\rightarrow$ 6) galactobiose	93% (0.244 $\pm$ 0.023 U mg $^{-1}$ )	23% (0.190 $\pm$ 0.011 U mg $^{-1}$ )	29% (0.193 $\pm$ 0.012 U mg $^{-1}$ )	27% (0.262 $\pm$ 0.001 U mg $^{-1}$ )

Protein extracts were prepared from cells grown either on glucose (Glc), lactose (Lac) or different GOS fractions (GOS with DP $\geq$ 2 or DP $\geq$ 3). Specific activities are presented as means and standard deviation of the means ( $n=3$ ) of three independent experiments

Glc glucose, Lac lactose, GOS galactooligosaccharide



**Fig. 5** Typical time course during synthesis of oligosaccharides with the *B. bifidum* recombinant  $\beta$ -galactosidases (BbgI, BbgII, BbgIII and BbgIV; data from HPLC and HPAEC-PAD analysis). *Multiplication*

*symbol* lactose, *filled triangle* glucose, *unfilled triangle* galactose, *unfilled square* DP=2, *filled square* DP $\geq$ 3

in *B. bifidum* DSM20082. Furthermore, the existence of transport systems upstream of BbgIV and downstream of the BbgII  $\beta$ -galactosidase genes in the *B. bifidum* NCIMB41171 genome has been previously demonstrated (Goulas et al. 2007b). However, whether these transport systems are expressed in the presence of different carbohydrates and whether they participate in the transport of these carbohydrates remains to be elucidated.

The different isoenzymes seem to act in a collaborative manner by being located in different cell positions or by breaking different sugar linkages and thus liberating sugar moieties from different GOS fractions. Amongst the isolated  $\beta$ -galactosidases, BbgIII contains all the necessary signal sequences for extracellular or cell wall bound location (Goulas et al. 2007b). Even though it had a relatively low activity towards GOS (<50%) in comparison

**Table 3** Carbohydrate composition of GOS mixtures synthesised by *B. bifidum* whole cells and each recombinant isoenzyme with 40% (w/v) lactose at time points where the optimum oligosaccharide concentration was observed (data from HPLC and HPAEC-PAD)

Enzyme source	GOS DP $\geq$ 3	GOS DP=2	Lac	Glc	Gal	Substrate conversion	Yield (w/w)
	Concentration (mg ml <sup>-1</sup> )						
<i>B. bifidum</i>	76.0	88.0	68.8	106.0	61.2	82.8	41.0
BbgI	70.2	97.6	93.4	87.2	51.5	76.6	41.9
BbgII	35.3	25.6	197.0	82.7	59.4	50.7	15.2
BbgIII	57.7	57.2	81.6	118.6	84.8	79.6	28.7
BbgIV	81.8	106.3	55.2	103.6	53.1	86.2	47.0

Substrate conversions at maximum oligosaccharide concentration were calculated based on the lactose concentrations determined by HPAEC-PAD. Yields calculated as the percent of lactose by weight converted to GOS

*Lac* lactose, *Glc* glucose, *Gal* galactose, *DP* degree of polymerisation

to lactose, BbgIII seems to contribute towards bacterial physiology by degrading extracellularly existing complex carbohydrates and thus liberating simpler di- or oligosaccharides and assisting intracellular incorporation of them. Intracellularly, the efficient degradation of these carbohydrates depends on the hydrolytic activity of the present  $\beta$ -galactosidases. Amongst the isoenzymes, BbgII showed a clear preference for different GOS fraction over lactose. This can explain the cells behaviour with GOS or lactose as substrates. If all the enzymes were more specific for lactose hydrolysis, then a preference of the bacterium for it would be expected. However, this bacterium showed similar or even better growth patterns in the presence of different GOS fractions than lactose. Subsequent analysis of the  $\beta$ -galactosidase expression profiles from cultures grown on the different carbohydrates indicated an apparently consistent expression of BbgI, BbgII and BbgIII isoenzymes and an inducible expression of BbgIV. However, besides BbgIV, whether the expression of the rest isoenzymes is further regulated cannot be determined under the experimental conditions used.

As shown before, BbgII had a clear preference for  $\beta$ -D-(1  $\rightarrow$  6) galactosides over  $\beta$ -D-(1  $\rightarrow$  4) galactosides and lactose (Goulas et al. 2009). The GOS mixture used for this study consists mainly of  $\beta$ -D-(1  $\rightarrow$  3) and to a lesser extent  $\beta$ -D-(1  $\rightarrow$  4) and  $\beta$ -D-(1  $\rightarrow$  6) linkages (Depeint et al. 2008). The hydrolytic activity of BbgII towards different fractions of GOS (DP=3 and DP=4) was higher over lactose indicating a preference of that isoenzyme towards  $\beta$ -D-(1  $\rightarrow$  3) galactosides. The preference of BbgII towards GOS is a characteristic described for other  $\beta$ -galactosidases belonging to glycosyl hydrolases 42 family. However, other characterised bifidobacterial isoenzymes (i.e. from *Bifidobacterium adolescentis*) have shown higher hydrolytic activity towards  $\beta$ -D-(1  $\rightarrow$  4) linked GOS fractions compared to  $\beta$ -D-(1  $\rightarrow$  3) and  $\beta$ -D-(1  $\rightarrow$  6) (Hinz et al. 2004). This suggests that the hydrolytic activities of the different isoenzymes present in the bacterial cell towards different GOS mixtures can reflect the bacterial capabilities for carbohydrate assimilation and may be a good starting point for identifying the most potent oligosaccharide mixture for strain rather than species specific design of prebiotics. The recent genome sequencing of the *B. bifidum* strain NCIMB41171 (Goulas et al. 2008) revealed the existence of a fifth  $\beta$ -galactosidase present in that microorganism. Although, whether this enzyme is expressed or not and which is its biological contribution to the bacterial physiology is unknown, based on amino acid (aa) sequence similarities searches with other known and characterised enzymes indicated that the enzyme share  $\approx$ 81% aa identity with the  $\beta$ -galactosidase (protein ID: AAR24113.1; Hinz et al. 2004) from *B. adolescentis* (mentioned above) indicating a similar biological role.

In order to identify which of the isoenzymes contributes the most to the GOS synthesis from lactose when the  $\beta$ -galactosidase activity of whole *B. bifidum* cells are used, GOS synthesis with each of the isoenzyme was performed separately. Although all the enzymes were able to some extent to synthesise GOS, based on patterns of the oligosaccharides formed and the yields obtained, it can be concluded that BbgIV and BbgI were mainly responsible for the synthesis of the specific GOS mixture. BbgIII and especially BbgII (a GOS hydrolysing enzyme) gave lower synthesis yields than the other enzymes and whole *B. bifidum* cells. Moreover, *B. bifidum* is usually precultured in a substrate combination of glucose and lactose for production of biomass which would be subsequently used for GOS synthesis (Goulas et al. 2007a). The expression profiles of  $\beta$ -galactosidases extracted from *B. bifidum* grown on lactose clearly indicates that BbgI, BbgIV and BbgIII are the main isoenzymes responsible for GOS synthesis when whole *B. bifidum* cells are utilised, whereas the presence of BbgII would apparently contribute to a reduction of the synthesised product through its hydrolytic activity.

A comparison of the results of the present study with the transgalactosylating properties of other enzymes would be interesting to better understand the enzyme efficiency for synthesis. However, the amount of the formed oligosaccharides depends upon several factors, including concentration of the substrate, the degree of conversion of the substrate and the reaction conditions (Mahoney 1998). Thus, comparison between different enzymes would be difficult, because the reaction conditions used in other studies have been different. Apparently, the results of this study suggest that enzymes with higher specificity constants ( $k_{\text{cat}}/K_m$ ) towards lactose (identified before; Goulas et al. 2009) exhibit better transgalactosylation properties. This can be further generalised by considering the protein family of the characterised isoenzymes (Goulas et al. 2007b). Enzymes belonging to glycosyl hydrolases 2 family (BbgI, BbgIII, BbgIV) showed better transgalactosylation properties than enzymes of glycosyl hydrolases 42 family (BbgII).

Overall, *B. bifidum* utilises more than one  $\beta$ -galactosidase for either GOS assimilation in growth cultures or GOS synthesis from lactose. These isoenzymes seem to contribute to bacterial physiology in a synergistic manner by breaking different carbohydrate linkages or by being located in different cell compartments. At the same time, the synthesis of GOS from lactose using whole *B. bifidum* cells seems to be the result of the collaborative action of three rather one  $\beta$ -galactosidases. Knowing the transgalactosylation properties of each of these isoenzymes and the GOS assimilation physiology of the specific strain can be of great assistance in order to develop prebiotic galactooligosaccharide mixtures targeting strain selectivity.



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