Original Research Article

Galactooligosaccharides Purification Using Microbial Fermentation and Assessment of Its Prebiotic Potential by *In Vitro* Method

Vikas Sangwan[1], Sudhir K. Tomar[1], Babar Ali[1], Ram R. B. Singh[2], and Ashish K. Singh[2], Surajit Mandal[1]

[1]Dairy Microbiology Division, National Dairy Research Institute, Karnal- 132001, India
[2]Dairy Technology Division, National Dairy Research Institute, Karnal- 132001, India
*Corresponding author

A B S T R A C T

Keywords

Galactooligos
accharides,
Prebiotics,
Microbial
Fermentation,
Probiotics

Galactooligosaccharides produced from the action of enzyme extracted from *Streptococcus thermophilus* using whey supplemented with lactose as a substrate was purified using three different organisms viz. *Sachharomyces cerevisiae* NCDC 50, *Kluyveromyces lactis* NCDC 115 and *Lactobacillus helveticus* NCDC 288. Amount of monosaccharides and disaccharides was reduced considerably using microbial fermentation. The purified oligosaccharide mixture was then assessed for its prebiotic activity using *in vitro* method. For this purpose, *Escherichia coli* NCDC 134 was used as a commensal pathogen and *Lactobacillus rhamnosus* GG was used as a probiotic organism. The substrate preferences of *L. rhamnosus* GG and *E. coli* were studied by incubating the bacteria with Glucose, Inulin and GOS. After inoculation the cell count of bacteria were taken at 0h and after 24 h and the prebiotic activity score was calculated. Increase in log count of *L. rhamnosus* GG was higher on both of the prebiotic tested as compared to *E. coli*. GOS was metabolized by both *L. rhamnosus* GG and *E.coli.* But the increase in the log count of *E. coli* was less as compared to *L. rhamnosus* GG, which showed that *L. rhamnosus* GG can utilize GOS more efficiently than *E. coli*, as a substrate.

Introduction

Prebiotics are emerging functional products generally useful for the improvement of the nutritional quality of foods, and are described as functional components of food which remain undigested through gastrointestinal tract and selectively fermented by the gastrointestinal microflora thereby confer benefits upon health and wellbeing of the host. Most of the research in the field on prebiotics has been done with inulin and fructooligosaccharides (FOS). But the recent momentum has been behind the Galactooligosaccharides (GOS). GOS are well documented to be as effective prebiotic ingredients which modulate intestinal microbiota, barrier functions, and other provide other beneficial health

effects such as stool improvement, mineral absorption, weight management, carcinogenesis, and allergy alleviation (Ohr et al., 2010; Figueroa-Gonzalez et al., 2011). GOS have been observed to stimulate the growth of species from Bifidobacterium and Lactobacillus genera in both *in vivo* and *in vitro* studies. (Macfarlane et al., 2008; Whisner et al., 2013). As a result, dietary GOS are being increasingly used in a wide range of functional foods such as infant foods, functional dairy products and fruit-based drinks (Sangwan et al., 2011).

GOS are industrially produced by transgalactosylation reactions using β-galactosidases from lactose, leading to a final product comprising oligosaccharides with a degree of polymerization (DP) of up to 8–9, and high amounts of mono- and disaccharides. The presence of mono- and disaccharides in prebiotic preparations is considered undesirable since they do not have the necessary fermentation selectivity and they are also absorbed in the small intestine increasing the calorific value of the products. In this sense, the removal of mono- and disaccharides fractions from a GOS mixture is necessary to both evaluate their functional properties, e.g., *in vitro* prebiotic activity, and determine their structures. On the other hand, the removal of these carbohydrates could help to expand the applications of the purified GOS in the food and pharmaceutical industries. Among these applications, there could be the inclusion of purified GOS as ingredients in (i) diabetic foods, which could allow diabetic people to utilize the beneficial properties of GOS, free from carbohydrates that increase the level of postprandial glucose, (ii) specialized foods for individuals intolerant to lactose and (iii) low calorie foods with a reduction in some mono- and

disaccharides (Hernandez et al., 2009). Different techniques viz. Size exclusion chromatography, activated charcoal treatment, diafilteration and yeast treatments are being used for the purification of GOS. Our aim was to purify the complex mixture of GOS using microbial intervention and assessment of prebiotic activity of purified product by *in vitro* method. Hernandez et al (2009) used *Saccharomyces cerevisiae* for GOS purification and reported utilization of all the monosaccharide content from GOS mixture whereas Gaulas et al (2007) only found a slight reduction of galactose during the incubation of GOS with *S. cerevisiae*. Kunova et al (2011) used *Lactobacillus helveticus* for this purpose and reported a considerable removal of both mono and disaccharides. In this study three different organisms (*S. cerevisiae, Kluyveromyces lactis, L. helveticus*) with variable carbohydrate utilization profile were used for the purification purpose. For the assessment of *in vitro* prebiotic activity, *Escherichia coli* was used as a commensal pathogen and *Lactobacillus rhamnosus* GG was used as a probiotic organism.

Materials and Methods

Bacterial strain

For partial purification:

S. cerevisiae NCDC 50, *K. lactis* NCDC 115 and *L. helveticus* NCDC 288 were used for the purification of oligosaccharide mixture. Yeast potato dextrose (YPD) broth and agar were used for *S. cerevisiae*, Yeast potato lactose (YPL) agar and broth were used for *K. lactis* and de Man Rogosa Sharpe (MRS) agar and broth were used for the growth of *L. helveticus*.

For *in-vitro* prebiotic assay

L. rhamnosus GG, *E. coli* NCDC 134 were used as a probiotic and enteric pathogen respectively for *in-vitro* assay. MRS agar and broth were used for the growth of *L. rhamnosus* GG and Brain Heart Infusion (BHI) broth and Violet Red Bile Agar (VRBA) were used for *E. coli.*

The cultures were inoculated in broth and thereafter streaked on their selective agar plates. Single colonies were picked up and again inoculated in their respective broth. The cultures were incubated 18-24 h at 37^0C. Cultures were stored as glycerol stock at -20^0C until use. The day prior to use, cultures were thawed, inoculated in their respective broth and grown overnight at $37^°$C.

Test substance

GOS was produced from the enzyme β-galactosidase extracted from native strains of *Streptococcus thermophilus* using whey supplemented with desirable amount of lactose as a substrate. High performance liquid chromatography (HPLC) was used for the detection and quantification of GOS.

Removal of Monosaccharides and Disaccharides from GOS Mixture

GOS was produced as a mixture of monosaccharides (Glucose and galactose), disaccharides (lactose) and oligosaccharides (GOS). Initially flask containing 100 mL of YPD broth was inoculated with activated culture of *S. cerevisiae* (at the rate of 1 %) and incubated at 37 ^{0}C for 18 h followed by centrifugation at 10000 rpm and 4 ^{0}C for 15 min. Supernatant was discarded and pellet was washed twice with 50 mM sodium phosphate buffer (pH 6.8) and centrifuged at 10000 rpm and 4 ^{0}C for 15 min. The pellet thus obtained was mixed with 10 mL of crude GOS mixture and incubated at 37 ^{0}C for 8 h. After incubation the mixture was centrifuged at 10000 rpm and 4 ^{0}C for 15 min and the resultant supernatant was filtered through 0.22 µm filter (Millipore) to remove the microbial cells and again mixed with cell pellet of *K. lactis* (obtained in a similar manner as that of *S. cerevisiae*) and incubated for 8 h at 37 ^{0}C. The mixture was centrifuged at 10000 rpm and 4 ^{0}C for 15 min and supernatant was filtered and mixed with cell pellet of *L. helveticus* (obtained as earlier) and incubated for 8 h at 37 ^{0}C followed by centrifugation at 10000 rpm and 4 ^{0}C for 15 min. The carbohydrate composition of the supernatant was estimated by using HPLC after each purification step.

In-vitro prebiotic assay

L. rhamnosus GG, and *E. coli* NCDC 134 were used for the study of prebiotic activity of GOS. Inulin was used as a standard prebiotic. Both of the cultures were activated before use in the experiment. The assay was carried out by adding 1% (vol/vol) of an overnight culture of *L. rhamnosus* GG to three different tubes containing 10 ml of complex medium (tryptone 10 g, peptone 10 g, yeast extract 5 g, Tween 80 1 ml, l-cysteine hydrochloride 0.5 g, distilled water 1 l, pH = 7). Each tube was supplemented with one carbon source i.e. glucose, GOS and inulin (2g each). The tubes were incubated at 37 ^{0}C. After 0 and 24 h of incubation, samples were enumerated on MRS agar using spread plating technique. For *E. coli*, the overnight culture 1% (vol/vol) was added to separate tubes containing 10 ml of M9 broth supplemented with glucose, GOS and inulin

(2g each) as a sole carbon source. The culture was incubated at 37^0C, and enumerated on VRBA after 0 and 24 h of incubation (Rada et al., 2008).

Prebiotic Activity Score

The prebiotic activity score was determined using the following equation:

Prebiotic activity score = {(probiotic log cfuml-^{1} on the prebiotic at 24h – probiotic log cfuml-^{1} on the prebiotic at 0h)/(probiotic log cfuml-^{1} on the glucose at 24h – probiotic log cfuml-^{1} on the glucose at 0h)} – {(enteric log cfuml-^{1} on the prebiotic at 24h – enteric log cfuml-^{1} on the prebiotic at 0h)/(enteric log cfuml-^{1} on the glucose at 24h – enteric log cfuml-^{1} on the glucose at 0h)} (Huebner et al. 2007). By definition, substrates with a high prebiotic activity score support good growth of the probiotic bacteria, with cell densities (cfu/ml) comparable with that when grown on glucose.

However, the cell densities of the enteric strains grown on the prebiotics should, in theory, be very low relative to growth on glucose. Using this equation, the prebiotic activity score of a particular oligosaccharide can be determined in relation to any given strain.

Statistical analysis

Values of *in vitro* prebiotic assay were expressed as means and standard error of three observations. Statistical analysis was carried out using Data was analyzed using Statistical Analysis System (SAS, V. 6, SAS Institute Inc). Results were analyzed and the significance level was calculated using the Duncan multiple range test and results are considered significant at P<0·05.

Results and Discussion

Partial purification of GOS

GOS mixture produced in the laboratory was analyzed by high performance liquid chromatography (HPLC). Along with the produced GOS, standards of glucose, galactose, lactose and commercial GOS (Vivinal GOS) were also analyzed for comparison. The peaks thus obtained with the produced GOS were compared with the chromatogram of standards and designated as glucose, galactose (peak 1), lactose (peak 2) and GOS (peaks 3, 4 and 5) (Fig. 1). As calculated from the peak area amount of monosaccharides and disaccharides in crude preparation was 117 g/l (39.28% of mixture) and 61.13 g/l (24.45% of mixture) respectively. *S. cerevisiae*, *K. lactis* and *L. helveticus* were used for the removal of mono and disaccharides from the GOS mixture. After fermentation step the resulting mixture was again analyzed by HPLC. The peaks correspond to glucose, galactose (peak 1) and lactose (peak 2) were reduced significantly (Fig. 2). After fermentation the amount of monosaccharides was reduced to 10.8 g/l (3.64% of mixture) and that of disaccharides was 12.03 g/l (4.63 % of mixture).

***In vitro* prebiotic assay**

L. rhamnosus GG and *E. coli* were inoculated in three different types of growth media having different carbon sources viz. glucose, inulin and GOS. After inoculation the cell count of bacteria were taken at 0h and after 24 h. At 0h counts of *L. rhamnosus* GG on glucose, inulin and GOS were 6.18±0.03, 6.12±0.02 and 6.12±0.02 cfu/ml respectively. After 24 counts of *L. rhamnosus* GG on glucose, inulin and

GOS were increased to 9.38±0.3, 8.85±0.02 and 8.97±0.03 cfu/ml respectively (Fig. 3). The increase in log count of *L. rhamnosus* GG on glucose, inulin and GOS was 3.12, 2.73 and 2.85 respectively. Growth of this bacterium on both of the prebiotics was less compared with growth on glucose. In case of *E. coli,* at 0h counts on glucose, inulin and GOS were 6.61 ±0.02, 6.49 ±0.02 and 6.61 ±0.02 cfu/ml respectively. *E. coli* counts after 24h on glucose, inulin and GOS were 9.62 ±0.03, 7.36 ±0.02 and 7.67 ±0.02 cfu/ml respectively (Fig. 4). The increase in log count of *E. coli* on glucose, inulin and GOS was 3.01, 0.87 and 1.06 respectively. The calculated prebiotic activity score of inulin was 0.56 and that of GOS was 0.54.

Partial Purification of GOS

GOS was prepared in our laboratory using β-galactosidase extracted from native strain of *S. thermophilus*. Whey supplemented with desirable amount of lactose was used as a cost effective substrate for the production. We optimized various experimental conditions to increase the product yield. The GOS thus produced was found to be similar in composition to the commercial sample as analyzed by high performance liquid chromatography (HPLC) (Unpublished Results). The product contains glucose, galactose, lactose and GOS. Fractionation of oligosaccharides has been previously carried out using different techniques including diafiltration, yeast treatment, activated charcoal adsorption and SEC. Size exclusion chromatography (SEC) has been widely used for the separation of carbohydrates by many research groups (Tzortzis et al., 2005; Shoaf et al., 2006; Huebner et al., 2007).

The aim of this study was to use the simplest method of microbial fermentation to purify the GOS mixture. Yeast has already been used in studies dealing with the purification of GOS using microbial fermentation. In a study carried out by Hernandez et al (2009), highest concentration and recovery of tri- and tetrasaccharides was obtained with yeast treatment. However, the major disadvantage of the yeast treatment for the purification of GOS is the incapacity of the yeast cells to remove the disaccharides fraction. Kunova et al (2011) successfully used cells of *L. helveticus* CCDM 40 for GOS purification and reported the removal of mono- and disaccharide fraction form the oligosaccharide mixture up to a non significant level.

In the present work, purification of the GOS mixture was attempted by fermenting the crude production with *S. cerevisiae* NCDC 50, *K. lactis* PL 2 and *L. helveticus* NCDC 288. The primary reaction in the removal of monosaccharides by yeast is anaerobic glycolysis in which the carbohydrate is converted into ethanol and CO_2 (Yoon et al., 2003). Cells of *S. cerevisiae* were inoculated in crude production and incubated for 1-18 h. The optimum time of incubation was found to be 8 h. Extension of incubation beyond 8 h was found to result into reduction in the amount of GOS, suggesting their probable fermentation. Accordingly, 8 h was taken as the optimum time of incubation for monosaccharide removal using *S. cerevisiae*. After fermentation with *S. cerevisiae* the mixture was fermented with *K. lactis* for lactose removal. *K. lactis* is one of the few yeasts that can use the milk sugar lactose as a carbon and energy source, which suggests that this yeast may have evolved under different and unique selection pressures, particularly for carbon

sources, than have many other yeasts including *S. cerevisiae*. *K. lactis* grows slightly more rapidly with lactose as a carbon source than with glucose, but at least in some strains, glucose is the preferred carbon source since it represses expression of the genes necessary for utilization of lactose or galactose (Dong and Dickson 1997). Fermentation with *K. lactis* considerably reduced the amount of lactose in the GOS mixture. The ability of the yeast *K. lactis* to metabolize lactose results from the presence of a lactose permease and a lactase (β-galactosidase) (Rubio-Texeira 2005). Lactose uptake by *K. lactis* is mediated by a transport system inducible by lactose and galactose and is an active process that permits the intracellular accumulation of lactose against a concentration gradient (Boze et al., 1987). After this step, most of the glucose and lactose were removed from the mixture yet a residual amount of galactose remained unutilized. Domingues et al (2010) mentioned that *S. cerevisiae* cannot assimilate lactose, yet it can utilize galactose. In our study the levels of galactose were reduced only slightly although *S. cerevisiae* has been reported to metabolise it completely (Yoon et al., 2003). The reason for this observation could be that it is necessary for the yeast to be grown in nutrients containing galactose as the sole carbohydrate source in order for the mechanism to be activated. In contrast, glucose uptake is regulated by a constitutive mechanism that does not require prior activation in order to be triggered. In the specific purifications, the abundant presence of glucose led the yeast cells to metabolise it initially (due to the already activated uptake mechanism) leaving galactose initially unaffected (mechanism repressed by glucose).

To remove galactose out of the mixture, it was then fermented with *L. helveticus*.

Within the LAB, *L. helveticus* is an industrially important thermophilic starter for the fermentation of food products. *L. helveticus* is able to ferment the glucose and galactose moieties of lactose and does not accumulate free galactose in the external medium. Hickey et al (1986) observed that there is no lactose or galactose phosphotransferase system in *L. helveticus* and suggested that the galactose moiety of the lactose is metabolized via the Leloir rather than the tagatose 6-phosphate pathway. In this study *L. helveticus* was found to remove galactose from the mixture and after fermentation partially purified GOS mixture was obtained containing 91.71% GOS in the mixture. Use of microbial fermentation, for the purification of GOS, compared to other available purification methods like nanofiltration (Goulas et al., 2002) has the advantage that purification can be performed directly on the synthesis mixtures without the need for significant dilution (with nanofiltration, dilution to 100–150 mg/mL total sugars is necessary). However, in the case of microbial fermentation, removal of the microbial cells is necessary in order to obtain the purified GOS.

In Vitro **Prebiotic Assay**

Prebiotics have the ability to influence the population of the gastrointestinal tract due to their preferential utilization by selected members of the gastrointestinal microflora. Organisms that rapidly ferment prebiotic sugars are enriched, presumably at the expense of those that do not. The goal of this study, therefore, was to quantify the extent to which prebiotic sugar (GOS) express this activity using selected strain of *L. rhamnosus*. Two different cultivation methods were reported in the past for the evaluation of the prebiotic potency of different sugar mixtures.

**Fig.1** HPLC chromatogram showing GOS production
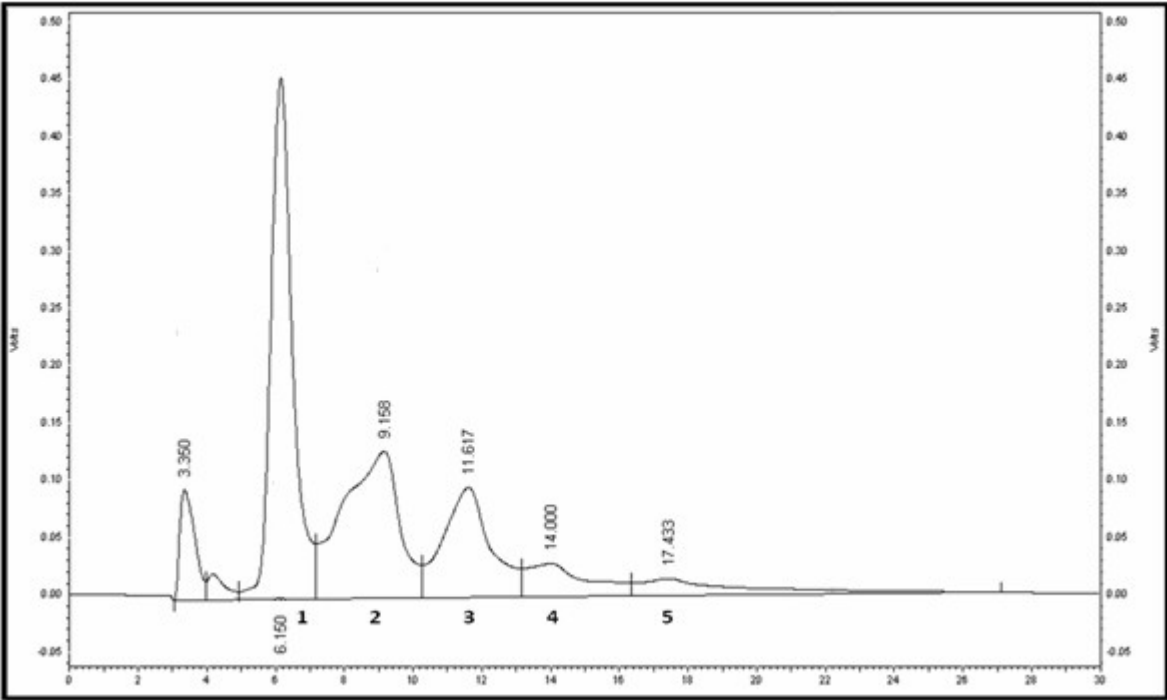
Peak 1: Combined peak of glucose and galactose; Peak 2: Lactose; Peak 3, 4 & 5: GOS

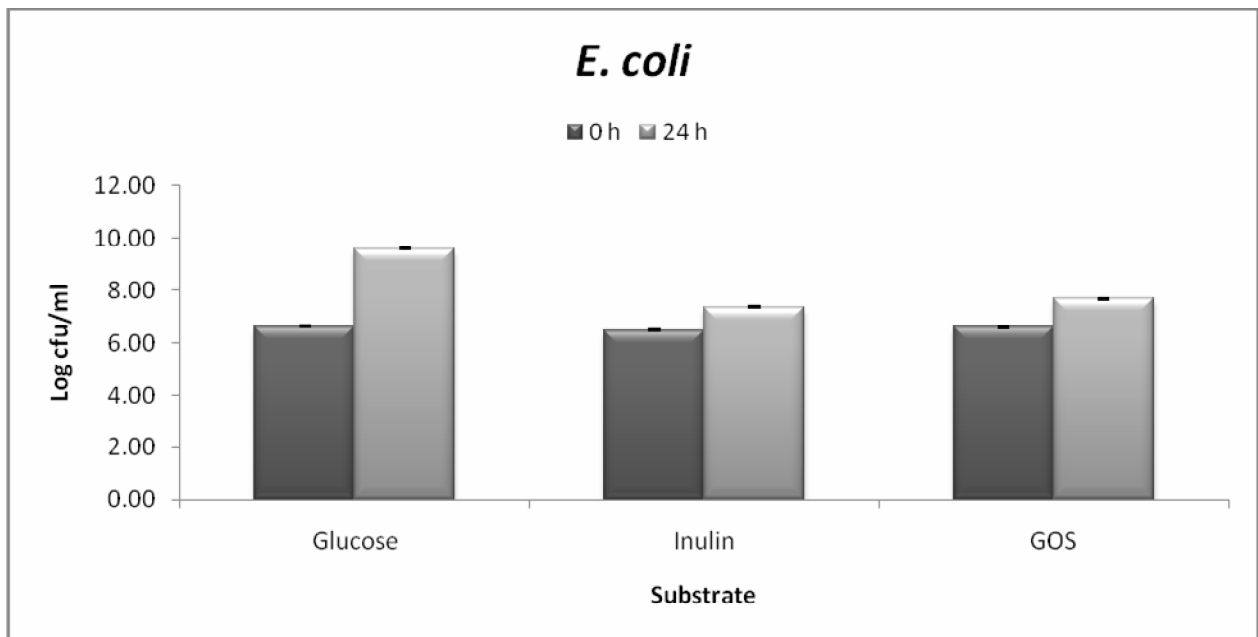**Fig.2** HPLC chromatogram of GOS Mixture after Microbial Fermentation
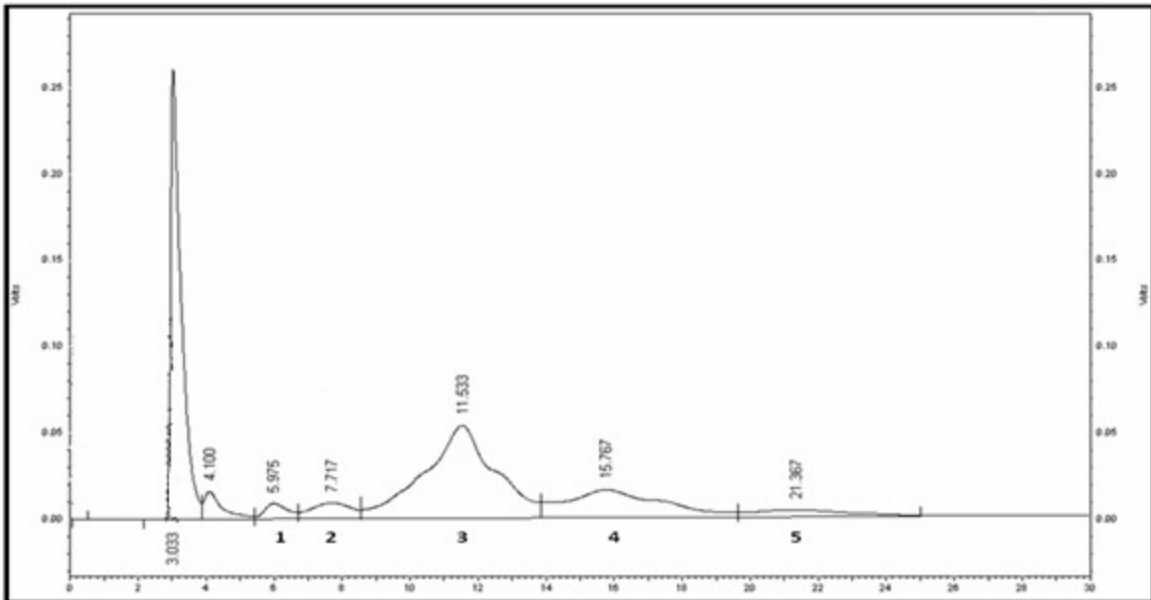
Peak 1: Combined peak of glucose and galactose; Peak 2: Lactose; Peak 3, 4 & 5: GOS

Fig.3 Growth of *L. rhamnosus* GG on different substrates

Values are mean ± SE (indicated error bars in graph) of 3 observations
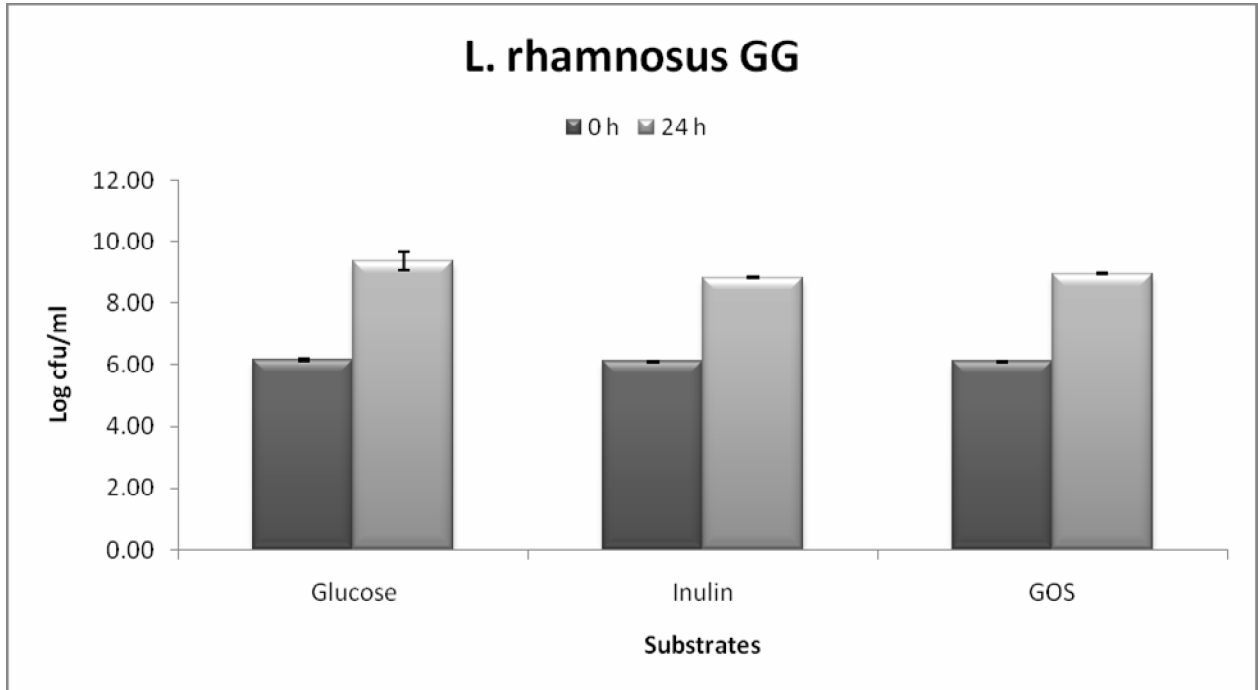
Fig.4 Growth of *E. coli* on different substrates

Values are mean ± SE (indicated error bars in graph) of 3 observations

A number of researchers employed pure culture fermentations to investigate the prebiotic effect (Kaplan and Hutkins 2000; Schrezenmeier and de Vrese 2001; Huebner et al., 2007), whereas others used a gut model to perform mixed culture fermentations (Sanz et al., 2005; Hughes et al., 2007). Gut model and faecal samples are not required in single culture fermentation and the fermentations are done in appropriate basal media depending on the probiotic strain and the increase in cell number is quantified by turbidometry or by viable cell count. In a number of *in vitro* studies (Vulevic et al., 2004; Sanz et al., 2005) prebiotic indices were based on changes in bacterial populations (of specific genera), substrate assimilation, growth rates, and/ or SCFA production, with each characteristic assigned a positive or negative effect on the calculated index. Unlike previous studies, our method to assess prebiotic activity evaluates the combination of a prebiotic with specific strains of putative probiotic bacteria and prebiotic index was calculated by comparison of the change in growth of the test strain on the prebiotic with the growth on glucose and to the growth of a mixture of commensal bacteria on the prebiotic and glucose. This method is relatively simpler than previously used methods because fecal samples are not required. In addition, it is a relatively quick way to evaluate a prebiotic's ability to be utilized by specific strains of bacteria.

There are a number of studies which used pure cultures of bifidobacteria, lactobacillus and pediococcus for the assessment of their ability to utilize different prebiotics including GOS, FOS and lactulose and it was reported that the ability to utilize prebiotic substrates was highly variable among organisms (van Laere et al., 2000; Gopal et al., 2001).

Among all the prebiotics GOS was found to be a preferred substrate over other prebiotics. Authors showed a perfect correlation between the ability of strains to utilize GOS and the presence of enzyme β-galactosidase. Rycroft et al (2001) used various prebiotics as substrates, in pH/temperature controlled anaerobic batch cultures inoculated with fecal homogenates, and showed that all prebiotics increased the numbers of bifidobacteria but GOS resulted in the largest decrease of clostridial population.

In this study the prebiotic effect of a GOS mixture free of monosaccharides (inconsiderable amount) and lactose was investigated using pure culture fermentation. *L. rhamnosus* GG and *E. coli* were used in pure culture fermentations to determine the prebiotic potency score on GOS mixture. As the commercially available GOS was not pure (containing glucose, galactose and lactose in considerable amount), well established prebiotic inulin (HiMedia) was used as a standard prebiotic for the comparison purpose. From the increase in cell number the prebiotic potency score was calculated. The substrate preferences of *L. rhamnosus* GG and *E. coli* were studied by incubating the bacteria with Glucose, Inulin and GOS. For a given sugar to have prebiotic activity, that sugar should be metabolized by a test strain as well, or nearly as well, as glucose is metabolized (Huebner et al., 2007). The other characteristic property of a prebiotic substrate is that it should be selective and not fermented by commensal organisms. Therefore, growth on each prebiotic was also determined for enteric bacteria *E. coli*, chosen to represent the enteric portion of the commensal flora. Increase in log count of *L. rhamnosus* GG was higher on both of the prebiotic tested as compared to *E. coli*. GOS was

metabolized by both *L. rhamnosus* GG and *E.coli*. But the increase in the log count of *E. coli* (1.06) was less as compared to *L. rhamnosus* GG (2.85), which showed that *L. rhamnosus* GG can utilize GOS more efficiently than *E. coli*, as a substrate. The growth of *E. coli* on inulin was less as compare to GOS. This may be due to the presence of residual monosaccharides and disaccharides in GOS mixture. The calculated prebiotic activity score of inulin was 0.56 and that of GOS was 0.54. The prebiotic activity scores reported in this study reflect the extent to which a given carbohydrate would promote selective growth of specific organism. This kind of activity can be extended to other commercially important probiotic organisms and the result thus obtained will provide a rational basis for identifying synbiotics for incorporation into dairy and other foods.

Huebner et al (2007) derived prebiotic activity score from the cell density values. The highest prebiotic activity scores were for *Lactobacillus paracasei* 1195 paired with Inulin-S, Raftiline HP, and Raftilose P95 (1.17, 1.10, and 0.99, respectively), followed by *Lactobacillus plantarum* 4008, *Lactobacillus acidophilus* 33200 and *L. acidophilus* NCFM grown on purified GOS, and *L. acidophilus* NCFM grown on Raftilose P95 (0.82, 0.70, 0.66, and 0.58, respectively). *L. plantarum* 12006 and *L. acidophilus* 33200 had prebiotic activity scores below zero when grown on all the prebiotics, except purified GOS. A low or negative prebiotic activity score was obtained if the test strain grew less well (based on cell densities) on the prebiotic compared with that on glucose and/or had less growth on the prebiotic than the growth of the enteric mixture on the prebiotic carbohydrate. Gugler (2008) reported the highest prebiotic potency

scores for *B. animalis lactis* Bif3, *L. reuteri* Lb46 and *B. animalis* Bifl when cultivated on their product GOS (8.45, 7.52, and 7.25, respectively) followed by *B. animalis lactis* Bif3 (6.92) and *L. reuteri* Lb46 (6.53) paired with Vivinal GOS. For *L. rhamnosus* Lb29, *L. paracasei* Lb 16 and *L. casei* Lb20 the lowest prebiotic potency scores were calculated when grown with all three prebiotic sugars. Also *B. longum* Bifl4 has a negative prebiotic potency score (-0.36) when grown on trans-galactooligosaccharides (TOS). The bifidobacteria strains in general had lower prebiotic potency scores paired with TOS in comparison to Vivnal GOS and GOS.

However, it is important to recognize two important factors. First, in the gastrointestinal tract, commensal organisms likely exist that, unlike the *E. coli* strains used in this study, will have better ability to utilize prebiotic carbohydrates. It now appears that apart from some well-known probiotic bacteria in the gut bacteroides and other resident members of intestinal microflora have the metabolic capacity to metabolize these substrates (Van der Meulen et al., 2006). In addition, the specific means by which metabolism of prebiotic carbohydrates occurs is also relevant with respect to their prebiotic activity. If, for example, a particular organism initiates metabolism of an oligosaccharide via extracellular hydrolysis, the products (mono- or disaccharides) that are released may then ''cross-feed'' other organisms.

Probiotic organism behaved quite differently in their capacity to utilize prebiotics which depends largely on their metabolic system. Utilization of prebiotics by bacteria requires the presence of specific hydrolysis and transport systems for the particular prebiotic (Kaplan and

Hutkins 2003). Therefore, genes coding for these metabolic systems may be present or absent in the different strains, resulting in varied prebiotic activity scores.

Presence of mono- and disaccharides in the product is highly undesirable and is the main reason of their low production throughout the world. We observed that use of microbes for the purification purpose is an efficient and cost effective method to improve the quality of the product. In this study in vitro method was used to assess the utilization of GOS by *L. rhamnosus* GG and *E. coli*. When compared with well know prebiotic inulin, GOS was found to have comparable effect on the growth of both the organisms. *In vitro* models aim at studying prebiotic effects independently from their passage through the upper parts of the GI tract even if digestion is sometimes partly simulated. These models are thus only indicative of a potential prebiotic effect, however, they do not prove the prebiotic attribute of a particular product as *in vivo* studies need to be performed to definitively demonstrate that the compound under investigation selectively stimulates the growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confers health benefits to the host.

Acknowledgement

References

Boze, H., Moulin, G., and Galzy, P. 1987. Galactose and lactose transport in *Kluyveromyces lactis*. Folia Microbiol. 32, 107-111.

Domingues, L., Guimaraes, P. M. R., and Oliveira, C. 2010 Metabolic engineering of *Saccharomyces cerevisiae* for lactose/whey fermentation. Bioeng Bugs. 1(3), 164-171.

Dong, J., and Dickson, R. C. 1997. Glucose represses the lactose–galactose regulon in *Kluyveromyces lactis* through a SNF1 and MIG1-dependent pathway that modulates galactokinase (GAL1) gene expression. Nucleic Acids Res. 25(18), 3657–3664.

Figueroa-Gonzalez, I., Quijano, G., Ramirez, G., and Cruz-Guerrero, A. 2011. Probiotics and prebiotics: perspectives and challenges. J Sci Food Agric. 91, 1341–1348.

Gopal, K. P., Sullivan, P. A., and Smart, J. B. 2001. Utilisation of galacto-oligosaccharides as selective substrates for growth by lactic acid bacteria including *Bifidobacterium lactis* DR10 and *Lactobacillus rhamnosus* DR20. Int Dairy J. 11, 19–25.

Goulas, A. K., Kapasakalidis, P. G., Sinclair, H. R., Rastall, R. A., and Grandison, A. S. 2002. Purification of oligosaccharides by nanofiltration. J Memb Sci. 209(1), 321-335.

Hernandez, O., Ruiz-Matutea, A. I., Olanob, A., Morenob, F. J., and Luz Sanza, M. 2009. Comparison of fractionation techniques to obtain prebiotic galactooligosaccharides. Int Dairy J. 19(9), 531–536.

Hickey, M. W., Hillier, A. J., and Jago, G. R. 1986. Transport and metabolism of

lactose, glucose, and galactose in homofermentative lactobacilli. Appl Environ Microbiol. 51, 825:831.

Huebner, J., Wehling, R. L., and Hutkins, R. W. 2007. Functional activity of commercial prebiotics. Int Dairy J. 17, 770–775.

Hughes, S. A., Shewry, P. R., Li, L., Gibson, G. R., Sanz, M. L., and Rastall, R. A. 2007. *In vitro* fermentation by human fecal microflora of wheat arabinoxylans. J Agricult Food Chem. 55, 4589-4895.

Kaplan, H., and Hutkins, R. W. 2000. Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria. Appl Environ Microbiol. 66(6), 2682-2684.

Kaplan, H., and Hutkins, R. W. 2003. Metabolism of fructooligosaccharides by Lactobacillus paracasei 1195. Appl Environ Microbiol. 69, 2217–2222.

Kunova, G., Rada, V., Lisova, I., Rockova, S., and Vlkova, E. 2011. *In vitro* Fermentability of Prebiotic Oligosaccharides by Lactobacilli. Czech J Food Sci. 29, S49–S54.

Macfarlane, G. T., Steed, H., and Macfarlane, S. 2008. Bacterial metabolism and healthrelated effects of galacto-oligosaccharides and other prebiotics. J Appl Microbiol. 104, 305–344.

Ohr, L. M. 2010. Health benefits of probiotics and prebiotics. Food Technol. 64, 59–64.

Rada, V., Nevoral, J., Trojanova, I., Tomankova, E., Smehilova, M., and Killer, J. 2008. Growth of infant faecal bifidobacteria and clostridia on prebiotic oligosaccharides in *in vitro* conditions. Anaerobe. 14, 205–208.

Rubio-Texeira, M. 2005. A comparative analysis of the *GAL* genetic switch between not-so-distant cousins: *Saccharomyces*

cerevisiae versus *Kluyveromyces lactis*. FEMS Yeast Res. 5, 1115-1128.

Rycroft, C. E., Jones, M. R., Gibson, G. R., and Rastall, R. A. 2001. A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides. J Appl Microbiol. 91, 878–887.

Sangwan, V., Tomar, S. K., Singh, R. R. B., and Ali, B. 2011. Galactooligosaccharides: novel components of designer foods. J Food Sci. 76, R103–R111.

Sanz, M. L., Polemis, N., Morales, V., Corzo, N., Drakoularakou, A., and Gibson, G. R. 2005. *In vitro* investigation into the potential prebiotic activity of honey oligosaccharides. J Agricult Food Chem. 53, 2914–2921.

Schrezenmeir, J., and Vrese, M. 2001. Probiotics, prebiotics and synbiotics-approaching a definition. Am J Clin Nutr. 73, 361-364.

Shoaf, K., Mulvey, G. L., Armstrong, G. D., and Hutkins, R. W. 2006. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. Infect Immun. 74, 6920–6928.

Tzortzis, G., Goulas, A. K., Gee, J. M., and Gibson, G. R. 2005. A novel galactooligosaccharides mixture increases the bifidobacterial population numbers in a continuous *in vitro* fermentation system and in the proximal colonic contents of pigs *in vivo*. J Nutr. 135, 1726-1731.

Van der Meulen, R., Makras, L., Verbrugghe, K., Adriany, T., and De Vuyst, L. 2006. *In vitro* kinetic analysis of oligofructose consumption by Bacteroides and Bifidobacterium spp. indicates different degradation mechanisms. Appl Environ Microbiol.

72, 1006–1012.

van Laere, K. M., Abee, T., Schols, H. A., Beldman, G., and Voragen, A. G. 2000. Characterization of a novel beta-galactosidase from *Bifidobacterium adolescentis* DSM 20083 active towards transgalactooligosaccharides. Appl Environ Microbiol. 66, 1379–1384.

Vulevic, J., Rastall, R. A., and Gibson, G. R. 2004. Developing a quantitative approach for determining the in vitro prebiotic potential of dietary oligosaccharides. FEMS Microbiol Lett. 236, 153–159.

Whisner, C. M., Martin, B. R., Schoterman, M. H. C., Nakatsu, C. H., McCabe, L. D., McCabe, G. P., Wastney, M. E., van den Heuvel, E. G. H. M., and Weaver, C. M. 2013. Galacto-oligosaccharides increase calcium absorption and gut bifidobacteria in young girls: a double-blind cross-over trial. Br J Nutr. 14, 1-12.

Yoon, S. H., Mukerjea, R., and Robyt, J. F. 2003. Specificity of yeast (*Saccharomyces cereisiae*) in removing carbohydrates by fermentation. Carbohydr Res. 338, 1127–1132.

.