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# Investigation on the prebiotic effect of a novel galacto-oligosaccharide mixture: using pure and mixed culture fermentation

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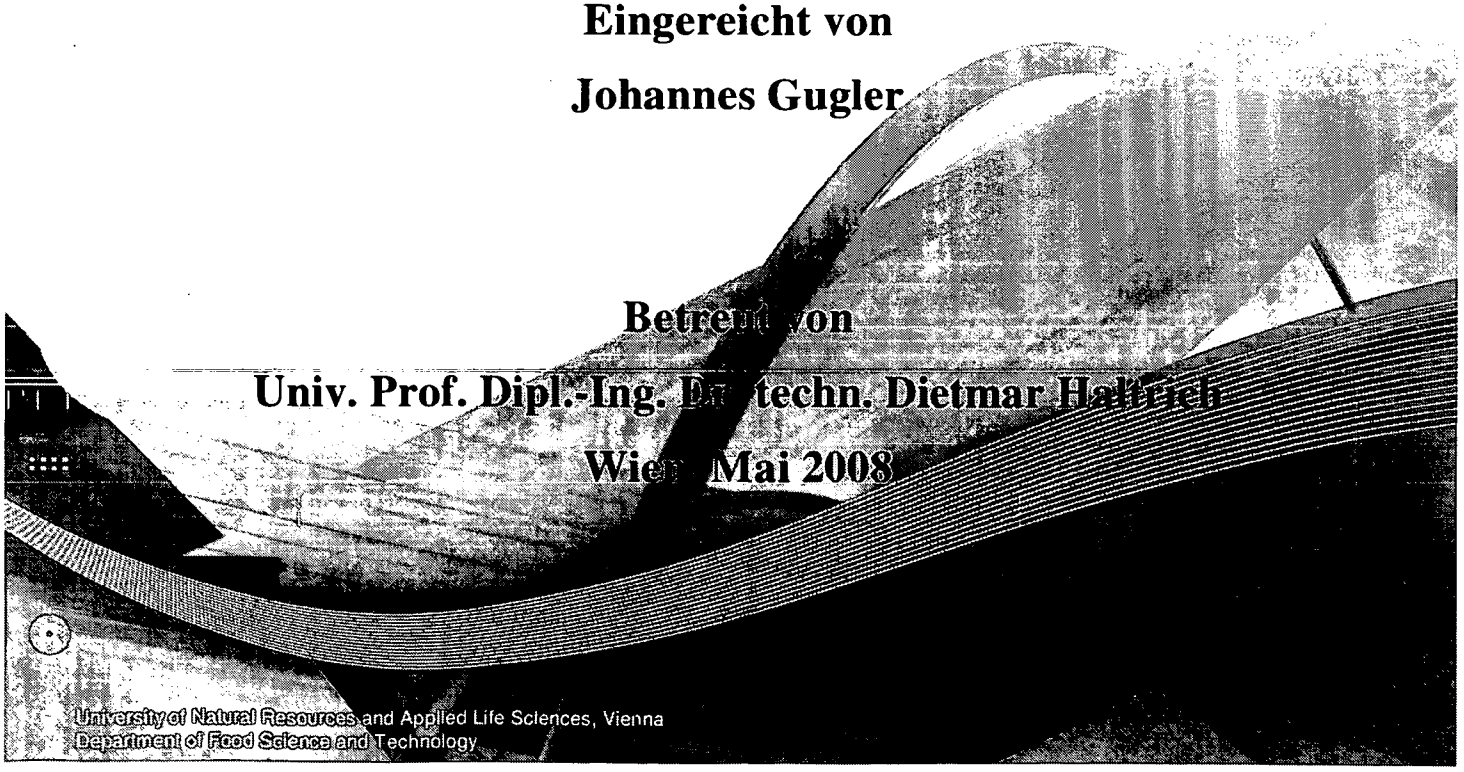

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ABTS | 2,2'-Azino-bis-(3-ethylenbenzthiazolin 6-sulfoniacid) |
| CDH | Cellobiose deyhdrogenase |
| CE | Capillary elecrophoresis |
| DCIP | 2, 6 Dichloroindophenol |
| FISH | Fluorescence *in situ* hybridization |
| FOS | Fructo-oligosaccharides |
| Gal | Galactose |
| Glu | Glucose |
| GOD | Glucose oxidase |
| GalOS | Galacto-oligosaccharides |
| GalOS60 | Galacto-oligosaccharides 60% conversion |
| HPAEC-PAD | High performance anion exchange chromatography with pulsed amperometric detection |
| HPLC | High performance liquid chromatography |
| Lac | Lactose |
| NADH | Reduced form of nicotinamide adenine dinucleotide |
| OD | optical density |
| *o*NP | *o*-Nitrophenyl |
| *o*NPG | *o*-Nitrophenyl-β-D-galactopyranoside |
| PBS | Phosphate buffer solution |
| POD | Peroxidase |
| SCFA | Short chain fatty acids |
| SDS | Sodium dodecyl phosphate |
| tp | time point |
| TLC | Thin layer chromatography |
| TOS | Trans-galacto-oligosaccharides |
| SD | Standard deviation |

# ABSTRACT

The work of this diploma thesis describes the production, purification of a novel galacto-oligosaccharide (GalOS) mixture and the evaluation of the prebiotic efficiency using pure and mixed culture fermentations.

Several galacto-oligosaccharide mixtures are known as prebiotics. Commercial available GalOS still contain significant amounts of monosaccharides and lactose. To evaluate the prebiotic effect of these oligosaccharides the remaining monosaccharides and lactose have to be removed. The novel GalOS were produced by a β-galactosidase from *Lactobacillus reuteri* L103 which is a probiotic bacterium. Biocatalytic approaches coupled with subsequent chromatographic steps were used to purify the GalOS mixture. Lactose was converted into lactobionic acid by a reaction system containing cellobiose dehydrogenase (CDH) and laccase and the redox mediator 2, 6 dichloroindophenol. Different cation and anion exchange chromatography steps were used to remove ions and monosaccharides. The composition of the monosaccharide and lactose free GalOS was analysed to be 33.5% di-, 60.5% tri- and 4.8% tetra- and 1.0% pentasaccharides with an inconsiderable amount of monosaccharides, lactose and lactobionic acid (0.3%). To evaluate the prebiotic efficiency bacterial growth characteristics in pure culture cultivations as well as in fermentations with human faeces were investigated. In these study fructo-oligosaccharides (FOS), trans-galacto-oligosaccharides (TOS), commercial available Vivinal GOS and "self made" galacto-oligosaccharides (GalOS) were compared. The increase of bacteria in pure cultures was measured by automated turbidimetry and microflora changes in batch fermentations were monitored by fluorescent *in situ* hybridization. Additionally, lactic acid and short chain fatty acids (SFCA) from batch fermentations were measured by high performance liquid chromatography. The prebiotic potency score was calculated for the pure culture cultivations referring to the changes in bacterial numbers which gave promising results. Using faecal batch fermentations significant increase in bifidobacteria population was observed after 5 h on all oligosaccharides. Combining other bacterial groups with the three sugar mixtures didn't show significant changes in growth. All cultures used here showed rises in total SCFA production over

fermentation time. The predominant SCFA was acetic acid in all three substrates. This suggests that monosaccharide and lactose free GalOS can effect the bacterial population in the intestine, especially bifidobacteria species.

# ZUSAMMENFASSUNG

Diese Arbeit beschäftigt sich mit der Produktion, Reinigung und der Bewertung der präbiotischen Wirkung von Galaktooligosacchariden im Vergleich zu bereits kommerziell erhältlichen präbiotischen Zuckern.

Galaktooligosaccharide sind eine Saccharidmischung mit unterschiedlicher Kettenlänge im Bereich von 2 bis 10 Molekülen. Aufgrund ihrer positiven Eigenschaften als Präbiotika werden sie bereits erfolgreich als Zusatzstoffe in der Lebensmittelindustrie eingesetzt. Galaktooligosaccharide werden durch die transgalaktosilierende Aktivität von β-Galaktosidasen hergestellt. Als Substrat dient das Disaccharid Laktose (Milchzucker). Die Zusammensetzung des Produktes wird von den Reaktionsbedingungen und von der Herkunft des Enzymes bestimmt. Folge dessen wird angenommen, dass eine Galaktooligosaccharidmischung, hergestellt von einer β-Galaktosidase, die aus einem probiotischen Organismus isoliert wurde, eine besser verwertbare Mischung für probiotische Bakterien darstellt. Probiotische Bakterien sind Bakterien, die natürlich im Magen- Darm- Trakt vorkommen.
In dieser Studie wurde eine β-Galaktosidase von dem probiotischen Stamm *Lactobacillus reuteri* L103 isoliert und für die Umsetzung von Laktose zu Galaktooligosacchariden verwendet. Das Produkt enthielt beträchtliche Mengen an Laktose, die mit Hilfe einer enzymatischen Reaktion zu Laktobionsäure oxidiert wurden und dann durch chromatische Verfahren abgetrennt werden konnten. Die noch in der Zuckermischung enthaltene Glukose und Galaktose wurden ebenfalls durch chromatographische Verfahren abgetrennt, wodurch schließlich eine Reinheit von 99,7% erzielt werden konnte. Die Untersuchung auf die präbiotische Wirkung der gefriergetrockneten GalOS erfolgte mittels Wachstumsversuchen mit Reinkulturen (durchgeführt von DI Thomas Maischberger) und *in-vitro* Fermentationenexperimenten. Bei den Reinkulturexperimenten wurden verschiedene Reinkulturen (probiotische und pathogene Kulturen) ausgetestet, ob diese auf den präbiotischen Substraten wachsen können. Zur Messung wurde eine automatische

Trübungsmessung verwendet und für die Vergleichbarkeit der präbiotischen Wirkung der verwendeten Zucker wurde der „Prebiotic Potency Score" (PPS) berechnet. Bei den Fermentationsexperimenten wurde ermittelt, ob ausgewählte Darmbakterien die Fähigkeit besitzen, GalOS als Substrat zu verwerten. Dafür wurden Fermentationsexperimente mit Humanfäces, durchgeführt. Bei den Fermentationen wurden an unterschiedlichen Zeitpunkten Proben genommen, und relevante Bakterienstämme mittels Fluoreszenz in- *situ hybridization* (FISH) quantifiziert. Mit Hilfe dieser Daten konnte auf die präbiotische Wirksamkeit der Saccharidmischung geschlossen werden. Weiters wurden spezifische Stoffwechselprodukte, wie kurzkettigen Fettsäuren und Milchsäure, analysiert. Um die präbiotische Wirkung vergleichen zu können, wurden Parallelversuche mit Fruktooligosacchariden und Transgalaktooligosacchariden, ebenfalls etablierte Präbiotika, durchgeführt. Um statistische Aussagen treffen zu können wurden alle Experimente als Dreifachbestimmung durchgeführt.

Die GalOS zeigten in den Reinkulturen einen beachtlichen PPS, im Besonderen bei den Bifidobakterien. Es konnte ebenfalls eine sehr gute präbiotische Wirkung bei den Fermentationsexperimenten nachgewiesen werden. Auch hier konnte ein signifikanter Anstieg an Bifidobakterien nachgewiesen werden. Auch andere Bakteriengruppen, wie die Gruppe der Laktobazillen oder Bakteroiden, konnten sich auf dem Substrat vermehren. Verglichen zu den etablierten Substraten konnten sich die selbst produzierten, monosaccharid- und laktosefreien GalOS als sehr gute Präbiotika durchsetzen.

Die kurzkettigen Fettsäuren, die Stoffwechselprodukte der Bakterien, zeigten ebenfalls einen erheblichen Konzentrationsanstieg. Obwohl sich einige Bakteriengruppen nicht signifikant vermehrten, konnte man über die spezifischen Stoffwechselprodukte erkennen, dass sie das Substrat metabolisieren können.

# AIM OF THE WORK

The aim of this project was

1. to produce a novel galacto-oligosaccharide mixture free of monosaccharides and lactose using the high galactosyl-transferase activity of the β-galactosidase from *Lb. reuteri* L103.

2. to use this prebiotic sugar mixture to compare the prebiotic potency with TOS, FOS and the commercial available Vivinal GOS in growth experiments using pure cultures and automated turbidimetry and small scale faecal batch fermentations.

MANUSCRIPT FOR A PUBLICATION

# INVESTIGATION ON THE PREBIOTIC EFFECT OF A NOVEL GALACTO-OLIGOSACCHARIDE MIXTURE: USING PURE AND MIXED CULTURE FERMENTATION

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## ABSTRACT

The prebiotic effect of a novel galacto-oligosaccharide mixture free of monosaccharides and lactose was investigated using pure and mixed culture fermentation. The prebiotic oligosaccharide mixture was produced from lactose using β-galactosidase from *Lactobacillus reuteri* L103. To remove the monosaccharides and lactose a biocatalytic oxidation was combined with several chromatographic separation steps. The composition of the finally obtained GalOS mixture was 33.5% di-, 60.5% tri- and 4.8% tetra- and 1.0% pentasaccharides with an inconsiderable amount of monosaccharides, lactose and lactobionic acid (0.3%). Eight lactobacilli and three bifidobacteria strains were used in pure culture fermentations to determine the prebiotic potency score on three different prebiotic sugar mixtures (GalOS, TOS, Vivinal GOS). From the increase in cell biomass the prebiotic potency score was calculated obtaining highest scores for *Lb. reuteri* Lb46 and the three bifidobacteria strains grown on these novel GalOS. Furthermore, mixed culture fermentations on human faecal samples were carried out using GalOS, TOS and FOS as carbohydrate source. Five different bacterial groups were enumerated by fluorescent in situ hybridisation and short chain fatty acids as well as lactic acid production were measured by high performance chromatography. GalOS and TOS seem to be the most promising prebiotic sugars by enhancing the population of bifidobacteria significantly. Lactic- and acetic acid were the main acids produced when cultivating the strains on media supplementation with all the different prebiotics substrates. These two cultivation methods were compared to their usefulness to determine the prebiotic efficiency of different oligosaccharides. The TOS used in single culture cultivation did not show a promising prebiotic effect on bifidobacteria compared to the usage in mixed culture fermentation. The supplementation of GalOS in mixed culture fermentation displayed moderate increase in lactobacilli, but high lactate production. When using this substrate in pure culture fermentation the increase in lactobacilli was capacious.



**Keywords:** Prebiotics, galacto-oligosaccharides, GOS, in vitro fermentation, SCFA, pure culture, prebiotic potency score

# INTRODUCTION

Inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GalOS), lactulose, lactosucrose, isomalto-oligosaccharides, soybean oligosaccharides, xylo-oligosaccharides (XOS), gentio-oligosaccharides and pyrodextrines can be subsumed under the term "prebiotics". Gibson et al. propose that "A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (1). These no-digestible, short-chain carbohydrates, mainly available in the Japanese, European and US markets, with their physiological importance and health benefit, have been reported extensively in several reviews on prebiotics. Prebiotic oligosaccharides have been found to alter the composition of the microbiota, both in the lumen and at the mucosal surface, by stimulation of the beneficial bacteria (such as lactobacilli and bifidobacteria) and inhibition of "undesirable" bacteria (such as clostridia and Bacteroides) (2, 3). Prebiotics also beneficially affect the bowel function (4, 5) enhance the absorption of calcium, magnesium and iron (6-11) and reduce the risk of colon cancer. In addition to these confirmed functional effects, postulated effects include reduction of intestinal disturbances (i.e. traveller's diarrhoea) and improvement of the well-being (12), reduction of the serum cholesterol level, improved bioavailability (3) and anti-inflammatory effects (13).

Beside these "healthy" effects prebiotics also act as carbon and energy sources for bacteria growing in the gut, where they are fermented to short chain fatty acid and are energy sources for different body tissues. Because of these benefits, prebiotic oligosaccharides are of great interest for both, human and animal nutrition.

The oligosaccharides GalOS, TOS and FOS are researched reasonably well and are already in use as food ingredients (14). FOS featuring a degree of polymerisation between 2- 60 (inulin) and 2- 20 (oligofructose) are one of the best known prebiotics (15). Related studies demonstrate the improvement of health and welfare in various perspectives for humans and animals (16, 17).

TOS are indigestible mixtures of oligosaccharides, which are produced from lactose by enzymatic transgalactosylation. TOS are linear oligosaccharides consisting of lactose and several galactose molecules in β-(1→6) and β-(1→4) bonds and cause disputed prebiotic impacts on the gut micro flora (18, 19).

GalOS occur naturally in human milk and are believed to influence the microflora in the gastro-intestinal tract (20). The bifidogenic effect of GalOS was confirmed in previous studies where the number of bifidobacteria of breast fed infants is higher than in bottle fed infants (21, 22). GalOS are produced enzymatically from lactose by the galactosyl-transferase activity of β-galactosidase (23). The galactosyl-transferase activity comes along with a hydrolysis activity and so commercial available GalOS mixtures normally contain great amounts of glucose and galactose (24, 25). For in vitro fermentations the purity of the used prebiotic carbohydrates is of particular importance.

Several studies have shown that the ability of probiotic strains to ferment prebiotic sugars is both strain and substrate specific (26-29) and several quantitative approaches were done to investigate the positive effect of different prebiotics during in vitro fermentations (30, 31). To quantify the prebiotic potency of indigestible carbohydrates there is a special need to have a closer look on the fermentation behaviour of specific single strains as well as on the degradation and metabolization of these oligosaccharides in mixed culture cultivations.

The aims of this project were to [1] produce GalOS, free of lactose and monosaccharides, using the high galactosyl-transferase activity of the β-galactosidase from *L. reuteri* L103 and [2] to use this prebiotic sugar mixture to compare the prebiotic potency with TOS, FOS and the commercial available Vivinal GOS (Friesland, Borculodomo Ingredients, Borculo, The Netherlands) in growth experiments using pure cultures with automated turbidimetry and small scale faecal batch fermentations.

# MATERIAL AND METHODS

## CHEMICALS

All chemicals were supplied by Sigma (St. Louis, MO, USA), Fluka (Buchs, Switzerland) and Oxoid (Basingstoke, UK). The testkit for the determination of D-galactose/lactose was from Megazyme (Bray, Ireland).

## BACTERIAL STRAINS

*Bifidobacterium animalis* Bf1, *B. animalis lactis* Bf3, *B. longum* Bf14, *Lactobacillus paracasei* Lb16, *Lb. acidophilus* Lb19, *Lb. casei* Lb20, Lb. reuteri Lb21, *Lb. rhamnosus* Lb29, *Lb. reuteri* Lb46, *Lb. acidophilus* Lb71, *Lb. acidophilus* Lb105, *Escherichia coli* Ec1, *Enterococcus faecium* En61, *Staphylococcus epidermis* St2 (Culture collection University of Natural Resources and Applied Life Sciences, Dept. of Food Sciences and Technology, Div. of Food Microbiology and Hygiene), *Klebsiella oxytoca* DSM 6673, *Citrobacter freundii* DSM 30039 (DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschwaig, Germany) were used for this study. The Bifidobacterium cultures were maintained in BHI broth medium (beef heart and calf brain 17.5 $g \cdot L^{-1}$, $Na_2HPO_4 \cdot 2H_2O$, peptone 10 $g \cdot L^{-1}$, NaCl 5 $g \cdot L^{-1}$, L-cysteine HCl 0.5 $g \cdot L^{-1}$) supplemented with 1% glucose and 15% (w/v) glycerol at -80°C. The lactobacilli cultures were maintained at -80°C in MRS broth (casein peptone 10 $g \cdot L^{-1}$, meat extract 8 $g \cdot L^{-1}$, yeast extract 4 $g \cdot L^{-1}$, $KH_2PO_4$ 2 $g \cdot L^{-1}$, di-ammonium hydrogen citrate 2 $g \cdot L^{-1}$, Sodium citrate$\cdot 3H_2O$ 5 $g \cdot L^{-1}$, $MgSO_4 \cdot 7H_2O$ 0.2 $g \cdot L^{-1}$, $MnSO_4 \cdot H_2O$ 0.02 $g \cdot L^{-1}$, Tween 80 1 $mL \cdot L^{-1}$, ) containing 2% lactose and 15% (w/v) glycerol. These eleven bacterial cultures are classified as probiotic bacteria. For the frozen stock cultures of *E. coli* Ec1, *K. oxytoca* and *C. freundii* nutrient broth (peptone 5 $g \cdot L^{-1}$, meat extract 3 $g \cdot L^{-1}$) with 15% (w/v) glycerol and for the strains *St. epidermis* and *E. faecium* corynebacterium broth (casein peptone 10 $g \cdot L^{-1}$, yeast extract 5 $g \cdot L^{-1}$, NaCl 5 $g \cdot L^{-1}$) supplemented with 0.5% glucose and 15% (w/v) glycerol was used. For this study these five strains are summarized under the term "pathogens". Gram staining was carried out to control the purity of the cultures.

## PREBIOTICS

The commercial prebiotic Vivinal GOS used in this study was obtained from Friesland, Borculodomo Ingredients (Borculo, The Netherlands) supplied with a purity of 60% (20% monosaccharides, 20% lactose, 19.8% disaccharides, 23.4% trisaccharides, 10.8% tetrasaccharides, 6% pentasaccharides).

The Fructo-oligosaccharides (FOS, Raftilose P95) composed of 95% of oligosaccharides, mainly β(1→2) fructan, and 5% of glucose, fructose and sucrose were purchased from Orafti (Tienen, Belgium)

The Trans-galacto-oligosaccharide (TOS) mixture with a purity of 99.9% was procured from Yakult Honsha (Tokyo, Japan). The composition of this sugar mixture was analysed to have 54.9% tri-, 31.4% tetra-, 11.6% penta- and 2.1% hexasaccharides, which are mainly β(1→4) linked.

The Galacto-oligosaccharides (GalOS) were produced and purified according to the procedure described by Maischberger et al. (32). For the production of the oligosaccharide mixture the β-galactosidase from the probiotic strain *Lb. reuteri* L103 was used (33). The reaction mixture contained 5 $U \cdot mL^{-1}$ of enzyme, 206.5 $g \cdot L^{-1}$ lactose, 50 mM sodiumphosphate and 1 mM $MgCl_2$. The pH value was set to 6.5 and the temperature was controlled at 23°C. The conversion was stopped when 73% of the lactose was converted. The composition of the newly formed oligosaccharide mixture was analysed by capillary electrophoreses (CE) and high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (48% (w/w) monosaccharides, 26.5% (w/w) lactose, 9.8% (w/w) non-lactose disaccharides, 14.7% (w/w) trisaccharides, and 1.0% (w/w) tetrasaccharides). To remove all lactose from the sugar solution the enzymatic conversion to lactobionic acid (lactobiono- δ-lactone) was used as described in Splechtna et al. (34). In a batch-wise reaction the enzyme cellobiose dehydrogenase (CDH) from the fungus *Sclerotium rolfsii* was used for the oxidation of lactose to lactobionic acid. This conversion is executed by a reaction system with CDH, laccase from *Trametes pubescens* MB 89 and the redox mediator 2,6-dichloro-indophenol (DCIP). The oxidation reaction is coupled to reduce DCIP, while the fungal laccase regenerates continuously the reduced DCIP by reducing molecular oxygen to water. The CDH-laccase batch was carried out at 30°C

with oxygen aeration containing 2.5 mM DCIP, 1 $U \cdot mL^{-1}$ CDH and 2.5 $U \cdot mL^{-1}$ laccase. The pH of 4.0 was kept constant during the whole reaction by adding 1 M acetic acid and 1 M sodium carbonate solution. After stopping the reaction the DCIP was removed by vacuum filtration through a filter paper coated with active coal powder.

By the use of a strong cation exchange resin, Lewatit® S 2528 (Lewatit, Bayer AG, Leverkusen) and a medium basic anion exchange resin, Lewatit® S4328 (Lewatit, Bayer AG, Leverkusen) ion exchange chromatography was performed to remove lactobionic acid as well as ions. The deionised GalOS solution was concentrated by vacuum evaporator at 60°C to a total sugar concentration of 60%. For the separation of the GalOS from D-glucose and D-galactose the strong cation- exchange material Unibead UBK- 530 (Mitsubishi Chemical Industries) was used. The purified GalOS fractions were pooled and analyzed by CE and HPAEC-PAD and subsequently freeze dried (32).

The composition of the highly pure GalOS mixture was made up of 33.5% di-, 60.4% tri-, 4.8% tetra and 1.0% pentasaccharides with an inconsiderable amount of monosaccharides, lactose and lactobionic acid. Formation of β-(1→3) and β-(1→6) linkages are preferred using the β-galactosidase from *Lb. reuteri* L103 for the GalOS production.

## *ENZYMATIC ASSAYS*

### β-GALACTOSIDASE ASSAY

The reaction was carried out at 30°C and initiated by adding 20 µL of enzyme solution to 480 µL of 22 mM oNPG (substrate) in 50 mM sodium phosphate buffer (pH 6.5) and stopped after 10 min by adding 750 µL of 0.4 M $Na_2CO_3$. The absorbance of oNPG was measured at 420 nm (35). One unit of oNPG activity (UoNPG) is defined as the amount of enzyme releasing 1 µmol of oNP per minute under the reaction conditions described above.

### CELLOBIOSE DEHYDROGENASE

For measuring the CDH activity the DCIP assay was performed by measuring the reduction of 300 µm DCIP in 50 mM sodium citrate buffer, pH 6.0, containing 30 mM

lactose at 520 nm and 30°C for 3 minutes. The absorption coefficient was determined experimentally to be 6.8 $mM^{-1}cm^{-1}$ (32).

### LACCASE

Laccase activity was detected at 436 nm using 2,2'-azinobis-(3 ethylenbenzthiazolin-6-sulfonic acid, ABTS) as electron donor ($\varepsilon_{420}$ = 43.2 $mM^{-1}cm^{-1}$) following the oxidation at 30°C for 3 minutes. The assay was performed in 100 mM sodium acetate buffer, pH = 4.0, containing 1 mM ABTS (36, 37). One unit of laccase activity was defined as the amount of enzyme oxidising 1 µmol of ABTS per minute under the indicated conditions.

Protein was determined by the method of Bradford (38) with the BioRad Coomassie Blue reagent using bovine serum albumin as the standard.

## *SUGAR ANALYSIS*

### GALOS

To analyze the composition of the GalOS mixture thin layer chromatography (TLC) high performance anion exchange chromatography with pulsed amperometric detection (HPAEC- PAD) and capillary electrophoreses (CE) was used.

TLC was carried out using high- performance TLC silica plates (Kieselgel 60 F245, Merck, Darmstadt, Germany). A diluted sample (~1 µL) containing ~40 $g \cdot L^{-1}$ sugar was applied to the plate and eluted twice in ascending mode with an n-butanol/n-propanol/ethanol/water mixture (2/3/3/2). Thymol reagent was used for detection.

HPAEC-PAD analysis was done on a Dionex DX-500 system (Dionex Corporation, Sunnyvale, CA USA). The sugars were detected using the ED 40 electrochemical detector with a gold working electrode and an Ag/AgCl reference electrode (Dionex). Separations were performed on a CarboPac PA-1 column (4 x 250 mm) connected to a CarboPac PA-1 guard column (Dionex).

CE analysis were carried out on a system with an UV-DAD detector (Agilent Technologies, Palo Alto, Ca, USA) using precolumn derivatized samples (2-aminopyridine) (32).

### D-GALACTOSE

D-galactose was determined using the lactose/D-galactose test kit from Boehringer Mannheim (Mannheim, Germany).

### D-GLUCOSE

For the D-glucose determination an assay based on glucose oxidase (GOD) and peroxidase (POD) was used (32).

## *PRELIMINARY GROWTH EXPERIMENTS USING PURE CULTURES AND AUTOMATED TURBIDIMETRY*

Before the growth experiments were carried out frozen cultures were streaked onto BHI agar containing 1 $g \cdot L^{-1}$ glucose for bifidobacterium cultures, or MRS agar supplemented with 2% lactose for the lactobacilli, or nutrient agar for *E. coli*, *K. oxytoca* and *C. freundii*, or corynebacterium medium containing 0.5% glucose for *St. epidermis* and *En. faecium*. After incubation at 37°C for 24 - 48 h one colony from each plate was streaked out again onto an appropriate agar plate. Lactobacilli and bifidobacteria were incubated under anaerobic conditions (85% $N_2$, 10% $CO_2$, 5% $H_2$) in an anaerobic chamber. All the other strains were grown at ambient atmosphere. For the inocula, biomass from each strain was transferred from the agar plates into the appropriate liquid media (without a carbohydrate source) and a series of dilutions was prepared. Aliquots of 100 µL of the bacterial dilutions and 200 µL of non inoculated medium were placed in the wells of honeycomb sterile plates. The carbohydrate (glucose or prebiotic sugar) concentration in each well was 0.5% and the bacterial starting $OD_{600}$ was diluted to be 0.001. For the cultivation of the anaerobic strains the enzyme Oxyrase® (Oxyrase, Inc, Ohio, USA) was added to a final concentration of 2% (v/v) and the reading plate was closed airtight. The inoculated honeycomb plates were placed in the reading chamber of a Bioscreen C (Labsystems, Labsystem France SA, Les Ulis, France). The cultivation temperature was 37°C and medium intensity shaking was set for 30 s before half-hourly optical density readings were done. The Bioscreen C was used to monitor the growth of the probiotic as well as of the pathogenic strains by reading OD at a wavelength of 600 nm for 24 hours. To

determine the growth on carbohydrate free medium no glucose or prebiotic carbohydrate source was added. All growth experiments were carried out in triplicates.

### PREBIOTIC POTENCY SCORE

The Prebiotic Potency Scores were calculated using the following equation:

$$PP = \frac{Pro_{prebiotic(\max)} - Pro_{prebiotic(\min)}}{[Pro_{glucose(\max)} - Pro_{glucose(\min)}] * [Pro_{blank(\max)} - Pro_{blank(\min)}]} - \frac{Pat_{prebiotic(\max)} - Pat_{prebiotic(\min)}}{[Pat_{glucose(\max)} - Pat_{glucose(\min)}] * [Pat_{blank(\max)} - Pat_{blank(\min)}]} \quad (1)$$

Where Pr*0* is standing for probiotic bacteria and Pat defines the group integrating the four pathogenic bacteria used in this study. For the calculation of the Prebiotic Potency Score the maximum (sample time) and minimum (inoculation) absorbences from the Bioscreen C $OD_{600}$ measurements were used. This equation assumes that an increase in the number of probiotic bacteria give a positive result while the increase in the population of pathogenic bacteria is giving a negative one. By entering the growth characteristics on glucose and on non supplemented media (blank) into the equation the population changes are normalized relatively to their ability to grow on glucose and on carbohydrate free media.

### *FAECAL SAMPLES AND IN VITRO FERMENTATION*

The fermentation reactors (Soham Scientific, Fordham, UK) with a working volume of 100 mL were equipped with a double jacket water cooling/heating unit. The temperature was controlled at 37°C and the pH was set to pH 6.8. Anaerobic fermentation conditions were supplied by aeration with nitrogen. The reactor was filled with culture medium (peptone water 2 $g \cdot L^{-1}$, yeast extract 2 $g \cdot L^{-1}$, NaCl 0.1 $g \cdot L^{-1}$, $K_2HPO_4$ 0.04 $g \cdot L^{-1}$, $KH_2PO_4$ 0.04 $g \cdot L^{-1}$, $MgSO_4 \cdot 7H_2O$ 0.01 $g \cdot L^{-1}$, $CaCl_2 \cdot 6H_2O$ 0.01 $g \cdot L^{-1}$, $NaHCO_3$ 2 $g \cdot L^{-1}$, haemin 0.005 $g \cdot L^{-1}$, L-cysteine HCl 0.5 $g \cdot L^{-1}$, bile salts 0.5 $g \cdot L^{-1}$, Tween 80 2 $ml \cdot L^{-1}$, vitamin K 10 $\mu L \cdot L^{-1}$, and resazurin solution of 0.025% (w/v) 4 $mL \cdot L^{-1}$). 1% (w/v) carbohydrates were partially dissolved in the medium prior to inoculation with 10% (w/v) of faecal slurry. This slurry was prepared by

homogenising fresh human faeces (10%, w/v) in phosphate buffered saline (PBS; NaCl 8 $g \cdot L^{-1}$, KCl 0.2 $g \cdot L^{-1}$, $Na_2HPO_4$ 1.15 $g \cdot L^{-1}$ and $KH_2HPO_4$ 0.2 $g \cdot L^{-1}$, pH 7.3) using a manual homogenizer (Fisher, Loughborough, United Kingdom) (39) All fermentations were done in duplicates and the sample time points were 0, 5, 10, 24 and 36 hours. The oligosaccharides FOS, TOS, GalOS were tested on their growth stimulating effect (prebiotic effect). The three faecal donors were male, healthy, aged between 25 and 30, had not received antibiotic treatment for at least three months prior to experimentation, had not knowingly consumed pre- or probiotic supplements and had no history of bowel disorders.

### *BACTERIAL ENUMERATION BY FLUORESCENT IN SITU HYBRIDISATION*

To enumerate the bacteria from the in vitro fermentations the fluorescent in situ hybridisation (FISH) technique was used (40). Samples (375 µl) were fixed for 4 h at 4°C with 4% (w/v) filtered paraformaldehyde (pH 7.2) in a ratio of 1:3 (v/v). After centrifugation at 13000 g for 5 min the samples were washed twice with filtered PBS and resuspended in 300 µl of a mixture of PBS and ethanol (1:1, v/v) and stored at -20°C. The hybridisation was carried out using genus- and group- specific 16S rRNA-targeted oligonucleotide probes labelled with Cy3 (MWG Biotech, Ebersberg, Germany) (41, 42). The following probes were used: Bif164, specific for Bifidobacterium (43); Bac303, specific for Bacteroides (44); Erec482 for the Clostridium coccoides- Eubacterium rectale group (40); Lab158 for Lactobacillus, Enterococcus (45); Ato291 for the Atopobium cluster, including most Coriobacteriaceae species (46); Eub338 for the total bacteria count (47, 48).

### *SCFA AND ORGANIC ACIDS ANALYSIS*

During the in vitro fermentation lactic-, acetic-, propionic-, isobutyric-, butyric-, isovaleric- and valeric acid were formed and analysed using high performance liquid chromatography. Analyses were carried out using the cation exchange column HPX-87H HPLC column (BioRad, Watford, UK). Samples were centrifuged at 13000 g for 20 minutes, the supernatant was passed through a 0.2 µm filter and separations were performed using 0.005 mM $H_2SO_4$ at a flow rate of 0.6 $mL \cdot min^{-1}$ (39, 49).

## *STATISTICAL ANALYSIS*

Preliminary growth experiments were repeated three times and in vitro fermentations were done in duplicates. The data were analysed using the SPSS (SPSS Inc. Chicago, Illinois; Version 11.0.0.). The Univariate analysis of variance (ANOVA) followed by the posthoc Tukey test were used to determine the significant difference among the increase in bacterial group populations, SCFA and lactic acid production.

# RESULTS

## *GALOS* PRODUCTION AND SEPARATION OF MONOSACCHARIDES AND LACTOSE

The sugar composition of the GalOS mixture after lactose conversion using the transgalactosylation activity of the β-galactosidase from *Lb. reuteri* L103 is shown in Table 1.

To remove the residual amount of lactose the disaccharide was oxidised into lactobionic acid using cellobiose dehydrogenase from *Sclerotium rolfsii*. After two ion exchange chromatography steps the monosaccharides were separated using size exclusion chromatography. The composition of the finally obtained GalOS mixture was 33.5% di-, 60.5% tri- and 4.8% tetra- and 1.0% pentasaccharides with an inconsiderable amount of monosaccharides, lactose and lactobionic acid (32).

**Table 1: Sugar composition of GalOS mixture after transgalactosylation reaction of lactose (206.5 $g \cdot L^{-1}$) with recombinant β- gal from *Lb. reuteri* L103**

| Name | Structure | Concentration [g/L] | Compostition [% w/w] |
|---|---|---|---|
| Glucose | | 62.4 | 30.2 |
| Galactose | | 36.8 | 17.8 |
| Lactose | β-D-Gal*p*-(1→4)-D-Glc | 54.7 | 26.5 |
| Allolactose | β-D-Gal *p* -(1→6)-D-Glc | 10.1 | 4.9 |
| | β-D-Gal *p* -(1→6)-D-Gal | 7.7 | 3.7 |
| | β-D-Gal *p* -(1→3)-D-Gal | 1.7 | 0.8 |
| | β-D-Gal *p* -(1→3)-D-Glc | 0.7 | 0.3 |
| 6'- galactosyl lactose | β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc | 17.8 | 8.6 |
| 3'- galactosyl lactose | β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc | 4.8 | 2.3 |
| Tetrasaccharides | | 2.1 | 1.0 |
| Unidentified | | 7.8 | 3.8 |

## GROWTH EXPERIMENTS WITH PURE CULTURES AND AUTOMATED TURBIDIMETRY

The maximum optical cell densities obtained for each species on 0.5% glucose, 0.5% prebiotic sugars and on carbohydrate free medium are shown in Table 2.

**Table 2: Maximum $OD_{600}$ values ± standard deviation obtained for each test strain grown with various carbohydrates**

| Bacterial culture | Glucose | Vivinal GOS | TOS | GalOS | Blank |
|---|---|---|---|---|---|
| *Lb. reuteri* Lb46 | 1.28 ± 0.04 | 1.26 ± 0.03 | 1.25 ± 0.01 | 1.38 ± 0.01 | 0.25 ± 0.00 |
| *Lb. reuteri* Lb21 | 1.30 ± 0.04 | 1.19 ± 0.02 | 1.19 ± 0.02 | 1.31 ± 0.01 | 0.34 ± 0.02 |
| *Lb. acidophilus* Lb19 | 1.43 ± 0.03 | 1.35 ± 0.04 | 1.19 ± 0.02 | 1.47 ± 0.01 | 0.40 ± 0.04 |
| *Lb. acidophilus* Lb71 | 1.47 ± 0.03 | 1.41 ± 0.03 | 1.36 ± 0.06 | 1.54 ± 0.01 | 0.43 ± 0.00 |
| *Lb. acidophilus* Lb105 | 1.44 ± 0.06 | 1.36 ± 0.04 | 1.39 ± 0.05 | 1.47 ± 0.05 | 0.38 ± 0.00 |
| *Lb. rhamnosus* Lb29 | 1.64 ± 0.01 | 1.01 ± 0.01 | 0.56 ±0.03 | 0.74 ±0.01 | 0.80 ± 0.03 |
| *Lb. paracasei* Lb16 | 1.55 ± 0.01 | 1.33 ± 0.01 | 0.55 ± 0.01 | 1.21 ± 0.01 | 0.71 ± 0.10 |
| *Lb. casei* Lb20 | 1.54 ± 0.00 | 1.33 ± 0.01 | 0.55 ± 0.02 | 1.21 ± 0.01 | 0.71 ± 0.02 |
| *B. animalis* Bif1 | 1.22 ± 0.02 | 0.77 ± 0.03 | 0.34 ± 0.04 | 0.95 ± 0.03 | 0.25 ± 0.02 |
| *B. animalis lactis* Bif3 | 1.05 ± 0.02 | 0.84 ± 0.04 | 0.35 ± 0.02 | 0.95 ± 0.03 | 0.24 ± 0.10 |
| *B. longum* Bif14 | 0.78 ± 0.03 | 0.69 ± 0.06 | 0.28 ± 0.02 | 0.79 ± 0.03 | 0.31 ± 0.01 |
| *E. coli* Ec1 | 0.41 ± 0.02 | 0.40 ± 0.01 | 0.69 ± 0.05 | 0.38 ± 0.01 | 0.95 ± 0.04 |
| *K. oxytoca* Kleb1 | 0.36 ± 0.04 | 0.35 ± 0.00 | 0.45 ± 0.06 | 0.33 ± 0.11 | 1.01 ± 0.10 |
| *C. freundii* Cit1 | 0.42 ± 0.09 | 0.51 ± 0.02 | 0.73 ± 0.01 | 0.34 ± 0.04 | 0.89 ± 0.04 |
| *En. faecium* En61 | 0.66 ± 0.01 | 0.72 ± 0.07 | 0.46 ± 0.01 | 0.88 ± 0.10 | 0.67 ± 0.19 |
| *St. epidermis* St2 | 0.96 ± 0.02 | 0.93 ± 0.02 | 0.75 ± 0.08 | 0.88 ± 0.04 | 0.66 ± 0.09 |

Sugars with prebiotic potency should be metabolised from a probiotic bacterial strain nearly as well as glucose. By feeding probiotic and non probiotic strains with prebiotic sugars and glucose as an internal reference the lactobacilli were divided into two groups. *Lb. rhamnosus* Lb29, *Lb. paracasei* Lb16 and *Lb. casei* Lb20 displayed highest growth on glucose, lowest growth on TOS and only moderate growth on GalOS compared to the other lactobacilli. These three species also showed the highest cell densities on carbohydrate free media in the group of lactobacilli. *Lb. rhamnosus* Lb29 displayed very poor growth on GalOS and TOS, although highest growth rates were seen on glucose among all lactobacilli. The other five lactobacilli strains showed

nearly the same growth characteristics, namely all displayed a preference for GalOS and glucose over TOS and Vivinal GOS. The growth on non supplemented media was low, reaching a maximum value of 0.43 (*Lb. acidophilus* Lb71). When cultivating the two *Lb. reuteri* strains Lb21 and Lb46 on the GalOS-containing media the maximum cell densities were reached after 13 h, while the three strains of *Lb. acidophilus* Lb19, 71, 105 needed nearly 27 h to reach their maximum $OD_{600}$ (data not shown).

In the bifidobacterium group *B. animalis* Bif1 displayed highest growth on glucose and GalOS followed by Vivinal GOS and TOS. *B. longum* Bif14 displayed the lowest growth on glucose and GalOS in the group of the bifidobacteria, nevertheless this species showed a preference for GalOS over glucose, but did not show growth in excess of the blank on TOS. All three bifidobacteria displayed relatively poor growth on TOS and grew well on Vivinal GOS.

Against the postulation that the growth of pathogenic strains on prebiotic sugars should be low, *E. coli* EC1, *K oxytoca* Kleb1 and *C. freundii* Cit1 grew well on carbohydrate free medium, followed by TOS, Vivinal GOS and glucose. These strains displayed relatively poor growth on GalOS. The growth of *En. faecium* En61 was enhanced most by GalOS followed by Vivinal GOS but did not show higher growth rates on glucose compared to the blank.

## PREBIOTIC POTENCY SCORE

The $OD_{600}$ values displayed in Table 2 were used for the calculation of the prebiotic potency score (Figure 1). The highest prebiotic potency scores were calculated for *B. animalis lactis* Bif3, *Lb. reuteri* Lb46 and *B. animalis* Bif1 when cultivated on GalOS (8.45, 7.52, and 7.25, respectively) followed by *B. animalis lactis* Bif3 (6.92) and *Lb. reuteri* Lb46 (6.53) paired with Vivinal GOS. For *Lb. rhamnosus* Lb29, *Lb paracasei* Lb16 and *Lb. casei* Lb20 the lowest prebiotic potency scores were calculated when grown with all three prebiotic sugars. Also *B. longum* Bif14 has a negative prebiotic potency score (-0.36) when grown on TOS. The bifidobacteria strains in general had lower prebiotic potency scores paired with TOS in comparison to Vivnal GOS and GalOS. Moreover the ratio of the prebiotic potency score for the different prebiotics in the bifidobacteria group is constant, only varying in the amount (*B. animalis lactis*

Bif3 over *B.animalis* Bif1 over *B. longum* Bif 14). The three *Lb. acidophilus* strains showed nearly the same prebiotic potency scores for the same prebiotic, unlike the two *Lb. reuteri* species. *Lb reuteri* Lb46 had higher scores compared with *Lb. reuteri* Lb21 for all of the prebiotics tested.

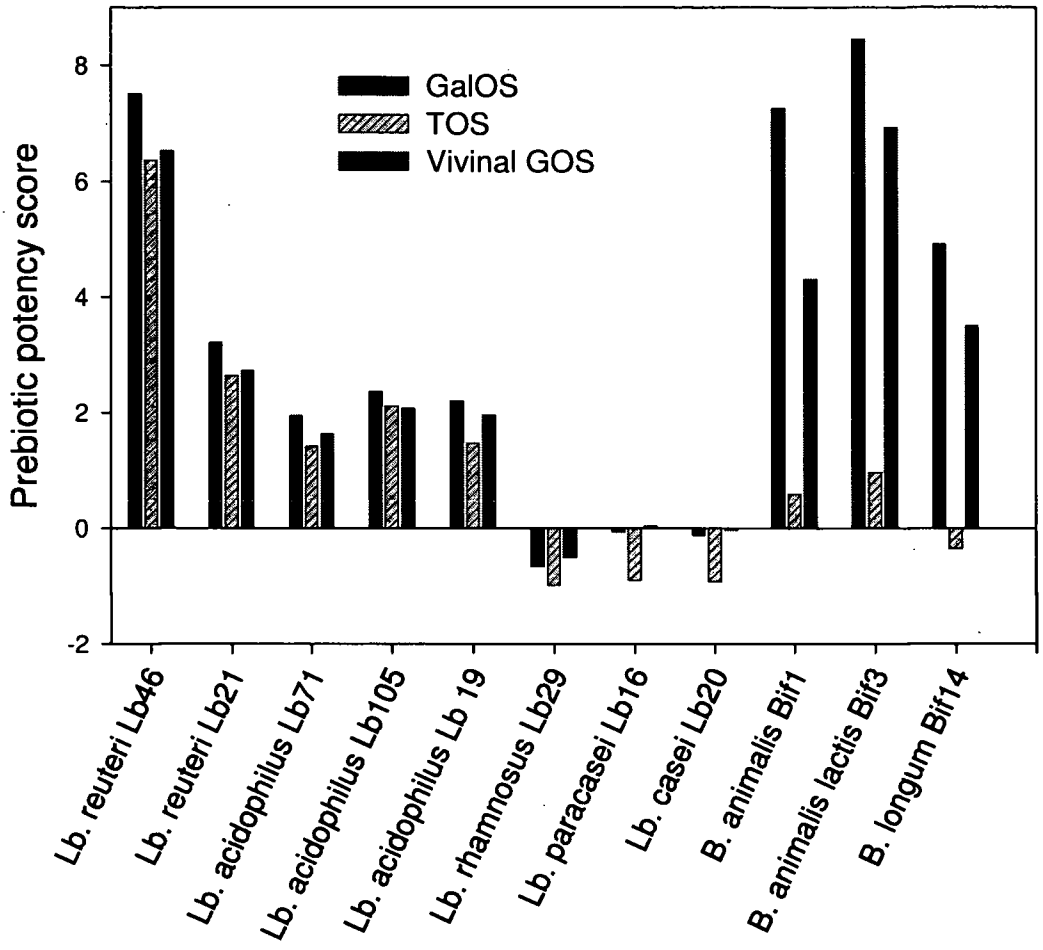



**Figure 1: Prebiotic potency score of various bacteria strains grown on prebiotic sugars**

## GROWTH EXPERIMENTS ON FAECAL SAMPLES USING GALOS, FOS AND TOS

Table 3 indicates the bacterial populations after 0, 5, 10, 24 and 36 h of inoculation in anaerobic batch cultures supplemented with GalOS, FOS and TOS which were enumerated by FISH.

**Table 3: Bacterial populations in log10 cells/g faeces ± standard error in small scale batch cultures at 0 (Inoculum), 5, 10, 24, 36 h using three different carbon sources*.**

| Prebiotic | time | total cells | Atobacterium | Bacteroides | Bifidobacterium | Eubacterium | Lactobacillus |
|---|---|---|---|---|---|---|---|
| | 0 | $9.45 \pm 0.02^{a}$ | $8.03 \pm 0.06^{a}$ | $8.52 \pm 0.11^{a}$ | $8.05 \pm 0.05^{a}$ | $8.60 \pm 0.03^{a}$ | $6.74 \pm 0.08^{a}$ |
| GalOS | 5 | $9.61 \pm 0.03^{a}$ | $8.02 \pm 0.16^{a}$ | $8.49 \pm 0.11^{a}$ | $9.01 \pm 0.11^{b}$ | $8.46 \pm 0.13^{a}$ | $6.81 \pm 0.10^{a}$ |
| | 10 | $9.74 \pm 0.04^{ab}$ | $7.94 \pm 0.13^{a}$ | $8.83 \pm 0.12^{a}$ | $9.19 \pm 0.15^{b}$ | $8.17 \pm 0.25^{a}$ | $6.86 \pm 0.25^{a}$ |
| | 24 | $9.61 \pm 0.04^{a}$ | $7.96 \pm 0.15^{a}$ | $8.68 \pm 0.17^{a}$ | $9.31 \pm 0.06^{b}$ | $8.27 \pm 0.18^{a}$ | $6.81 \pm 0.15^{a}$ |
| | 36 | $9.49 \pm 0.05^{a}$ | $7.76 \pm 0.21^{a}$ | $8.56 \pm 0.16^{a}$ | $9.04 \pm 0.10^{b}$ | $8.28 \pm 0.06^{a}$ | $6.67 \pm 0.13^{a}$ |
| TOS | 5 | $9.50 \pm 0.04^{a}$ | $8.19 \pm 0.11^{a}$ | $8.74 \pm 0.15^{a}$ | $8.82 \pm 0.15^{b}$ | $8.35 \pm 0.31^{a}$ | $6.80 \pm 0.27^{a}$ |
| | 10 | $9.76 \pm 0.11^{a}$ | $8.12 \pm 0.14^{a}$ | $8.89 \pm 0.14^{a}$ | $9.14 \pm 0.22^{b}$ | $8.26 \pm 0.29^{a}$ | $6.95 \pm 0.40^{a}$ |
| | 24 | $9.65 \pm 0.07^{a}$ | $8.06 \pm 0.20^{a}$ | $8.99 \pm 0.13^{a}$ | $9.36 \pm 0.11^{b}$ | $8.79 \pm 0.10^{a}$ | $6.61 \pm 0.33^{a}$ |
| | 36 | $9.59 \pm 0.07^{a}$ | $7.97 \pm 0.26^{a}$ | $8.82 \pm 0.11^{a}$ | $9.19 \pm 0.10^{b}$ | $8.40 \pm 0.24^{a}$ | $6.60 \pm 0.22^{a}$ |
| FOS | 5 | $9.56 \pm 0.08^{a}$ | $8.29 \pm 0.19^{a}$ | $8.75 \pm 0.05^{a}$ | $8.94 \pm 0.17^{b}$ | $8.34 \pm 0.24^{a}$ | $6.59 \pm 0.26^{a}$ |
| | 10 | $9.70 \pm 0.05^{a}$ | $8.12 \pm 0.24^{a}$ | $8.98 \pm 0.06^{a}$ | $9.11 \pm 0.27^{b}$ | $8.31 \pm 0.66^{a}$ | $6.84 \pm 0.46^{a}$ |
| | 24 | $9.58 \pm 0.06^{a}$ | $8.17 \pm 0.28^{a}$ | $8.89 \pm 0.03^{a}$ | $9.19 \pm 0.12^{b}$ | $8.43 \pm 0.28^{a}$ | $6.84 \pm 0.48^{a}$ |
| | 36 | $9.36 \pm 0.12^{a}$ | $7.95 \pm 0.42^{a}$ | $8.57 \pm 0.16^{a}$ | $9.04 \pm 0.08^{b}$ | $8.47 \pm 0.09^{a}$ | $6.57 \pm 0.42^{a}$ |

*Univariate ANOVA and Tukey tests were used to determine significant differences for each population (n= 60). The letters indicate significant differences ($p < 0.05$) between initial population (0 h) and different time points.

Table 3 gives an overview of the bacterial numbers during the small scale batch fermentations.

Independent of the carbohydrate source it can be seen that total cell numbers are increasing until time point 10. After 10 h of fermentation the total cell number decreased again. This rise in total bacterial cell number was statistically significant ($p < 0.05$) only for the treatment with the GalOS mixture.

The only considerable and sustained proliferation among different bacteria groups gave bifidobacteria. It increased significantly between time point 0 h and time point 5 h in all treatments. The increase after time point 5 h is detectable but not significant anymore. The population of bifidobacteria showed a faster increase in GalOS and in

FOS treatment compared to the TOS feeding. However, TOS supplementation gave the highest count with 9.36 log10 cells·$g^{-1}$ faeces over GalOS with 9.31 log10 cells·$g^{-1}$ and FOS 9.19 log10 cells·$g^{-1}$.

A slight increase in cell number occurred in the bacteroides group in all prebiotic treatments. Using GalOS and FOS as carbohydrate source an increase in cell number was enumerated for the first 10 h. In comparison the growth on TOS was lasting for 24 h.

The lactobacillus group was growing until to the $10^{th}$ fermentation hour regardless of the prebiotic supplemented. The highest cell density was reached when grown on TOS (6.95 log10 cells·$g^{-1}$ faeces).

Eubacteria group decreased in all substrates until time point 10 (without significance). After this time point eubacteria number increased a bit, especially with TOS and decreased again.

Atobacteria displayed an immediate decrease when supplemented with GalOS. TOS and FOS enhanced the growth of this group until fermentation hour 5.

## *Analysis of Short Chain Fatty Acids and Lactic Acid Production*

Short chain fatty acids and lactic acid are end products of carbohydrate fermentation and were analyzed by HPLC. The concentrations measured at time point 0 h, 5 h, 10 h, 24 h and 36 h are presented in Table 4.

Lactate and acetate were the principal SCFA produced on GalOS and FOS, the exception being lactic acid on TOS where butyric acid was higher compared to the other two prebiotics. In all fermentations acetic acid was predominantly produced by bacteroides, bifidobacteria, lactobacilli and/or clostridia. Lactate is mainly metabolized by lactic acid bacteria including bifidobacteria which are dominant in all fermentations. Although the number of lactobacilli did not increase during fermentation, they still metabolize carbohydrates.

Bacteroides are known as producer of propionic acid. Especially with the GalOS mixture as substrate the produced propionic acid can be related to the population growth. Fermentations with TOS displayed no significant increase in the bacteroides

group but a respectable production in propionate. FOS as substrate was acting similar to TOS.

The concentration of the butyric acid was very low for all sugars, which correlates well to the marginal growth of the eubacteria group. Clostridia, which are also known as butyrate producer, were not enumerated. The highest significant amount was measured using TOS as carbohydrate source (8.92 mM at time point 24 h).

Isobutyric acid, valeric acid and isovaleric acid were also included in the measurements. Valeric acid was found in insignificant amounts and branched chain fatty acids were not found at all (data not shown).

**Table 4: SCFA and lactic acid concentrations (mM) ± standard error in small scale batch culture at 0 (Inoculum), 5, 10, 24, 36 h using 3 different substrates***

| | | | SCFA | | |
|---|---|---|---|---|---|
| Treatment | time | Lactic acid | Acetic acid | Propionic acid | Butyric acid |
| | 0 | $0.19 \pm 0.05^{a}$ | $1.62 \pm 0.05^{a}$ | $0.67 \pm 0.04^{a}$ | $0.21 \pm 0.02^{a}$ |
| GalOS | 5 | $22.92 \pm 2.92^{b}$ | $36.52 \pm 7.69^{b}$ | $12.11 \pm 3.83^{a}$ | $0.56 \pm 0.32^{a}$ |
| | 10 | $23.57 \pm 3.41^{b}$ | $51.38 \pm 5.00^{b}$ | $21.32 \pm 6.63^{a}$ | $1.42 \pm 0.71^{a}$ |
| | 24 | $13.26 \pm 6.11^{ab}$ | $51.78 \pm 5.86^{b}$ | $9.65 \pm 4.43^{a}$ | $5.95 \pm 2.04^{ab}$ |
| | 36 | $9.00 \pm 5.76^{ab}$ | $52.49 \pm 5.60^{b}$ | $10.40 \pm 4.61^{a}$ | $8.58 \pm 2.01^{b}$ |
| TOS | 5 | $10.20 \pm 1.50^{a}$ | $25.21 \pm 6.36^{ab}$ | $9.70 \pm 5.24^{a}$ | $1.20 \pm 0.92^{ab}$ |
| | 10 | $8.71 \pm 2.99^{a}$ | $43.93 \pm 1.72^{b}$ | $20.84 \pm 9.33^{a}$ | $3.25 \pm 2.25^{ab}$ |
| | 24 | $0.00 \pm 0^{a}$ | $58.45 \pm 6.51^{b}$ | $14.51 \pm 8.44^{a}$ | $8.92 \pm 1.57^{b}$ |
| | 36 | $0.00 \pm 0^{a}$ | $51.30 \pm 8.13^{b}$ | $14.71 \pm 8.10^{a}$ | $8.64 \pm 1.40^{b}$ |
| FOS | 5 | $15.46 \pm 2.16^{a}$ | $26.61 \pm 7.41^{ab}$ | $9.44 \pm 3.55^{a}$ | $0.62 \pm 0.41^{a}$ |
| | 10 | $20.17 \pm 5.12^{a}$ | $45.84 \pm 10.16^{b}$ | $19.47 \pm 9.72^{a}$ | $1.57 \pm 1.23^{a}$ |
| | 24 | $15.77 \pm 7.88^{a}$ | $50.42 \pm 10.75^{b}$ | $9.55 \pm 4.98^{a}$ | $2.72 \pm 1.54^{a}$ |
| | 36 | $7.48 \pm 4.61^{a}$ | $48.86 \pm 9.81^{b}$ | $9.93 \pm 5.07^{a}$ | $5.77 \pm 1.06^{a}$ |

* Univariate ANOVA and Tukey tests were used to determine significant differences for acid concentration (n= 60). The letters indicate significant differences ($p < 0.05$).

# DISCUSSION

Carbohydrates which are indigestible by humans but metabolisable by probiotic bacterial strains can be grouped as prebiotics. These sugars can influence the microbiota of the gastrointestinal tract by enhancing the population number of the "healthy" strains. To evaluate the prebiotic potency of different sugar mixtures two different cultivation methods were reported in the past. Huebner et al., Kaplan & Hupkins and Schrezenmeier & de Vrese were using pure culture fermentations to investigate the prebiotic effect (26, 28, 50), whereas Palfram et al., Sanz et al., and Hughes et al. were using a gut model to perform mixed culture fermentations (39, 51, 52). Single culture fermentation do not need the gut model nor the faecal samples. The fermentations are done in appropriate basal media depending on the probiotic strain and the increase in cell number is quantified by turbidimetry or by viable cell count. Mixed culture fermentations using faecal samples and the gut model are enumerated by the FISH technique. Due to specific primers all the bacteria will be put into several groups. This combination of cultivation and enumeration enables the investigation on the interaction of these different bacterial groups. It is known that on the one hand the prebiotics can be transported directly into the cell using different transportation channels (53-55) and on the other hand a few extra cellular enzymes produced by probiotic bacteria were reported having hydrolytic activities (56). If, for example, GalOS are cleaved by extra cellular β-galactosidases in easily digestible monosaccharides like glucose and galactose, these sugars can be metabolized by every bacterial strain in the human gut.

In general, comparative studies of oligosaccharide application including mixed culture experiments, pure culture studies as well as application *in vivo* are still limited. Thus, results between studies seem to be difficult to compare and no overall conclusion concerning prebiotic efficiency of different oligosaccharide types are reported. For this study pure and mixed culture fermentations were carried out to compare the prebiotic potency of the new oligosaccharide product (GalOS) compared with those oligosaccharides which were already known for their prebiotic effects (FOS). All the substrates used in this work, except Vivinal GOS, containing about 40% of

monosaccharides and lactose, were highly pure oligosaccharides. The novel GalOS mixture was the product of the galactosyltransferase activity of the purified β-galactosidase from the probiotic strain *Lb. reuteri* L103, purified from monosaccharides and lactose to a degree of 99.7% and investigated here in both pure and mixed culture fermentations. This enzyme was shown to have a high specificity for the formation of β(1→6) and β(1→3) linkages (35). It is assumed that the structure of the GalOS components is related to its prebiotic activity and it led to the speculation that GalOS produced with an enzyme isolated from a probiotic strain might enhance the growth of probiotic strains. Such a preference was seen in the pure culture fermentation used here. The highest prebiotic potency score was observed cultivating the three bifidobacteria strains and *Lb. reuteri* Lb46 on media supplemented with GalOS. Although the TOS mixture, used in this study, which was also produced by a β-galactosidase showed significantly lower prebiotic potency scores when combined with the bifidobacteria. These mixture was analysed to have mainly β(1→4) linked sugars what confirms the studies from Rycroft and Sanz (42, 51) that the linkage type and/or the molecular weight of the prebiotic oligosaccharides affect the growth of human probiotic strains. β-galactosidase of bifidobacteria was reported to possess a specificity for cleavage of β(1→6) and β(1→3) linkages (57). This would be one explanation for the enhanced growth of bifidobacteria with GalOS produced with β-gal from *Lb. reuteri*.

The prebiotic efficiency of the novel GalOS was further investigated in a gut model using faecal samples. In a comparative in vitro study xylooligosaccharides and lactulose produced highest increase in bifidobacteria whereas fructo-oligosaccharides resulted in highest populations of lactobacilli (42). Tzortzis et al. tested the impact of a novel GalOS mixture, synthesized with whole cells of *Bifidobacterium bifidum*, in a three vessel gut model on four different bacterial groups, where the bifidobacterium group was enhanced most in growth followed by lactobacilli (58). From all the substrates tested here, fermentation with TOS and GalOS resulted in highest increase of bifidobacterial growth after 24 hours fermentation time (9.36 log10 cells and 9.31 log10 cells, respectively). Lactic acid concentrations were lower with TOS than with GalOS at time point t= 24 and 36 h. It might have been metabolized by other species. Highest final acetic acid concentration, the main fermentation product of bifidobacteria, was seen at fermentation with TOS followed with GalOS.

Rossi et al. compared the bifidobacterial growth of pure and faecal cultures on FOS and inulin. Pure culture fermentations showed higher growth rates on FOS compared to inulin as substrate, however faecal culture fermentations did not display significant differences in cell number between FOS and inulin as substrate (29). Furthermore, Tannock et al. has demonstrated in a recent study on the prebiotic effect of galacto-oligosaccharides that changes in metabolic activities can be detected, whereas changes in the composition of the microbial population can not (59). This could explain the increase in pure lactobacilli cultures with GalOS in contrast to the insignificant increase of lactobacilli using faecal culture fermentations although the lactic acid concentration was increased significantly.

We showed that structurally related carbohydrates of different linkage types like GalOS and TOS affect the increase in cell numbers using pure culture fermentation. This observation was not approved in faecal culture cultivations determining only small differences on the two substrates. These mostly unknown and unpredictable interactions of different prebiotic bacteria in the human colon enable a better consumption of nutrients, which are no good substrate in pure culture fermentation. A particular organism metabolises an oligosaccharide via extra cellular hydrolysis, the products that are released may feed other bacterial strains or species. On the other hand it is of particular importance that the bacteria chosen for the pure culture fermentations are belonging to the group of prebiotics. Due to the extremely low or even negatively prebiotic potency score of *Lb. rhamnosus* Lb29, *Lb. paracasei* Lb16 and *Lb. casei* Lb20 these three lactobacilli do not seem to belong to the group of "healthy" microbiota of the human gut.

The prebiotic potency scores, calculated from the different pure culture growth characteristics on several substrates, reflect the potential of a given carbohydrate promoting the selective growth of specific organisms. Thus, synbiotics for food and feed industry can be figured out and optimised to a certain extent.

To award a novel sugar mixture the title "prebiotic" at least pure and mixed culture fermentations to determine the prebiotic efficiency should be used.

# APPENDIX

## A 1 GENERAL INTRODUCTION

### *A 1.1 PRE- AND PROBIOTICS*

In recent years numerous investigations have been focused on the field of prebiotics. Prebiotic oligosaccharides are perhaps the most promising tools for modulation of the activities of the colonic microbiota. A prebiotic sugar is defined as "a non- digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (60).

The most interesting prebiotics are non-digestible oligosaccharides (61). These non-digestible oligosaccharides can have a range of positive impacts on the colonic microbiota. These "health promoting" organisms which are also known as probiotics can selectively metabolize different prebiotic sugars.

Probiotics are defined as "live microbial feed supplement, which beneficially affects the host by improving its intestinal microbial balance" (62). The usual target micro-organisms are bifidobacteria and lactobacilli at the expense of less desirable groups such as clostridia. In general, a bacterium should meet the following criteria to be defined as probiotic:

- it should be of human origin and non-pathogenic
- it has to withstand incorporation into a foodstuff and maintain its effectiveness for the duration of the shelf life of the product
- it must overcome transit through the gastrointestinal tract, including low gastric pH, bile secretions and nutrient competition with the resident microbiota
- it must be able to proliferate and colonize the digestive tract
- it should be associated with other health benefits (63).

Prebiotic intake can result in health outcomes. Prebiotic supplementation can have a positive effect on different diseases like acute gastroenteritis by blocking the causative agents including shigellae, salmonellae, *Yersinia enterocolitica* or other pathogens like sulphate reducers and clostridia.

## *A 1.2 OLIGOSACCHARIDES*

### A 1.2.1 GALACTO-OLIGOSACCHARIDES

GalOS occur naturally in human milk. The bifidogenic effect of GalOS was confirmed in previous studies where the number of bifidobacteria of breast fed infants is higher than in bottle fed infants (21, 22). GalOS are produced enzymatically by the galactosyl-transferase activity of β-galactosidase using lactose as substrate (23). Transgalactosylation is thought to involve intermolecular as well as intramolecular reactions. Intramolecular or direct galactosyl transfer to glucose yields regioisomers of lactose. The glycosidic bonds of lactose [β(1→4)] is cleaved and immediately formed again at different position of the glucose molecule before it diffuses out of the active site. This is how allolactose [β(1→6)] can be formed even in the absence of significant amounts of free glucose.

By intermolecular transgalactosylation di-, tri-, tetra- and pentasaccharides and eventually higher oligosaccharides are produced. Any sugar molecule in the reaction mixture can be the nucleophil to accept the galactosyl moiety from the galactosyl-enzyme complex. The newly formed GalOS can be regarded as kinetic intermediates as they are also substrates for hydrolysis. For all these reasons the GalOS yield and composition change dramatically with reaction time. It is hard to predict what happens. The chemical structure and composition of GalOS greatly depend on the enzyme source. We speculate that ß-gal from lactobacilli forms GalOS for their own proliferation. These enzymes catalyse also the hydrolysis of lactose.

The reaction scheme is shown in Figure 2. As described above, galactosyl-transferase activity comes along with a hydrolysis activity and so commercial available GalOS mixtures normally contain high amounts of glucose and galactose (24, 25).

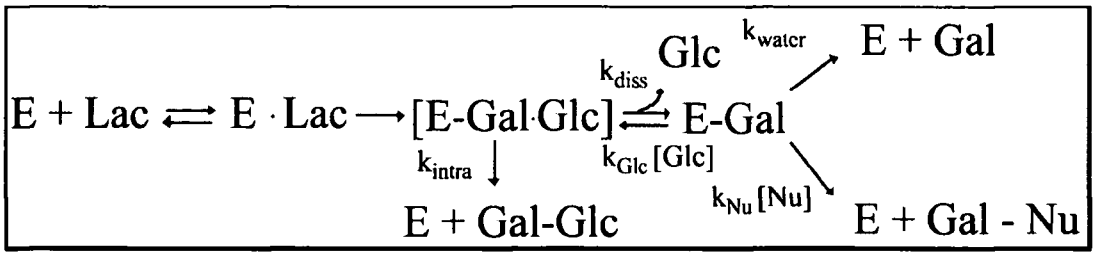



**Figure 2: Hydrolysis and galactosyl transfer reactions during the conversion of lactose catalyzed by β-galactosidase. E, enzyme; Lac, lactose; Gal, galactose; Glc, glucose; Nu, nucleophile (64)**

Studies document that GalOS induce a clearly enhanced absorption of calcium, magnesium and also iron which affects bone mineral content and bone structure. Fructo-oligosaccharides (FOS) shows similar effects (65, 66). They are already in use as food ingredients and commercially available most notably in Japan (14).

Trans-galacto-oligosaccharides (TOS) are galacto-oligosaccharides, which are produced from lactose by enzymatic transgalactosylation. TOS are linear oligosaccharides consisting of lactose and several galactose molecules in β(1→6) and (β1→4) joined linkages and cause disputed prebiotic impacts on the gut micro flora (18, 19).

### A 1.2.2 FRUCTO-OLIGOSACCHARIDES

FOS are one of the best known prebiotics. There are many sources of fructans, principally from plants (chicory, Jerusalem artichoke, Dahlia, onion...). The amount of fructans and the DP (degree of polymerisation) vary greatly with source and storage conditions. These oligofructans feature a degree of polymerisation between 2- 60 (Inulin) and 2- 20 (Oligofructose) (15). FOS are synthesized enzymatically by different processes. They can be produced by the transfructosylation action of a β-fructofurinase from the fungi *Aspergillus niger*, *Aureobasidium pullunans* or *Aspergillus japonicus*. Another way is a controlled enzymatic hydrolysis of inulin extracted from chicory roots by the enzyme inulase (23). Related studies are showing the improvement of health and welfare in various perspectives for humans and animals (16, 17).

## A 1.3 MICROBIOTA* IN THE LARGE INTESTINE

Microbiota occurs along the whole length of the human alimentary tract. The population numbers and species distribution is characteristic for particular regions of the gut and is a large and complex ecosystem (67). This microflora in the large intestine is able to perform complex hydrolytic and digestive functions (68). This can be the breakdown of dietary components (complex carbohydrates), but also the hydrolysis of some proteins, which were not hydrolyzed nor absorbed in the upper digestive tract.

There are about 500 – 800 different species of indigenous bacteria present in the adult large intestine comprising around $10^{12}$ bacteria per gram dry weight. A summary of most numerous bacteria with description and nutrition is shown in Table 5. The colonic microflora is largely anaerobe. These non- sporing, strict anaerobe bacteria like *Bifidobacterium*, *Bacteroides* and *Eubacterium*; less dominant are *Atopobium*, the peptococci, clostridia and other families in the clostridia mega-genus (e.g. *Clostridium leptum*, *Clostridium histolyticum*) dominate the colonic microflora. Some facultative anaerobe are far less numerate and include *Lactobacillus* spp., streptococci, enterococci, and *Enterobacteriaceae* (69, 70). Yeasts can be found in low amounts in the gut. In general, the lactate producing bacteria (bifidobateria and lactobacilli) are considered to be health promoting (71).

These bacteria perform two main types of fermentation; the saccharolytic and the proteolytic fermentation. Saccharolytic fermentation involves the breakdown of complex carbohydrates and the proteolytic fermentation means the breakdown of proteins that are not hydrolyzed nor absorbed in the upper digestive tract. The end products of the fermentations are absorbed by the host. Generally, gut bacteria can be divided on the basis of health promoting, benign or potentially harmful activities in their host. (72)

* Microbiota refers to all microorganisms (i.e. bacteria and fungi); microflora refers only to the bacteria of an environment.

**Table 5: Most numerous predominant anaerobes in the human large intestine**

| Bacteria | Description | Numbers reported in faeces (Log10 per g dry wt) | | Nutrition | Fermentation products* |
|---|---|---|---|---|---|
| | | Mean | Range | | |
| Bacteroides | Gram negative rods | 11.3 | 9.2-13.5 | Saccharolytic | A, P, S |
| Eubacteria | Gram positive rods | 10.7 | 5.0-13.3 | Saccharolytic, some amino acid fermenters | A, B, L |
| Bifidobacteria | Gram positive rods | 10.2 | 4.9-13.4 | Saccharolytic | A, L, f, e |
| Clostridia | Gram positive rods | 9.8 | 3.3-13.1 | Saccharolytic, some amino acid fermenters | A, P, B, L, e |
| Lactobacilli | Gram positive rods | 9.6 | 3.6-12.5 | Saccharolytic, | L |
| Fusobacteria | Gram negative rods | 8.4 | 5.1-11.0 | Amino acid fermenters, carbohydrate assimilated | B, A, L |
| Ruminococci | Gram positive rods | 10.2 | 4.6-12.8 | Saccharolytic | A |
| Peptostreptococci | Gram positive rods | 10.1 | 3.8-12.6 | Saccharolytic, some amino acid fermenters | A, L |
| Peptococci | Gram positive rods | 10.0 | 5.1-12.9 | Amino acid fermenters | A, B, L |
| Propionibacteria | Gram positive rods | 9.4 | 4.3-12.0 | Saccharolytic, lactate fermenters | A, P |
| Actinomyces | Gram positive rods | 9.2 | 5.7-11.1 | Saccharolytic | A, L, S |
| Streptococci | Gram positive rods | 8.9 | 3.9-12.9 | Carbohydrate and amino acid fermentation | L, A |
| Escherichia | Gram negative rods | 8.6 | 3.9-12.3 | Carbohydrate and amino acid fermentation | Mixed acid |
| Desulvobibrios | Gram negative rods | 8.4 | 5.2-10.9 | Various (e. g. SO4, H2, CO2) | A |
| Methanobrevibacter | Gram positive cocci bacilli | 8.8 | 7.0-10.5 | Chemolithotrophic | CH4 |

* A, acetate; P, propionate; B, butyrate; L, lactate; S, succinate; f, Formate; e, ethanol (72)

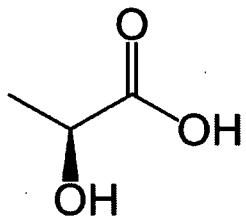
## A 1.4 SHORT CHAIN FATTY ACIDS AND LACTIC ACID

Short chain fatty acids are organic fatty acids with the length between 2 and 6 carbon atoms and are the principal end products together with gases ($CO_2$, $CH_4$, $H_2$) from bacterial fermentation of polysaccharide, oligosaccharide, proteins, peptide and glycoprotein precursors in the intestine. A numerous of bacteria are saccharolytic fermenters. SCFA are supplier of energy for the host and have also specific physiological functions in the body. It is estimated that about 60 % of the energy of a carbohydrate that is fermented in the large intestine is absorbed as SCFA, and that the absorption and subsequent oxidation of SCFA from the colon may contribute up to 8% of total energy usage. The major SCFA produced by gut bacteria are acetate, propionate and butyrate which are avidly absorbed by the intestine. Various publications showed that SCFA production is in order of acetate > propionate > butyrate, the relative ratio is about 1 acetate : 0.31 propionate : 0.15 butyrate. Other SCFA are valerate and lactate, which is a hydroxyl acid.

The absorption of SCFA is localized in the caecum and colon. Only 5-10% of produced SCFA can be found in faeces. The absorption is associated with enhanced sodium absorption and bicarbonate excretion.

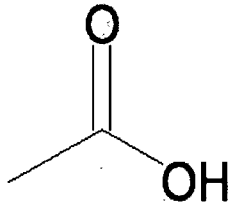
SCFA play a key role in colonic health and may have specific impacts in prevention and management of certain diseases.

### A 1.4.1 LACTIC ACID



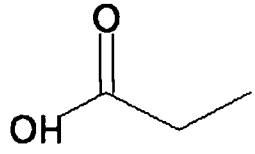

Lactic acid is a hydroxy acid and one of the main end products of carbohydrate fermentation in the colon of lactic acid bacteria included bifidobacteria. Both the D- and L- enantiomers are formed, although much more bacteria groups producing the L- from than the D- form. Lactate is poorly absorbed by the large intestine, but concentrations in the gut are kept low by bacteria fermenting lactic acid to SCFA and other products. Normally, lactate is a product of amino acid fermentation but during the fermentation of carbohydrates it is used as electron sink intermediate product. From there, different SCFA are produced (73-76),

### A 1.4.2 ACETIC ACID



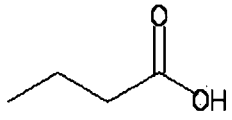

Acetate is fermented of a large number of different bacteria like bacteroides, bifidobacteria, eubacteria etc. (Table 6) and is the most produced SCFA. Acetate is readily absorbed and transported to the liver or is metabolised in muscle, kidney, heart and brain. Uptake by liver is generally enhanced when fermentation of large quantities of carbohydrate is occurring in the large intestine. This is the only SCFA that reaches the systematic circulation in significant amount. It is also an important product for synthesis of long chain fatty acids like glutamine, glutamate and beta-hydroxybutyrate. Acetate is the major anion buffer in many total parenteral nutrition regimens and it is also used as correction of acidosis due to diarrhoea disorders. In human studies, acetate is often an important monitor for colonic events, because it is the main SCFA in the blood (73-76),

### A 1.4.3 PROPIONIC ACID



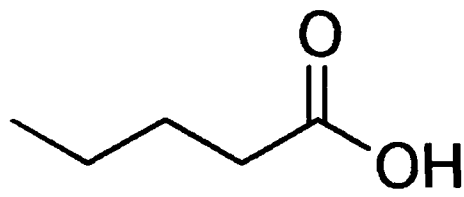

Propionate is a metabolite of bacteroides, propionibacteria, and veillonella and it is formed in two main pathways: the dicarboxylic acid pathway and the acrylate pathway. A large part of propionate is probably used for glycogen synthesis by the indirect pathway in the liver; otherwise the utilization of propionate carbon for lipogenesis is probably very limited. Propionate is also reported to inhibit cholesterol synthesis in the liver. This cholesterol lowering effect may be a secondary consequence to propionate production in the colon. Generally, propionate is present in lower concentrations than acetate in portal blood but on a quantity basis, it is taken up more than acetate. (73-76)

### A 1.4.4 BUTYRIC ACID




Butyrate producers are clostridia, fusobacteria, butyrivibrio, eubacteria, peptostreptococci but not lactic acid bacteria. The absorption is generally low because it is produced in smaller quantities than the other SCFA. Butyrate is the most important acid in colonic metabolism. A large part of butyrate is used by colonic mucosal cells during absorption. It has a regulatory function in the control of the machinery regulating apoptosis and cellular proliferation and differentiation. Acetate and propionate are less zealously metabolized than butyrate. This acid has lots of different effects in gene expression via phosphorylation and acylation of histone protein. Butyrate may have a role in preventing gastrointestinal disorders and various types of cancer. Butyrate also stimulates the immunogenic of cancer cells (73-76),

## A 1.4.5 VALERIC ACID




Valeric acid occurs in the colon in smaller amounts than prior acids. It has a circumstantial relevance in human colon.

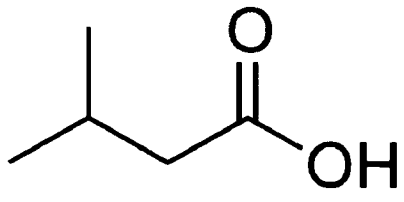
**Table 6: Predominant products of carbohydrate metabolism in the human colon (60).**

| End product | Bacterial group involved | Metabolic fate |
|---|---|---|
| Acetate | Bacteroides, bifidobacteria, eubacteria, lactobacilli, clostridia, ruminococci, peptococci, veillonella, peptostreptococci, propionibacteria, fusobacteria, butyrivibrio | Metabolised in muscle, kidney, heart and brain |
| Propionate | Bacteroides, propionibacteria, veillonella | Cleared by the liver, possible gluceogenic precursor, suppresses cholesterol synthesis |
| Butyrate | Clostridia, fusobacteria, butyrivibrio, eubacteria, peptostreptococci | Metabolised by the colonic epithelium, regulator of cell growth and differentiation |
| Ethanol, succinate, lactate, pyruvate | Bacteroides, bifidobacteria, lactobacilli, eubacteria, peptostreptococci, clostridia, ruminococci, actinomycetes, enterococci, fusobacteria | Absorbed, electron sink products, further fermented to SCFA |
| Hydrogen | Clostridia, ruminococci, fusobacteria | Partially excreted in breath, metabolised by hydrogenotrophic bacteria |

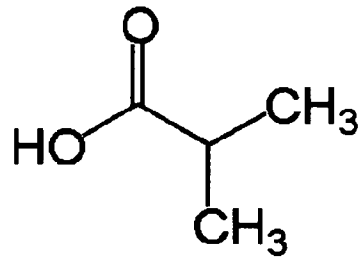
## A 1.4.6 BRANCHED CHAIN FATTY ACIDS

Anaerobic fermentation of proteins and amino acids by proteolytic bacteria gives rise to the branched chain fatty acids isobutyric (C4), isovaleric (C5) and isocapric (C6) and also $CO_2$, $CH_4$, $H_2$, phenols and amines. These acids are found in small amounts in the human large intestine by comparison with SCFA. There is a concern about a possible toxicity of some triglycerides, mostly linked with C4, C5 and C6 fatty acids which may percolate the blood- brain barrier. Generally, branched chain fatty acids have disputed roles in the host health (73-76).

e. g. Branched chain fatty acids:




Isovaleric acid




Isobutyric acid

# A 2 APPENDIX OF THE MANUSCRIPT

## *A 2.1 ACCESSORILY INFORMATION TO MATERIAL AND METHODS*

### A 2.1.1 THIN LAYER CHROMATOGRAPHY FOR MEASUREMENT OF MONOSACCHARIDES

The removal of monosaccharides was carried out by several chromatographic steps. Thin layer chromatography was used for fast detection of the sugars (Figure 3).

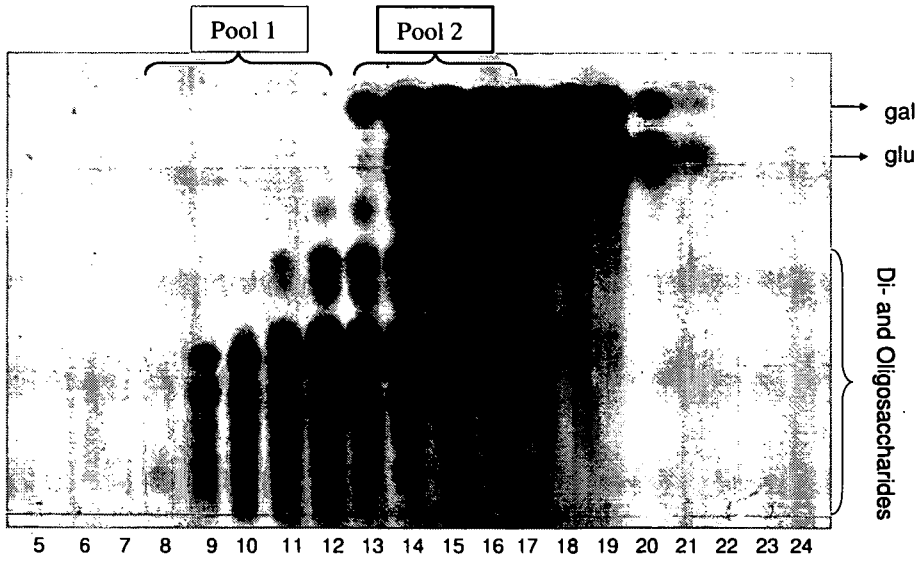



**Figure 3: TLC analysis of fractions after separation of monosaccharides**

To obtain GalOS free of monosaccharides the fractions were pooled. Fractions 8 to 12 were pooled together (pool 1), fractions from 13 to 16 were pooled to pool 2. The first pool contained GalOS free of monosaccharides. Pool 2 still contained monosaccharides and so this pool was applied a second time to the size exclusion chromatography resin.

### A 2.1.2 ACID/BASE USE IN SMALL SCALE BATCH CULTURES.

0.25 mM NaOH and 0.25 mM HCl were used for the control of the pH in small batch fermentations. The acid or base were filled into 50 mL syringes and connected to a squeeze pump. The use of NaOH and HCl for the different batch fermentations can be seen in Table 7, Table 8 and Table 9.

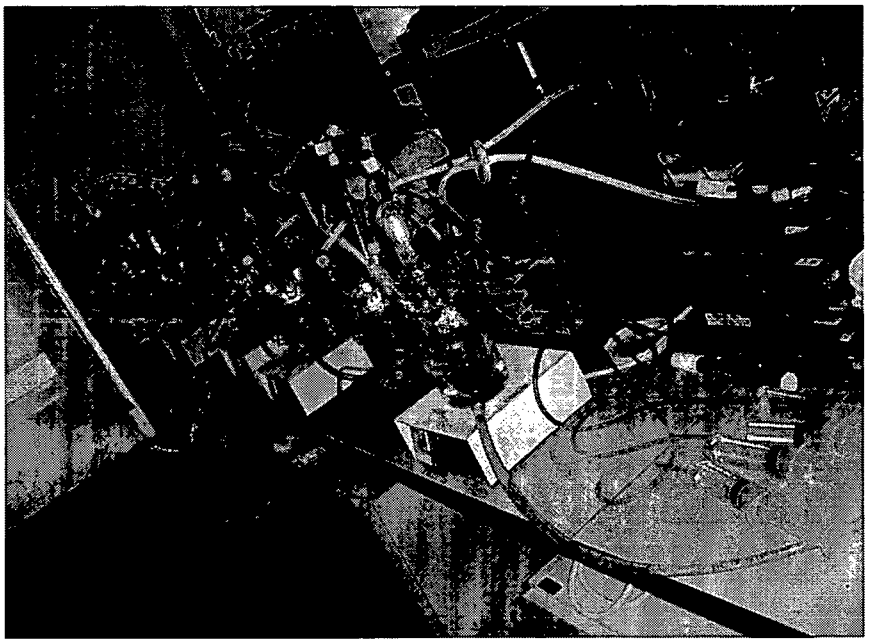


**Figure 4: Small scale faecal batch cultures**

**Table 7: Use of NaOH and HCl in batch 1**

| | GalOS 60 | | | GalOS 60 | | | GalOS | | | GalOS | | | TOS | | | TOS | | | FOS | | | FOS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH |
| Tp 0 | 0 | 0 | 6.70 | 0 | 0 | 6.70 | 0 | 0 | 6.78 | 0 | 0 | 6.77 | 0 | 0 | 6.72 | 0 | 0 | 6.76 | 0 | 0 | 6.78 | 0 | 0 | 6.77 |
| Tp 5 | 10 | 21 | 6.90 | 11 | 22 | 6.70 | 11 | 22 | 6.73 | 10 | 25 | 6.71 | 9 | 22 | 6.71 | 12 | 23 | 6.73 | 15 | 32 | 6.69 | 11 | 25 | 6.73 |
| Tp 10 | 10 | 26 | 6.90 | 12 | 24 | 6.90 | 12 | 24 | 6.85 | 11 | 28 | 6.88 | 10 | 25 | 6.88 | 13 | 26 | 6.86 | 15 | 36 | 6.84 | 11 | 26 | 6.88 |
| Tp 24 | 13 | 26 | 6.90 | 12 | 24 | 6.88 | 13 | 24 | 6.80 | 11 | 28 | 6.92 | 10 | 25 | 6.86 | 13 | 26 | 6.87 | 15 | 36 | 6.84 | 11 | 26 | 6.89 |
| Tp 36 | 13 | 26 | 6.88 | 12 | 24 | 6.90 | 13 | 24 | 6.90 | 11 | 28 | 6.80 | 10 | 25 | 6.86 | 13 | 26 | 6.82 | 15 | 36 | 6.82 | 11 | 26 | 6.88 |

**Table 8: Use of NaOH and HCl in batch 2**

| | GalOS 60 | | | GalOS 60 | | | GalOS | | | GalOS | | | TOS | | | TOS | | | FOS | | | FOS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH |
| Tp 0 | 11 | 10 | 6.87 | 10 | 10 | 6.90 | 9 | 12 | 6.78 | 10 | 10 | 6.84 | 11 | 10 | 6.84 | 9 | 11 | 6.83 | 11 | 9 | 6.87 | 10 | 9 | 6.81 |
| Tp 5 | 12 | 21 | 6.78 | 15 | 25 | 6.83 | 9 | 26 | 6.84 | 10 | 14 | 6.76 | 11 | 15 | 6.76 | 10 | 18 | 6.75 | 11 | 15 | 6.78 | 13 | 28 | 6.69 |
| Tp 10 | 13 | 33 | 6.71 | 19 | 41 | 6.88 | 9 | 32 | 6.82 | 10 | 29 | 6.72 | 11 | 24 | 6.74 | 10 | 24 | 6.76 | 11 | 28 | 6.75 | 14 | 34 | 6.76 |
| Tp 24 | 13 | 33 | 6.79 | 30 | 52 | 6.80 | 10 | 33 | 6.78 | 10 | 29 | 6.80 | 11 | 27 | 6.89 | 10 | 27 | 6.87 | 12 | 29 | 6.88 | 14 | 34 | 6.84 |
| Tp 36 | 14 | 33 | 6.80 | 44 | 65 | 6.89 | 11 | 33 | 6.79 | 10 | 29 | 6.78 | 11 | 27 | 6.80 | 12 | 28 | 6.78 | 12 | 40 | 6.80 | 15 | 34 | 6.81 |

**Table 9: Use of NaOH and HCl in batch 3**

| | GalOS 60 | | | GalOS 60 | | | GalOS | | | GalOS | | | TOS | | | TOS | | | FOS | | | FOS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH | HCl (mL) | NaOH (mL) | pH |
| Tp 0 | 12 | 10 | 6.86 | 8 | 10 | 6.73 | 14 | 12 | 6.80 | 12 | 9 | 6.75 | 13 | 13 | 6.70 | 12 | 12 | 6.76 | 11 | 12 | 6.84 | 14 | 13 | 7.31 |
| Tp 5 | 12 | 30 | 6.78 | 17 | 40 | 6.71 | 14 | 31 | 6.74 | 12 | 29 | 6.74 | 23 | 40 | 6.76 | 12 | 29 | 6.76 | 11 | 21 | 6.78 | 42 | 47 | 6.53 |
| Tp 10 | 14 | 30 | 6.83 | 21 | 43 | 6.86 | 14 | 31 | 6.77 | 13 | 30 | 6.86 | 23 | 44 | 6.89 | 14 | 31 | 6.86 | 11 | 31 | 6.79 | 0 | 0 | 6.48 |
| Tp 24 | 15 | 30 | 6.82 | 21 | 43 | 6.82 | 14 | 31 | 6.83 | 14 | 30 | 6.82 | 24 | 44 | 6.84 | 14 | 31 | 6.86 | 11 | 31 | 6.81 | 0 | 0 | 0.00 |
| Tp 36 | 15 | 30 | 6.79 | 21 | 43 | 6.90 | 14 | 31 | 6.87 | 14 | 30 | 6.82 | 24 | 44 | 6.89 | 14 | 31 | 6.90 | 11 | 31 | 6.88 | 0 | 0 | 0.00 |

### A 2.1.3 FISH: FLUORESCENT IN SITU HYBRIDIZATION

Fluorescent in situ hybridization (FISH) is an established molecular technique for detection of prokaryotes. According to this technique rRNA molecules are labelled with oligonucleotide probes. The probes are labelled with fluorescent dyes. 16S rRNA molecules are ideal target molecules because they are ubiquitously distributed and have a high degree of conservation which makes them suitable to differentiate between strains of a given species. During the last years many rRNA- targeted oligonucleotide probes for in situ detection of prokaryotes have been published (77, 78).

In this work the FISH technique was used with fluorochrome Cy 3 which has an excitation of 514 nm and an emission of 566 nm. The fluorescence colour was orange-red as can be seen in Figure 6 and Figure 7.

FISH with rRNA- targeted probes consists following steps:

- Fixation and dehydration of the diluted samples.
- Hybridization of the samples with the probes and washing.
- Microscope evaluation.

## A 2.1.4 ENUMERATION OF DIFFERENT BACTERIAL GROUPS

### A 2.1.4.1 Preparation

Samples of the in vitro fermentations had to be prepared before accomplishing FISH. Samples (375 µl) were obtained at different time points from each vessel and fixed for 4 h at 4°C with 4% (w/v) filtered paraformaldehyde (pH 7.2) in a ratio of 1:3 (v/v). The fixed samples were centrifuged at 13000 g for 5 min and were washed twice with 1 mL filtered PBS and resuspended in 300 µl of a mixture of PBS and ethanol (1:1, v/v) and stored at -20°C.

### A 2.1.4.2 Hybridization and Washing

The fixed and frozen samples were shook vigorously and adequate dilutions were prepared. 20 µL were added to a 6 well plate and dried for ~15 min at 40- 50°C in a desktop plate incubator. For dehydration the slides were dipped into 50, 80 and 96% ethanol (3 minutes in each) and then dried again. 50 µL of the hybridization mix solution which included 10% (v/v) probe solution in hybridization buffer (contained per mL: 180 µL 5 M NaCl, 20 µL 1 M TRIS-HCl, 1 µL 10% SDS 799 µL $H_2O$) was added on each well which contained the dehydrated samples. After that the samples were incubated in an incubation oven for 4 hours at the appropriate hybridization temperature (Table 10). Once the hybridization was completed the slides were transferred into the washing buffer for exact 15 min at the appropriate temperature. The washing buffer contained per L: 180 mL 5 M NaCl, 20 mL 1 M TRIS-HCl and 800 mL $H_2O$. For cleaning the slides from buffer they were dipped into ice cold distilled water for 2-3 seconds. To dry the slides as quick as possible steam of compressed air was used. Then ~5 µL antifade (Pro® Long Gold, Invitrogen, USA) was added on each well and a coverslip was placed on top of the slide. The slides were stored a lightproof box in the fridge and had to be counted within 3 days.

| Probe name | Sequence (5′ to 3′) | Hybridization pre-treatment | Temperature (°C) | |
|---|---|---|---|---|
| | | | Hybridization | Washing |
| Ato291 | GGTCGGTCTCTCAACCC | None | 50 | 50 |
| Bac303 | CCAATGTGGGGGACCTT | None | 46 | 48 |
| Bif164 | CATCCGGCATTACCACCC | None | 50 | 50 |
| Erec482 | GCTTCTTAGTCARGTACCG | None | 50 | 50 |
| Lab158 | GGTATTAGCAYCTGTTTCCA | Lysozyme/ Lipase | 50 | 50 |
| EUB338 | GCTGCCTCCCGTAGGAGT | None | 46 | 48 |

**Table 10: Sequences of oligonucleotide probes and hybridization conditions which were used in this work**

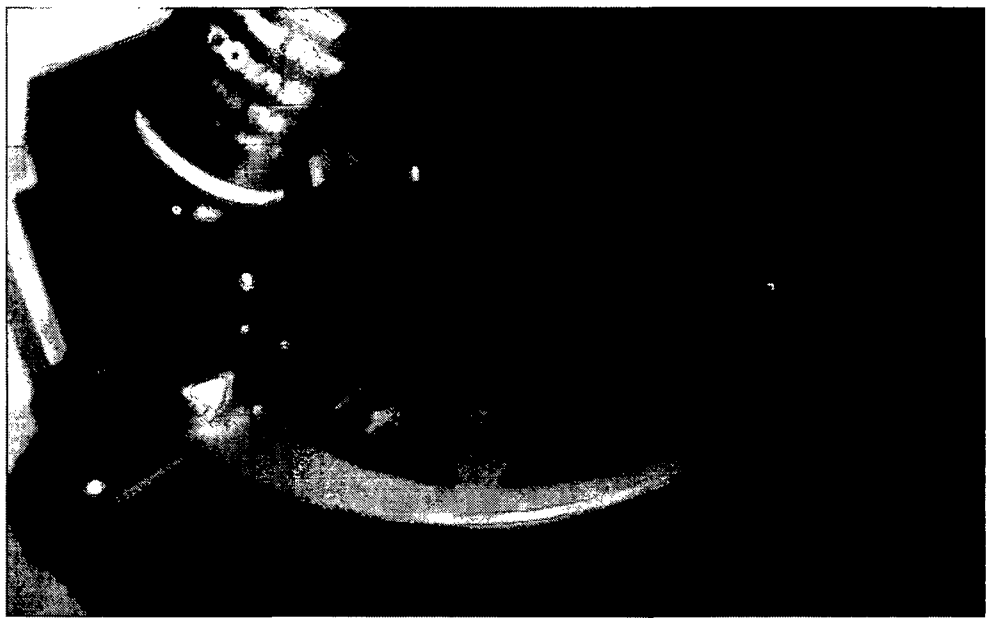


**Figure 5: Slide with 6 wells clamped on the microscope desk.**

### A 2.1.4.3 Counting and calculating of the bacterial numbers

The slides were viewed under a fluorescent microscope (Eclipse 400 fluorescent microscope, Nikon, Kingston upon Thames Surrey, UK) and visualised with the aid of a DM 575 filter. The number of bacteria per millilitre of sample was calculated as follows:

$$BN = DF * ACC * 6732.42 * 50 * DF_{SAMPLE}$$

BN = bacterial number
DF = dilution factor = 0.8
ACC = average cell count
$DF_{SAMPLE}$ = used dilution

The dilution factor of the sample was calculated by taking into account the concentration of the original sample (375 µL to 300 µL= 0.8 →dilution factor). Average cell count was determined by counting 15 fields of view assuming a normal distribution for all counts. The number 6732.42 is obtained by the division of the area of the well and the area of the field of view. Multiplying by 50 takes the count back to millilitre of sample (20 µL of sample was put on the slide). $DF_{SAMPLE}$ refers to the dilution of sample used with a particular probe or stain.

**Figure 6: FISH image of EUB338 under the microscope; dilution 1:300**

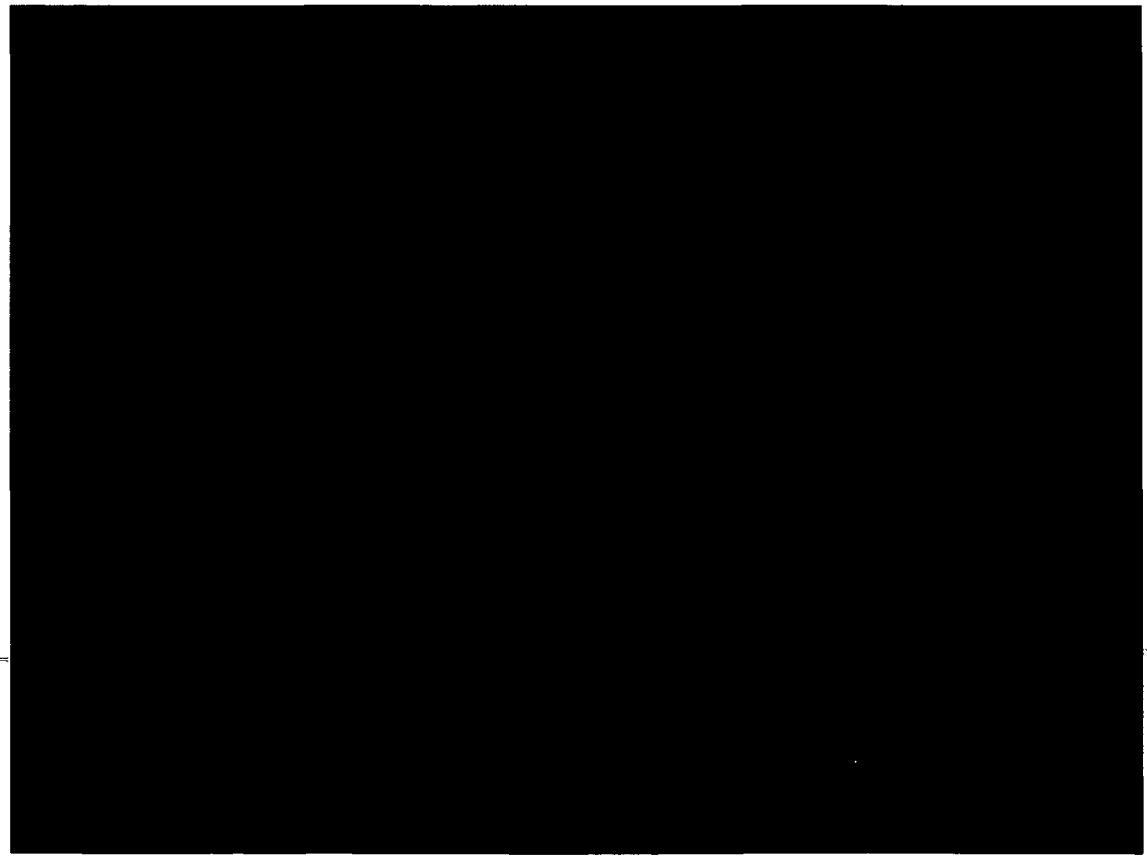


**Figure 7: FISH image of BIF 64 under the microscope; dilution 1:300**

## A 2.1.5 ACCESSORILY INFORMATION TO SHORT CHAIN FATTY ACIDS AND LACTIC ACID PRODUCTION

Short chain fatty acids and lactic acid which are end products of fermentation were determined by HPLC analysis. Following acids were analyzed:

- Lactic acid
- Acetic acid
- Propionic acid
- Isobutyric acid
- Butyric acid
- Isovaleric acid
- Valeric acid

Materials, methods and results are described in the Manuscript (- 24 -, - 26 -). Isobutyric, Isovaleric and Valeric acid were also measured by HPLC without finding. These acids are normally produced by fermentation of proteins due to that they are inexistent. The acids were quantified by external calibration curves using 100 mM, 75 mM, 50 mM, 25 mM and 12.5 mM.

## *A 2.2 ACCESSORILY INFORMATION TO RESULTS*

### A 2.2.1 GROWTH EXPERIMENTS ON FAECAL SAMPLES

Figure 8 shows the growth experiments in a graph. Bacterial numbers are listed in Table 3.

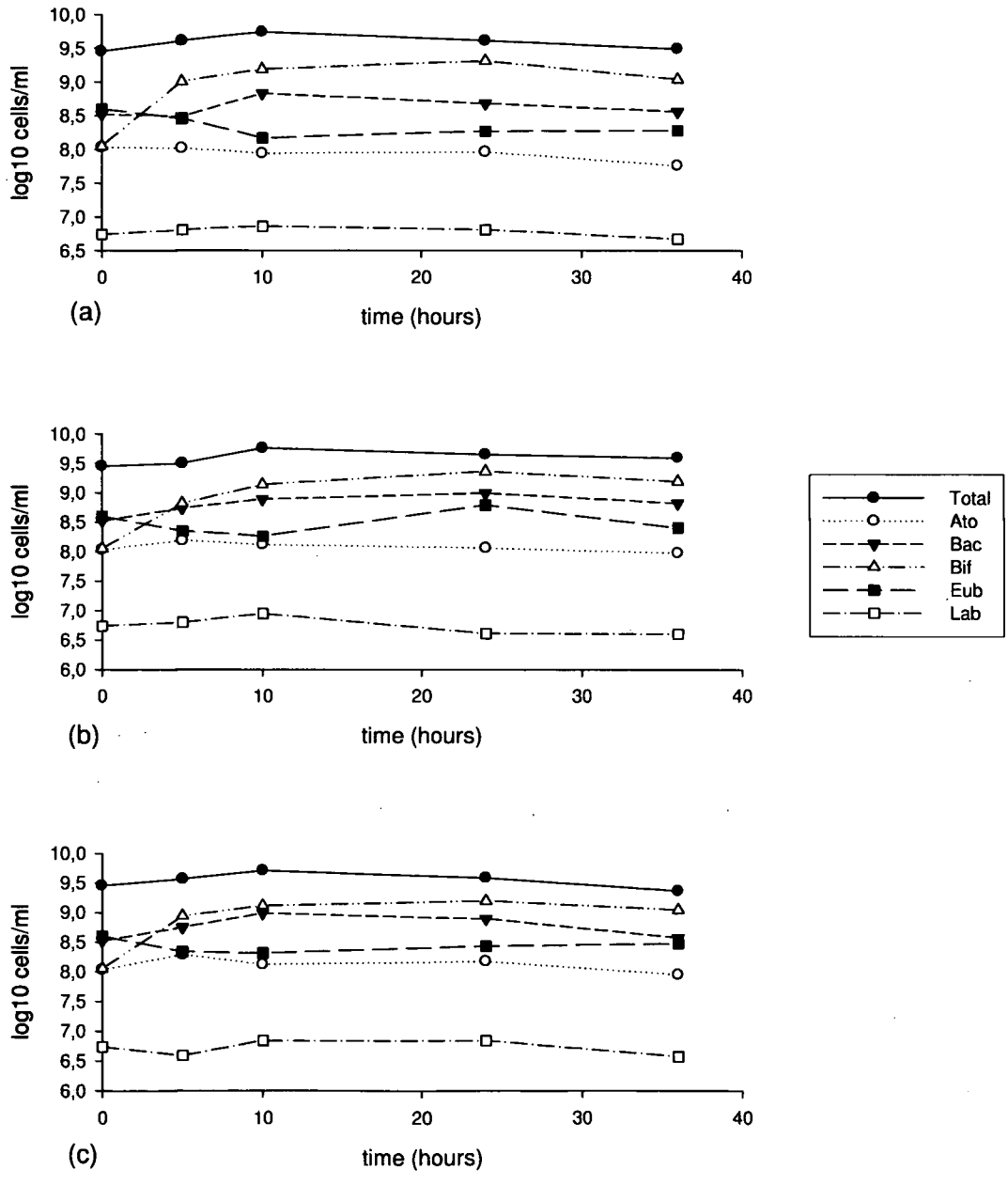



**Figure 8: Growth outputs of different bacterial groups determined in percentage of 1% (w/v): (a) GALOS; (b) TOS; (c) FOS as detected by FISH.**

## A 2.2.2 ANALYSIS OF SCFA AND LACTATE PRODUCTION IN GROWTH EXPERIMENTS

Following Figure 9 demonstrates the acid production during the experiments in graphs. Concentrations are listed in Table 4.

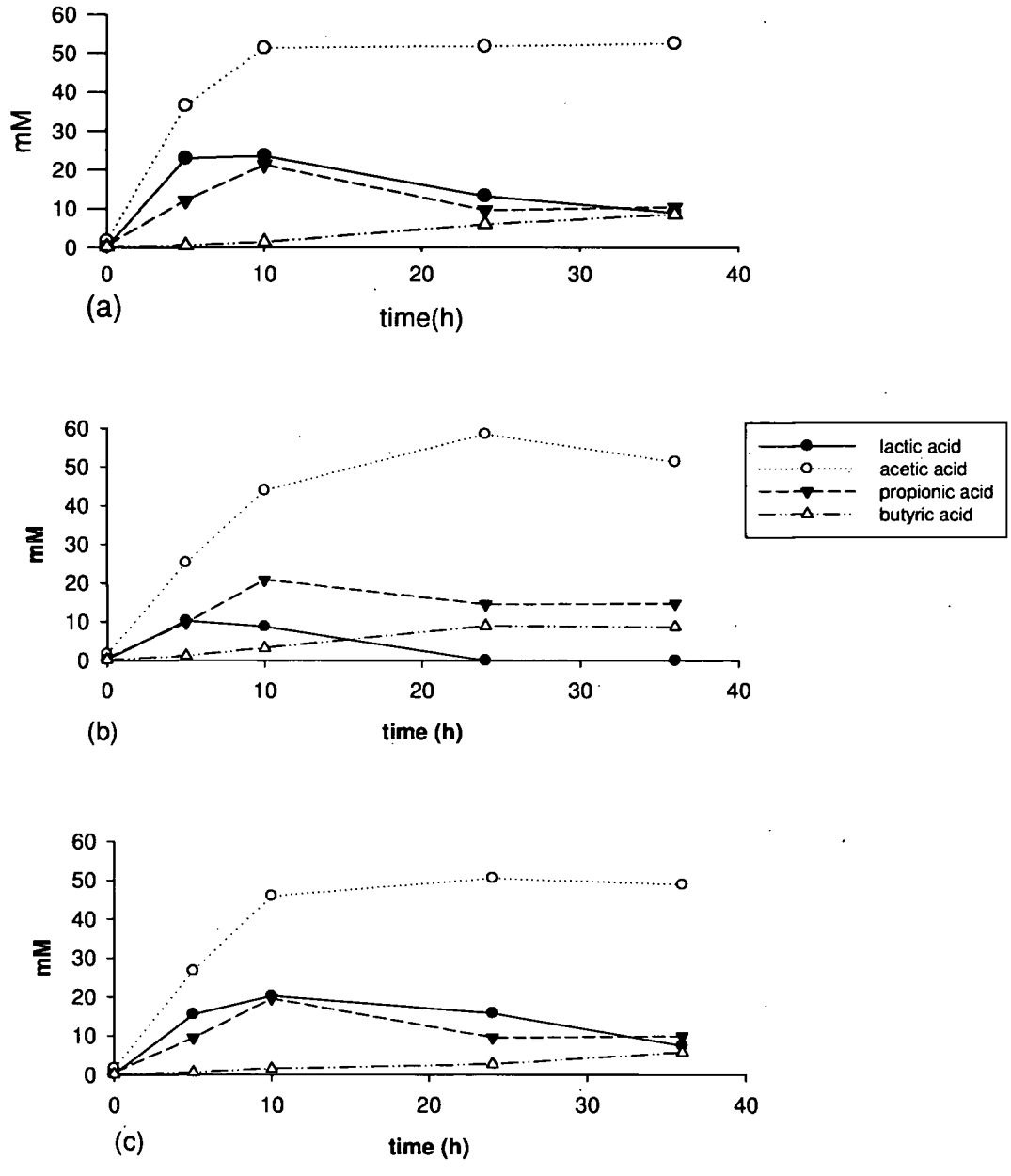



**Figure 9: Short chain fatty acid and lactic acid concentrations during the *in- vitro* fermentation experiments: (a) GALOS; (b) TOS; (c) FOS, measured by HPLC analysis.**

## *A 2.3 PRODUCTION OF GALOS60*

A second mixture of GalOS was produced by conversion of lactose. The reaction was stopped after 60% lactose conversion. The intention was to produce another GalOS mixture with a different composition of prebiotic sugars. The final composition of this GalOS mixture was 7.0% tetrasaccharides, 60.7% trisaccharides, 31.1% disaccharides and 1.2% monosaccharides and lactose after all purification steps. The slight differences in composition did not result in a significant difference of bacterial growth. Table 11 and Figure 10 show the bacterial growth with GalOS60 supplementation.

**Table 11: Bacterial populations in log10 Cells/g faeces ± standard error in small scale batch cultures at 0 (Inoculum), 5, 10, 24, 36 h using GalOS60*.**

| treatment | time | total cells | Atobacterium | Bacteroides | Bifidobacterium | Eubacterium | Lactobacillus |
|---|---|---|---|---|---|---|---|
| | 0 | $9.45 \pm 0.02^{a}$ | $8.03 \pm 0.6^{a}$ | $8.52 \pm 0.11^{a}$ | $8.05 \pm 0.05^{a}$ | $8.60 \pm 0.03^{a}$ | $6.74 \pm 0.08^{a}$ |
| GALOS60 | 5 | $9.62 \pm 0.04^{ab}$ | $8.2 \pm 0.17^{a}$ | $8.63 \pm 0.11^{a}$ | $8.99 \pm 0.12^{b}$ | $8.5 \pm 0.12^{a}$ | $6.94 \pm 0.12^{a}$ |
| | 10 | $9.69 \pm 0.07^{b}$ | $8.13 \pm 0.19^{a}$ | $8.78 \pm 0.10^{a}$ | $9.12 \pm 0.14^{b}$ | $8.42 \pm 0.28^{a}$ | $6.8 \pm 0.12^{a}$ |
| | 24 | $9.58 \pm 0.06^{ab}$ | $7.98 \pm 0.25^{a}$ | $8.71 \pm 0.10^{a}$ | $9.17 \pm 0.12^{b}$ | $8.35 \pm 0.25^{a}$ | $6.75 \pm 0.13^{a}$ |
| | 36 | $9.47 \pm 0.04^{ab}$ | $7.91 \pm 0.23^{a}$ | $8.62 \pm 0.10^{a}$ | $9.06 \pm 0.17^{b}$ | $8.26 \pm 0.17^{a}$ | $6.6 \pm 0.18^{a}$ |

*Univariate ANOVA and Tukey tests were used to determine significant differences for each population (n= 60). The letters indicate significant differences ($P < 0.05$) between the initial population (0 h) and different time points.

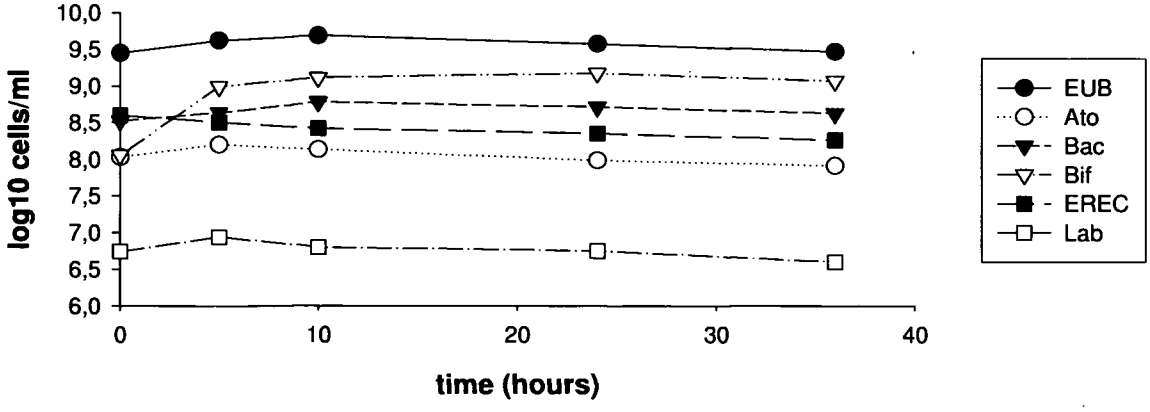



**Figure 10: Growth outputs of different bacterial groups determined in percentage of 1% (w/v) of GalOS60 as detected by FISH.**

Short chain fatty acids and lactic acid were analyzed by HPLC as well. Samples were taken at time points 0 h, 5 h, 10 h, 24 h and 36 h. Data are shown below.

**Table 12: SCFA and lactic acid concentrations (mM) ± standard error in small scale batch culture at 0 (Inoculum), 5, 10, 24, 36 h using GalOS60***

| | | | SCFA | | |
|---|---|---|---|---|---|
| Treatment | time | Lactic acid | Acetic acid | Propionic acid | Butyric acid |
| | 0 | 0.19 ± 0.05$^{ac}$ | 1.62 ± 0.05$^{a}$ | 0.67 ± 0.04$^{a}$ | 0.21 ± 0.02$^{a}$ |
| GALOS60 | 5 | 20.51 ± 2.55$^{b}$ | 31.47 ± 5.87$^{b}$ | 10.87 ± 2.94$^{ab}$ | 0.25± 0.38$^{a}$ |
| | 10 | 18.70 ± 2.42$^{b}$ | 43.79 ± 3.11$^{b}$ | 19.30 ± 5.47$^{b}$ | 0.79 ± 1.34$^{a}$ |
| | 24 | 6.78 ± 4.32$^{c}$ | 42.12 ± 3.68$^{b}$ | 10.10 ± 5.05$^{ab}$ | 2.91 ± 2.56$^{ab}$ |
| | 36 | 1.56 ± 1.56$^{c}$ | 40.52 ± 4.32$^{b}$ | 10.98 ± 5.13$^{ab}$ | 7.02 ± 2.23$^{b}$ |

* Univariate ANOVA and Tukey tests were used to determine significant differences for acid concentration. The letters indicate significant differences (P< 0.05) between initial concentration and different time points.

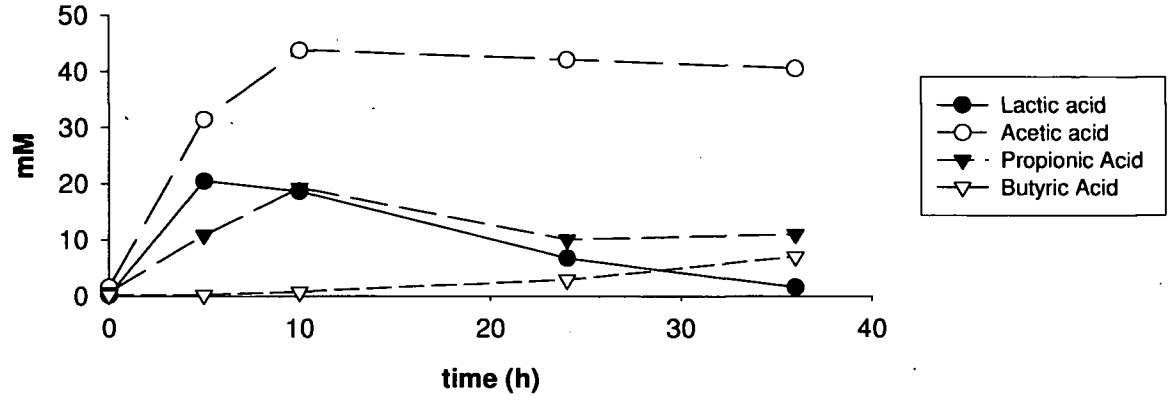



**Figure 11: Short chain fatty acid and lactic acid concentrations during the in- vitro fermentation experiments from GalOS60, measured by HPLC analysis.**

By comparison of the two GalOS mixtures, it was found that GalOS60 had almost the same effect on bacterial growth and on SCFA and lactic acid production. The differences in the composition of the two GalOS mixtures were too low to lead into different effects.

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