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High purity galacto-oligosaccharides enhance specific *Bifidobacterium* species and their metabolic activity in the mouse gut microbiome

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Abstract

Prebiotics are selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon the host health. The aim of this study was to evaluate the influence of a β(1-4)galacto-oligosaccharides (GOS) formulation consisting of 90% pure GOS (GOS90), on the composition and activity of the mouse gut microbiota. Germ-free mice were colonised with microbiota from four pathogen-free wt 129 mice donors (SPF), and stools were collected during a feeding trial in which GOS90 was delivered orally for 14 days. Pyrosequencing of 16S rDNA amplicons showed that *Bifidobacterium* and specific *Lactobacillus*, *Bacteroides* and Clostridiales were more prevalent in GOS90-fed mice after 14 days, although the prebiotic impact on *Bifidobacterium* varied among individual mice. Prebiotic feeding also resulted in decreased abundance of Bacteroidales, *Helicobacter* and *Clostridium.* High-throughput quantitative PCR showed an increased abundance of *Bifidobacterium adolescentis, Bifidobacterium pseudocatenulatum, Bifidobacterium lactis* and *Bifidobacterium gallicum* in the prebiotic-fed mice. Control female mice showed a higher diversity (phylogenetic diversity (PD) = 15.1±3.4 in stools and PD = 13.0±0.6 in intestinal contents) than control males (PD = 7.8±1.6 in stool samples and PD = 9.5±1.0 in intestinal contents). GOS90 did not modify inflammatory biomarkers (interleukin (IL)-6, IL-12, IL-1β, interferon gamma and tumour necrosis factor alpha). Decreased butyrate, acetate and lactate concentrations in stools of prebiotic fed mice suggested an increase in colonic absorption and reduced excretion. Overall, our results demonstrate that GOS90 is capable of modulating the intestinal microbiome resulting in expansion of the probiome (autochtonous commensal intestinal bacteria considered to have a beneficial influence on health).

Keywords: mouse gut microbiome, prebiotics, *Bifidobacterium*

1. Introduction

Gut microorganisms live in a symbiotic interaction with the host and co-exist in a dynamic equilibrium while contributing to host physiology. The colon microbiota comprises populations of about 10^{11} cells/g that represent between 500 and 1000 different species which are involved in the host's metabolic, physiological and immune processes (Sekirov *et al.*, 2010). Recent studies have correlated diseases like inflammatory bowel diseases (IBD), obesity, colorectal cancer, diabetes and allergies with imbalances in the composition of the gut microbial community (dysbiosis) (Dibaise *et al.*, 2012; Rowland, 2009; Russell *et al.*, 2012; Wen *et al.*, 2008). These findings have increased the interest in modulation of the gut microbial communities through dietary strategies to maintain health. One of these strategies is the consumption of prebiotics, which are currently defined as 'selectively fermented ingredients

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that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon the host health' (Gibson *et al.*, 2004). Dietary interventions with prebiotics have been shown to selectively stimulate the growth and/or activity of health-promoting intestinal bacteria, such as species of *Bifidobacterium* and *Lactobacillus* (Ben *et al.*, 2008; Davis *et al.*, 2011; Sangwan *et al.*, 2011; Watson *et al.,* 2013).

Galacto-oligosaccharides (GOS) are prebiotics recognised for their health benefits like modulation of the intestinal microbiota, alleviation of constipation, enhancing mineral absorption, prevention of carcinogenesis and mitigation of allergy (Macfarlane *et al.*, 2008; Tzortzis and Vulevic, 2009). GOS can also reduce the adherence of enteropathogens to the epithelial intestinal cells by functioning as receptor decoys that mimic mucosal cell surface glycans (Quintero *et al.*, 2011; Searle *et al.*, 2009; Shoaf *et al.*, 2006; Tzortzis *et al.*, 2005). Moreover, GOS are currently added to infant formula to stimulate growth of intestinal bifidobacteria and lactobacilli in order to obtain the health benefits of a 'breastfed-like' microbiota (Ben *et al.*, 2008).

Studies on GOS modulation of the gut microbiome showed that the prebiotic increased bifidobacteria populations in the human gastrointestinal tract of healthy individuals at the expense of less beneficial groups of Gram-negative bacteria such as *Bacteroides* and *Desulfovibrio* spp (Davis *et al.*, 2010, 2011; Vulevic *et al.*, 2013; Walton *et al.*, 2012). Additionally, a study conducted by Silk *et al.* (2009) showed that GOS specifically stimulated gut bifidobacteria and alleviated symptoms like flatulence and abdominal pain in irritable bowel syndrome patients. Moreover, a study conducted in overweight adults fed GOS showed increased secretory immunoglobulin A and decreased faecal inflammatory markers, such as calprotectin and plasma C reactive protein, as well as reduced metabolic syndrome markers, such as insulin, total cholesterol (TC), triglycerides and TC:high density lipoprotein cholesterol ratio (Vulevic *et al.*, 2013), suggesting that GOS feeding stimulates the immune response and decreases the risk of developing cardiovascular diseases.

GOS molecules are synthesised enzymatically by β-galactosidases in a reaction known as transgalactosylation (Tzortzis and Vulevic, 2009). However, the concentration and composition of GOS can vary with the method and enzyme used for their generation, which can influence their effect on the intestinal microbiota (Rastall and Maitin, 2002). For instance, it has been reported that mono- and di-saccharides are the preferred substrate for *Lactobacillus rhamnosus*, while *Bifidobacterium lactis* grows better in tri- and tetra-saccharides, since it possesses specific transport systems that will import these more efficiently (Sangwan *et al.*, 2011). In a previous study, we heterologously expressed a synthetically generated version of the hexosyltransferase gene (*Bht*) from *Sporobolomyces singularis* (Dagher *et al.*, 2013). The *Bht* gene encodes a glycosyl-hydrolase (EC 3.2.1.21) that acts as galactosyltransferase, able to catalyse a one-step conversion of lactose to GOS. Commercial GOS formulations such as Vivinal GOS (Friesland Domo), Oligomate (Yakult) and others contain only approximately 50% GOS (w/w), and also contain residual glucose, lactose and galactose. The high purity GOS (GOS90) preparation contains 90% GOS and 10% lactose (w/w), with no residual glucose or galactose.

The aim of the present study was to evaluate the influence of GOS90 on the composition of the transient and adherent components of the microbiota and their metabolic activity in wt 129 mice.

2. Materials and methods

We hypothesised that the prebiotic would shift the composition and metabolic activity of the colon microbiota toward a more health-promoting community enriched with lactobacilli and bifidobacteria. To prove our hypothesis, mice of the 129 background were fed GOS90 over 14 days. Stool samples were collected at three time points and intestinal contents were collected at the end of the trial in order to characterise and compare their bacterial communities by 16S amplicon pyrosequencing. In addition, we developed and optimised a high-throughput qPCR method using the BioMark platform by Fluidigm (San Francisco, CA, USA) to identify and quantify *Bifidobacterium* species. Targeted metabolomic and cytokine analysis were carried out to determine the impact of prebiotic feeding in the production of short-chain fatty acids (SCFAs) and inflammatory markers.

Animal housing, treatment, and sample collection

Thirteen germ-free wt 129 mice (4 males and 9 females, 8-12 weeks old) were transferred to specific pathogen-free (SPF) conditions by colonisation using gavage and rectal swabs prior to the experiment without any antibiotic treatment. Numbers of animals were based on minimum that would provide statistical power to validate differences between control and treatment. This was based on experience and published literature (McCafferty *et al.*, 2013). The colonisation inoculum consisted of stool samples from four SPF wt 129 mice born/raised not humanised. Nine stool pellets were suspended in 5 ml of phosphate buffered saline (PBS). The particulate matter was allowed to settle for approximately 5 min before inoculation. 9 days after colonisation, mice were randomly divided into two groups: the control standard (Purina Prolab RMH3000) diet group consisting of 4 male and 3 female mice (cages 1 and 3) and 6 GOS90-fed female mice (cages 2 and 4). We allowed 9 days for the microbiota to stabilise based on a previous study that showed that after 1 week, the gut microbiota

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of gavage-colonised animals were more similar to the donor community than animals that had acquired their microbiota without intervention (McCafferty *et al.*, 2013). Originally, the groups were planned to be 4 males and 3 females receiving GOS90, and 6 control females, but 3 of 4 male mice would not provide stool samples on the day of the prebiotic treatment and consequently they were placed in the control group. Stool samples for those 3 mice were collected at day 2. 50 µl of GOS90 syrup, the equivalent of 0.26 g/kg bodyweight of GOS (not including lactose), was delivered daily orally by pipette to the prebiotic-fed group for 14 days. The prebiotic dose was selected based on a previous study (Everard *et al.*, 2011). No carrier solution (placebo) was provided to the control mice.

All faecal samples from prebiotic-fed mice were collected at three time points during the experiment: day 1 (no dietary modulation), day 7 and day 14. Day 1 was the first day of GOS feeding and stools were collected before delivering the prebiotic. All samples were stored at -80 °C immediately after collection. Animals were euthanised by CO_2 inhalation followed by cervical dislocation. Colons were removed, flushed with PBS, and cut into 3 sections – proximal, mid, and distal colon. These were each frozen in their own tube and kept at -80 °C until analysis. All mouse protocols were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Generation of GOS90

Production of highly pure GOS (90% GOS, 10% lactose, w/w) was carried out by standard transgalactosylation reactions utilising a recombinant β-hexosyltransferase (BHT) from *Sporobolomyces singularis* expressed in *Pichia pastoris* as previously described (Dagher *et al.*, 2013). Since this preparation was generated using a BHT and not a β-galactosidase, the generated product was a highly-pure β(1-4)GOS free of residual glucose and galactose. Reactions were carried out using standardised amounts of enzyme (0.5 U/g lactose) in 5 mM sodium phosphate buffer (pH 5.0) and a similarly buffered solution containing lactose (200 g/l) at 30 °C. Purity of the GOS90 product used in this study was determined as previously described (Bruno-Barcena and Azcarate-Peril, 2015; Dagher *et al.*, 2013).

DNA isolation from faecal and intestinal content samples

All samples were kept frozen at -80 °C until use. Prior to DNA extraction of the distal colon samples, the mucosal layer was gently scraped with a razor blade. The scraped material was placed in a microtube. At that time, DNA from faecal samples and intestinal samples (scraped material) was extracted using the QIAmp DNA Stool kit (Qiagen, Hilden, Germany). Modifications in the disruption step were made to the supplied protocol to ensure lysis of Gram-positive bacteria. In short, 200 mg of sample were transferred to a microtube containing 0.2 g of autoclaved glass beads (11 micron in diameter, Sigma) and 1.4 ml of ASL solution (Qiagen). Next, samples were homogenised in a TissueLyser II instrument (Qiagen Inc., Valencia, CA, USA) at 25 Hz for 2 min. The following steps were performed according to the manufacturer's protocol.

Barcoded 16S rRNA PCR and pyrosequencing

Amplicon pyrosequencing of the 16S ribosomal gene was performed as previously described (Devine *et al.*, 2013; Thompson *et al.*, 2015). Amplification of the V1-V2 region of the bacterial 16S rDNA gene was performed on total DNA from faecal and intestinal samples. Master mixes for these reactions included the Qiagen HotStar HiFidelity Polymerase Kit (Qiagen Inc.) with a forward primer composed of the Roche Titanium Fusion Primer A (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'), a 10 bp Multiplex Identifier (MID) sequence (Roche, Indianapolis, IN, USA) unique to each sample and the universal bacteria primer 8F (5'-AGAGTTTGATCC-TGGCTCAG-3') (Edwards *et al.*, 1989). The reverse primer was composed of the Roche Titanium Primer B (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3'), the identical 10 bp MID sequence as the forward primer and the reverse primer 338R (5'-GCTGCCTCCC-GTAGGAGT-3') (Fierer *et al.*, 2008). We also performed amplicon sequencing of fragments generated by PCR amplification using the *Bifidobacterium*-specific primer B-8F (5'-AGGGTTCGATTCTGGCTCAG-3') (Martinez *et al.*, 2010) paired with the 338R as the reverse primer for specific detection of this genus. For intestinal content samples we used a combination (4:1) of the primers 8F and Bifido-8F with 338R as the reverse primer as described (Martinez *et al.*, 2010). PCR products were gel-purified individually using the E-Gel Electrophoresis System (Life Technologies, Invitrogen, Carlsbad, CA, USA), and standardised prior to pooling. The 16S rDNA amplicons from the pooled sample were sequenced on a Roche 454 Genome Sequencer FLX Titanium instrument in the Microbiome Core Facility (Chapel Hill, NC, USA) using the GS FLX Titanium XLR70 sequencing reagents and protocols.

Amplicon sequencing data analysis

16S rRNA amplicon sequencing data was analysed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso *et al.*, 2010). Sequences were de-multiplexed, filtered for quality control and grouped into Operational Taxonomic Units (OTUs) at a 97% level to approximate species-level phylotypes using UCLUST with standard parameters (Edgar, 2010). Next, chimeras and singletons were removed using ChimeraSlayer within QIIME (Haas *et al.*, 2011). To ensure an even sampling

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depth, a random selection of 2,700 sequences (matching the sample with lowest number of reads) from each sample was used for rarefaction analysis to determine alpha diversity metrics (observed species and phylogenetic diversity indexes) on rarefied OTU tables. Beta diversity and principal coordinates analysis (PCoA) were also calculated within QIIME using weighted and unweighted unifrac distances (Lozupone *et al.*, 2006) between samples at a depth of 2,700 sequences per sample to evaluate dissimilarities between samples. Sequencing data obtained in this work has been registered as a bioproject in NCBI (BioProject ID PRJNA291486).

PICRUSt analysis of 16S amplicon sequencing data

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (http://picrust.github.com/picrust/) is a tool that predicts metagenome functional content from 16S rRNA amplicon sequencing input data. OTUs were picked to be used in PICRUSt using a closed reference OTU picking against the reference genome Greengenes database Version 13_8 (http://greengenes.secondgenome.com/). OTUs were assigned at 97% identity within QIIME using the UCLUST method (Edgar, 2010).

Bifidobacterium species detection by 24.192 Dynamic array

The access array AA 24.192 (Fluidigm Corporation, San Francisco, CA, USA) in the Advanced Analytics Core at UNC (NC, USA) was used to detect specific *Bifidobacterium* species. The bacterial strains listed in Table 1 were used as controls in the study. All strains were grown in De Man, Rogosa and Sharpe broth (BD Difco, Franklin Lakes, NJ, USA) + 0.5% cysteine at 37 °C under anaerobic conditions (80% N_2, 10% CO_2, and 10%, H_2) in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA). Primers defined in previous studies (Hermann-Bank *et al.*, 2013; Junick and Blaut; Kwon *et al.*, 2006; Matsuki *et al.*, 2004) were used to amplify the 16S ribosomal RNA and the chaperonin *gro*EL genes (Table 2).

Primers targeted the following: domain Bacteria, phylum Actinobacteria, genus *Bifidobacterium* and the *Bifidobacterium* species listed in Table 2. All primers were tested by standard PCR and conventional quantitative PCR using pure cultures of the strains listed in Table 1 and a pool of the strains at the same concentration. Standard PCR was performed using Qiagen HotStar HiFidelity PCR reagents and 50 ng of genomic DNA per reaction. PCR reactions were performed in duplicate under the following conditions: initial denaturation at 95 °C for 1 min, followed by 35 cycles of 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, followed by a final 5 min extension at 72 °C. Conventional qPCR was performed using an ABI Prism 7000 (Applied Biosystems, Carlsbad, CA, USA). The reaction mixture (20 µl) contained 2× power SYBRGreen PCR Master Mix, 200 nM of each primer and 2 µl of the DNA template. qPCR reactions were performed in triplicate under the following conditions: initial holding stage at 50 °C for 2 min and 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s and 60 °C for 1 min.

DNA from stool samples at a concentration of 25 ng/µl was used for high-throughput qPCR. To increase the template target yield, a pre-amplification (specific target amplification: STA) was performed before the Fluidigm Real-Time PCR according to recommendations from the manufacturer. This step consisted of a multiplex PCR reaction with a pool of the 13 primer sets. The reaction was performed using the following conditions: initial annealing at 95 °C for 2 min, followed by 12 cycles of 95 °C for 15 s and 60 °C for 4 min. After the STA reaction, products were diluted 5 times with Tris-EDTA buffer. Microfluidic qPCR was performed using a BioMark HD reader with a Dynamic Array 24.192 chip (Fluidigm). The 24.192 chip was processed following the manufacturer instructions. Briefly, 3 µl of the sample premix (2× SsoFast Evagreen supermix, 20× DNA binding dye and 1.35 µl 5-fold diluted STA products) and 3 µl of the assay premix (2× assay loading buffer, 1× DNA suspension buffer, and 10 µM each primer) were loaded into the chip. Finally, real-time PCR was performed following the next conditions: 95 °C for 60 s, followed by 35 cycles of 96 °C for 5 s and 60 °C for 45 s.

Table 1. *Bifidobacterium* strains used in the present study as positive and negative controls.

Bifidobacterium strain	Sample original ID	Origin
B. longum longum	NCK 1575	kindly provided by T. Klaenhammer (NC State University)
B. longum infantis	ATCC 15697	ATCC
B. pseudocatenulatum	human stool sample	kindly provided by T. Keku (UNC-Chapel Hill)
B. bifidum	ATCC 29521	ATCC
B. breve	ATCC 15700	ATCC
B. lactis	NCK 1573	kindly provided by T. Klaenhammer (NC State University)
B. adolescentis	human stool sample	kindly provided by T. Keku (UNC-Chapel Hill)
B. bifidum	NCK 2083	kindly provided by T. Klaenhammer (NC State University)

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Table 2. Specific primers used in the study to target different bacterial groups and species of *Bifidobacterium* based on the 16S and *gro*EL gene.

Target species	Primer	Target gene	Sequence (5' to 3')	Amplicon size	Reference
All bacteria	Uni_V4_F	16S	CAGCAGCCGCGGTAATAC	389	Hermann-Bank *et al.* (2013)
	Uni_V4_R		CCGTCAATTTCTTTGAGTTT		
All bacteria	Uni_F	16S	GTGSTGCAYGGYYGTCGTCA	194	Maeda *et al.* (2003)
	Uni_R		ACGTCRTCCMCNCCTTCCTC		
All bacteria	8F	16S	AGAGTTTGATCCTGGCTCAG	330	Edwards *et al.* (1989), Fierer
	8F_B		AGGGTTCGATTCTGGCTCAG		*et al.* (2008), Martinez *et al.*
	Uni_338_R		GCTGCCTCCCGTAGGAGT		(2010)
Actinobacteria	Actino_235_F	16S	GCGKCCTATCAGCTTGTT	283	Hermann-Bank *et al.* (2013)
	Actino_567_R		CCGCCTACGAGCYCTTTACGC		
Bifidobacteriaceae	Bifidobac_F	16S	CTCCTGGAAACGGGTGG	442	Hermann-Bank *et al.* (2013)
	Bifidobac_R		CTTCACACCRGACGCG		
Bifidobacterium	Bifido_5	16S	GATTCTGGCTCAGGATGAACGC	236	Gueimonde *et al.* (2004)
	Bifido_3		CTGATAGGACGCGACCCCAT		
B. adolescentis	Biado_1a		CTCCAGTTGGATGCATGTC	279	Matsuki *et al.* (1999)
	Biado_1b	16S	TCCAGTTGACCGCATGGT		
	Biado_2		CGAAGGCTTGCTCCCAGT		
B. longum	Longum_5	16S	TTCCAGTTGATCGCATGGTCTTCT	277	Gueimonde *et al.* (2004)
	Longum_3		GGCTACCCGTCGAAGCCACG		
B. catenulatum group	BiCATg_1	16S	CGGATGCTCCGACTCCT	285	Matsuki *et al.* (1999)
	BiCATg_2		CGAAGGCTTGCTCCCGAT		
B. bifidum	Bifidum_F	16S	TGA CCG ACC TGC CCC ATG CT	110	Rinne *et al.* (2005)
	Bifidum_R		CCC ATC CCA CGC CGA TAG AAT		
B. breve	BiBRE_F	16S	CCGGATGCTCCATCACAC	288	Matsuki *et al.* (1999)
	BiBRE_R		ACAAAGTGCCTTGCTCCCT		
B. animalis	ISR_lactis_F	ISR	ATCCGAACTGAGACCGGTT	382	Kwon *et al.* (2006)
	ISR_lactis_R		GCATGTTGCCAGCGGGTGA		
B. angulatum	BiAng_1	16S	CAGTCCATCGCATGGTGGT	280	Matsuki *et al.* (1999)
	BiAng_2		GAAGGCTTGCTCCCCAAC		
B. dentium	BiDEN_1	16S	ATCCCGGGGGTTCGCCT	387	Matsuki *et al.* (1999)
	BiDEN_2		GAAGGGCTTGCTCCCGA		
B. gallicum	BiGAL_1	16S	TAATACCGGATGTTCCGCTC	303	Matsuki *et al.* (1999)
	BiGAL_2		ACATCCCCGAAAGGACGC		
B. adolescentis	B_ado_F	*gro*EL	CTCCGCCGCTGATCCGGAAGTCG	268	Junick and Blaut (2012)
	B_ado_R		AACCAACTCGGCGATGTGGACGACA		
B. longum	B_lon_F	*gro*EL	CGGCGTYGTGACCGTTGAAGAC	259	Junick and Blaut (2012)
	B_lon_R		TGYTTCGCCRTCGACGTCCTCA		
B. catenulatum group	B_cat_F	*gro*EL	GGCTATCGTCAAGGAGCTCA	188	Junick and Blaut (2012)
	B_cat_R		AGTCCAGATCCAAACCGAAAC		
B. bifidum	B_bif_F	*gro*EL	CTCCGCAGCCGACCCCGAGGTT	233	Junick and Blaut (2012)
	B_bif_R		TGGAAACCTTGCCGGAGGTCAGG		
B. breve	B_bre_F	*gro*EL	GCTCGTCGTTGCCGCCAAGGACGTT	272	Junick and Blaut (2012)
	B_bre_R		ACAGAATGTACGGATCCTCGAGCACG		
B. animalis	B_ani_F	*gro*EL	CACCAATGCGGAAGACCAG	184	Junick and Blaut (2012)
	B_ani_R		GTTGTTGAGAATCAGCGTGG		
B. angulatum	B_ang_F	*gro*EL	CTGTCCTCCCAGCAGGACGTGGTC	97	Junick and Blaut (2012)
	B_ang_R		GCGCTTCGCCGTCAACGTCTTCGG		
B. dentium	B_den_F	*gro*EL	GGCCCAGTCTTTGGTGCATGAAGGCC	364	Junick and Blaut (2012)
	B_den_R		GTCTTCGAGCACCGCGGTCTGGTCC		
B. gallicum	B_gal_F	*gro*EL	AGCTCGTCAAGTCCGCCAAGC	188	Junick and Blaut (2012)
	B_gal_R		CATACCTTCGGTGAACTCGAGG		

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In order to validate the method and obtain the Pearson's correlation between the two different primer sets (16S and *gro*EL) used in this study, DNA from pure bacterial cultures of *Bifidobacterium* species (Table 1) at three different concentrations (25 ng/µl, 2.5 ng/µl and 0.25 ng/µl) were analysed with their respective primers sets.

High-throughput qPCR data analysis

Raw data were normalised using the Livak method (Schmittgen and Livak, 2008). C_q values for each sample were normalised against their respective C_q value obtained from universal primers using the equation:

Ratio (reference/target) = $2^{-C_t\,(ref)\,-\,C_t\,(target)}$

Targeted metabolome analysis

Target metabolites included 5 SCFAs (acetate, propanoate, isobutyrate, butyrate, valerate) and other relevant metabolites (succinate, glycerol, lactate, glucose, aminobutyrate and galactose). Briefly, approximately 40 mg of faecal samples were weighed and extracted with 1 ml of water. 4-chlorophenylalanine and ^{13}C-labelled hexanoic acid were used as internal standards. After centrifugation, a volume of 500 µl was used for SCFA analysis with propyl-chloroformate derivatisation, and a volume of 150 µl was used for analysis of other metabolites with methoxyamine and bistrimethylsilyltrifluoroacetamide derivatisation. The derivates were analysed with gas chromatograph-mass spectrometer (GC-MS) analysis at UNCG Metabolomics Facility (Kannapolis, NC, USA).

Cytokine analysis

Colon tissues (proximal and medium regions) were collected in 700 µl of sterile PBS supplemented with complete EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) and 0.1% IGEPAL. Colon tissues weighing between 300 and 600 mg were homogenised in a TissueLyser (Qiagen Inc.) for 5 min at 30 Hz at room temperature. Colon homogenates were centrifuged twice at 15,000×*g* for 20 min at 4 °C to remove cell debris, and the supernatants were aliquoted and stored at -80 °C. Supernatants were used for cytokine analysis using an ELISA kit (Qiagen Inc.) following the procedures recommended by the manufacturer. The cytokines analysed were interferon gamma (IFN-γ), interleukin (IL)-1β, IL-12, IL-6, IL-10 and tumour necrosis factor alpha (TNF-α). Values were expressed as pg/mg protein. The mucosal protein content was determined using the Bradford assay (BioRad, Richmond, CA, USA).

Statistical analyses

To identify bacterial taxa and biological pathways significantly impacted by prebiotic feeding, we used the non-parametric Mann-Whitney and Steel Dwass All Pairs test, which accounts for multiple comparisons, in JMP Genomics (SAS JMP Genomics 5.0, Cary, NC, USA). Differences in the abundance of bacterial taxa and pathways were analysed between control and GOS90 at day 7 and at day 14. To test differences in microbial communities we performed Analyses of Similarity (ANOSIM) to calculate R and *P* values using the phylogeny-based unweighted UniFrac distance metric. For metabolomics and cytokine analysis, unpaired t-tests were used to compare metabolites concentration between feeding groups (SAS JMP Genomics 5.0). *P*-values of less than 0.05 were considered significant.

3. Results

Microbiome composition of stools and intestinal contents of wt 129 mice

To assess the influence of GOS90 on the gut microbiome, we performed a community structure analysis of faecal samples of wt 129 SPF mice whose diet was supplemented or not (control) with 1% (0.26 g/kg/day) of the prebiotic for 14 days (Figure 1). Pyrosequencing of the regions V1-V2 of the 16S rRNA gene from 39 faecal samples using the universal bacterial primers 8F and 338R resulted in an average of 4,316±1,095 sequences per sample. A total of 172,636 sequences were assigned to 3,366 OTUs at ≥97% similarity, clustering into 78 genera, 18 classes and 9 phyla. The overall composition of the faecal microbiome without dietary supplementation (n=27) was dominated by the phyla Firmicutes (59.2±22.4%) and Bacteroidetes (37±23.6%) (Figure 2A), in accordance with previous reports of the gut microbiome composition of wt 129 mice (Ohland *et al.*, 2013). Other phyla detected included Deferribacteres (1.9±2.0%), Proteobacteria (0.8±0.6%) and Actinobacteria (0.6±0.6%) (Figure 2A). Representatives of the Tenericutes and TM7 phyla were also detected at low levels (<0.5%). At the order level, the predominant groups were Lactobacillales (40±25.8%), Bacteroidales (37±23.6%) and Clostridiales (17.6±15.4%) (Figure 2B). Composition of the gut microbiome of individual mice showed that Bacteroidetes was the phylum predominant in males, while Firmicutes was more abundant in female mice at day 1, with the exception of mice C2M3 and C4M3 (Supplementary Figure S1).

Sequencing of 16S rRNA amplicons from intestinal samples using a mixture (4:1) of forward primers 8F and B8F for the specific detection of *Bifidobacterium* as described by Martinez *et al.* (2010) yielded a total of 50,716 sequences that were assigned to 362 OTUs at ≥97% similarity. As in stools, Firmicutes and Bacteroidetes were the dominant

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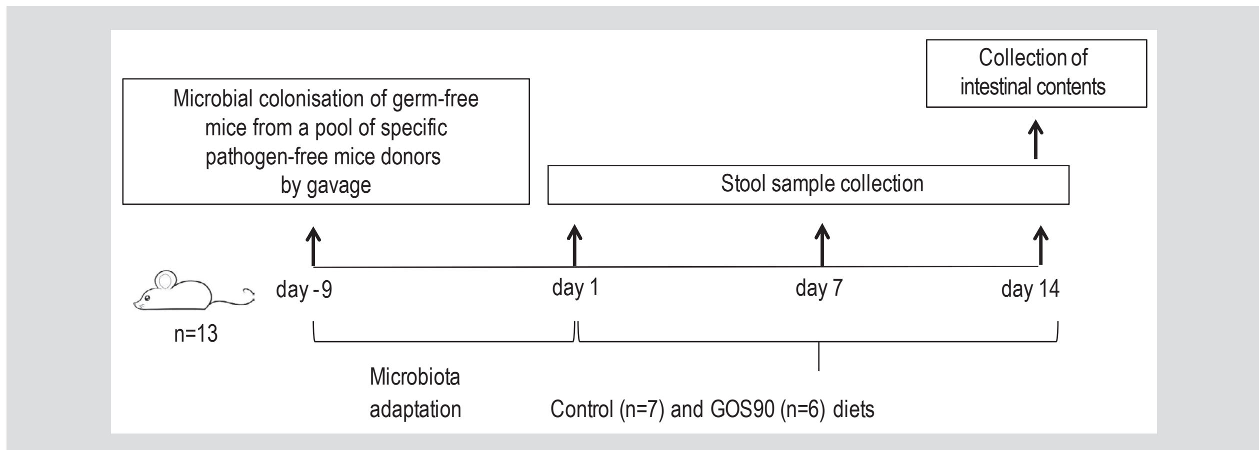

Figure 1. Study experimental design. Thirteen germ-free mice were colonised by gavage from a pool of specific pathogen-free donors. After 9 days, GOS90 was delivered orally to six mice for 14 days. Faecal samples were collected at three time points (1, 7 and 14 days) and intestinal contents were collected at day 14. Day in negative numbers indicate days before starting the feeding trial.

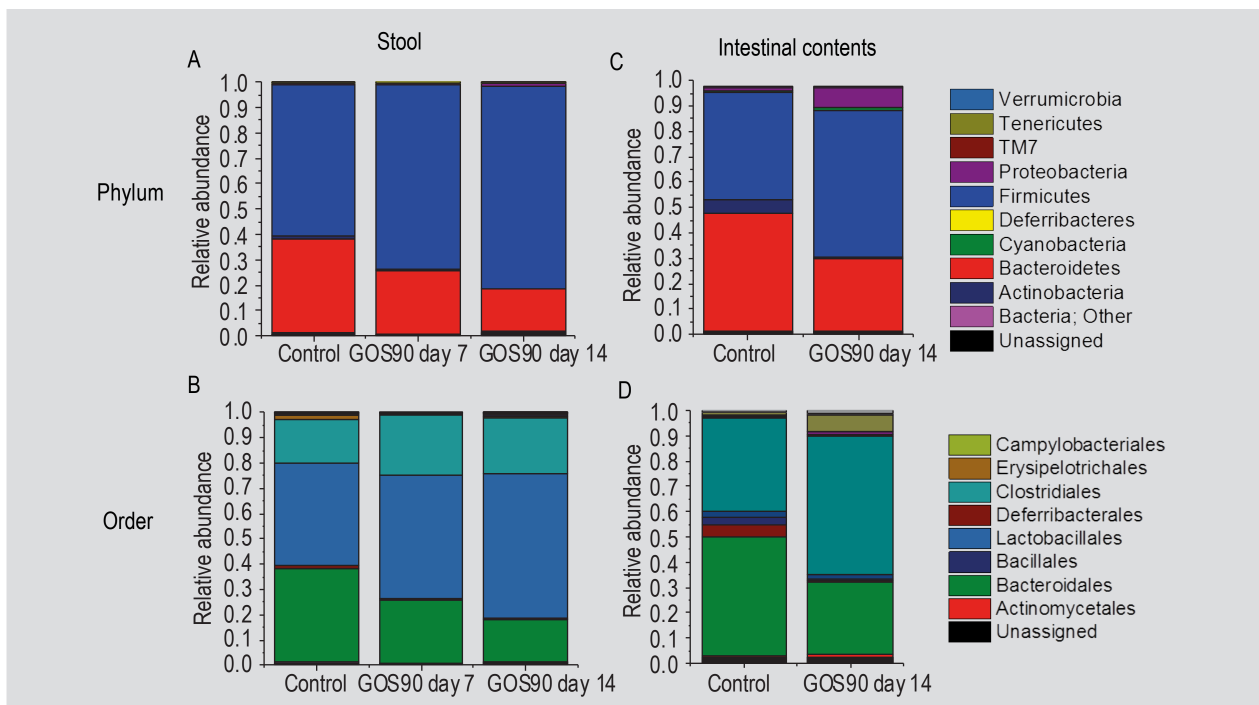

Figure 2. Microbiome composition of stools and intestinal contents. Phylum and order level distributions of faecal (A, B) and intestinal contents (C, D) microbiota by feeding group (control and GOS90) at day 7 and 14 of the study. The control group included all stool samples from mice not fed the prebiotic. Universal 8F-338R primers were used for stool samples and a combination of the universal and *Bifidobacterium*-modified primer set were used for the intestinal contents.

taxa but proportions of both phyla were similar (46.7±15.2% and 42.5±18.3%, respectively; Figure 2C). Abundance of Deferribacteres (5.1±8.9%) and Proteobacteria (1.6±1.3%) was higher in intestinal contents than faecal samples (1.9±2.0% and 0.7±0.6%, respectively) although differences did not reach statistical significance. Interestingly, abundance of the order Lactobacillales in intestinal contents was low (2.5±2.1%) compared to stool samples (40±17.5%), while abundance of Clostridiales was higher in intestinal contents (36.0±16.3%) than in faecal samples (17.0±12.3%).

Alpha diversity analysis performed in 2,700 randomly selected sequences per sample showed that the faecal microbiome diversity and richness increased over time in prebiotic-fed mice (Table 3). A small, but statistically significant effect on variables was observed for treatment suggesting that the faecal microbiome composition was

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Table 3. Phylogenetic diversity and species richness of control and GOS90 samples at days 7 and 14. Universal 8F-338R primers were used for 16S amplicon sequencing of stool samples and a combination of the universal and *Bifidobacterium*-modified primer set (4:1) were used for intestinal contents.

	Stool samples		Intestinal contents	
	Phylogenetic diversity	Species richness	Phylogenetic diversity	Species richness
Control	15.0±6.1	138.2±72.9	10.6±2.0	117.0±56.8
GOS90 day 7	16.5±4.7	157.8±60.8	-	-
GOS90 day 14	17.5±4.3	170.5±65.0	10.9±2.9	124.4±59.9

modified by the prebiotic (ANOSIM R=0.1127, *P*=0.025) (Figure 3).

Influence of sex on the faecal microbiota of wt 129 mice

In our study, unweighted PCoA analysis of samples showed a clear clustering of samples by sex (Supplemental Figure S2A and S2B). However, since we applied the treatment per pen, and we had only one pen per interaction of factors, data was insufficient to draw significant conclusions. Control males had significantly lower bacterial diversity (PD=7.8±1.6 in stools and PD=9.5±1.0 in intestinal contents) compared to control females (PD=15.1±3.4 in stools and PD=13.0±0.6 in intestinal contents). We identified significant differences in the abundance of fifteen genera belonging to the phyla Actinobacteria, Proteobacteria, Bacteroidetes, Firmicutes and Tenericutes between genders (Supplemental Figure S2). Specifically, the relative abundance of nine genera (*Anaeroplasma*, *Helicobacter*, *Butiricicoccus*, *Roseburia*, *Coprococcus*, *Lactobacillus*, *Alistipes*, *Odoribacter* and *Asaccharobacter*) was significantly higher (between 1- and 80-fold) in females than males. In contrast, abundance of eight genera (*Parasuterella*, *Coprobacillus*, *Anaerostipes*, *Anaerovorax*, *Hydrogenoanaerobacterium*, *Eubacterium*, *Enterococcus* and *Bacteroides*) was higher (between 1.3- and 10-fold) in the microbiota of male mice. Since no male mice were included in the prebiotic-fed group and to identify the GOS90-specific impact on the gut microbiota, only samples from female mice (mice without dietary modification (n=15), GOS90-fed mice at days 7 (n=6) and 14 (n=6) were considered for further analysis.

Gut microbiome composition of control and prebiotic-fed mice

GOS90 feeding resulted in statistically insignificant increases in relative abundance of Firmicutes (Control=70±20.8%; GOS90=80±17.5%), especially Lactobacillales (Control=48±29.11%; GOS90=57±29.8%), and decreases of Bacteroidetes (Control=25±20.9%, GOS90=16.7±15.7%), especially the order Bacteroidales (Figure 2). We observed a high inter-individual variation,

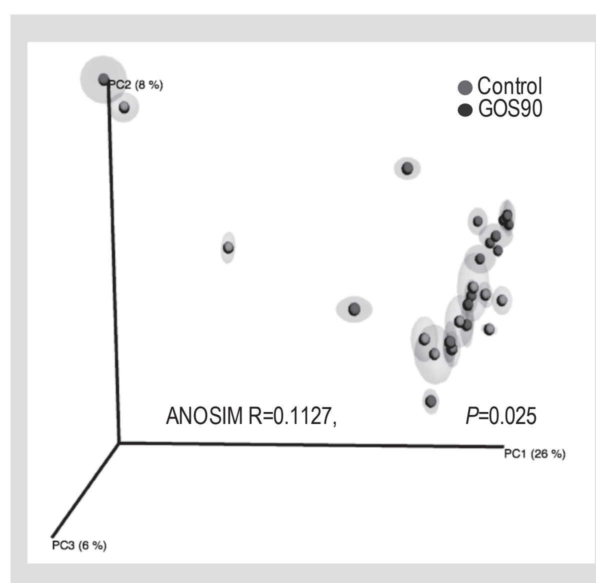

Figure 3. Unweighted Principle Coordinate Analysis plot of faecal microbiome from control (n=15) and prebiotic-fed female mice (n=12). Each dot represents one faecal sample. All time points were included in the analysis.

but despite this variation, GOS90 feeding was associated with an underrepresentation of three Bacteroidales taxa: unclassified Bacteroidales (Steel Dwass All Pairs, *P*=0.02), *Parabacteroides* (*P*=0.1) and unclassified Rikenellaceae (*P*=0.07) after 14 days (Figure 4). In addition, *Helicobacter* (*P*=0.005), *Ruminococcus* (*P*=0.02) and *Clostridium* (*P*=0.1) were underrepresented at days 7 and 14 compared to the control.

Analysis at OTU level showed that 14 out of 15 *Lactobacillus* OTUs impacted by GOS showed an increased abundance (Supplementary Figure S3). Likewise, although total proportional amounts of Bacteroidetes (Bacteroidales) declined after 14 days, GOS90 increased abundance of specific *Bacteroides* OTUs at days 7 and 14. Additionally, within Clostridiales we observed an increased abundance of six Lachnospiraceae OTUs, three Ruminococcaceae OTUs, and one *Clostridium* OTU at the end of the feeding trial.

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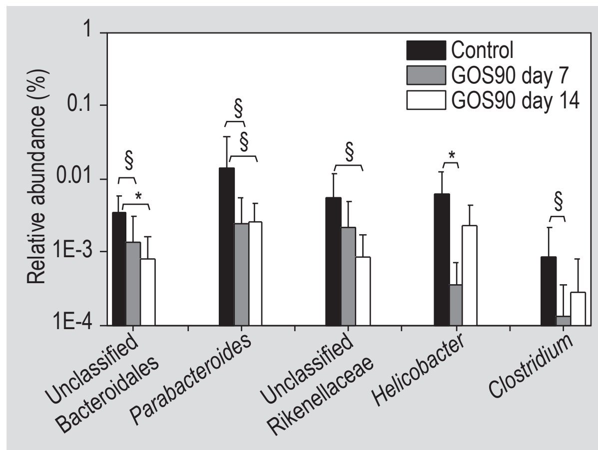

Figure 4. Mean relative abundance of underrepresented genera at days 7 (n=6) and 14 (n=6) of GOS90 feeding compared to control samples (n=15). Error bars indicate standard deviations (Steel-Dwass All Pairs, * *P*≤0.05, § *P*≤0.1).

The analysis of intestinal contents with a combination of universal and *Bifidobacterium*-specific primers resulted in similar changes at the phylum level as stools, with increased abundance of Firmicutes and decreased Bacteroidetes in prebiotic-fed mice. Due to the small sample size (n=5) owing to depletion of 4 samples (original n=9 female mice) we did not perform statistical analyses on abundance of bacterial taxa of intestinal samples from female mice. However, we observed that, unlike stools that showed an increase in the order Lactobacillales, in intestinal contents there was an increased abundance of the order Clostridiales (Figure 2D) and, similar to stool samples, we observed a marked reduction in the abundance of unclassified Bacteroidales (control=0.02±0.007; GOS90=0.005±0.002).

Faecal microbiome of prebiotic-fed mice using *Bifidobacterium* specific primers

Since the universal primers used in our first 16S amplicon sequencing experiment have been reported to under-detect *Bifidobacterium* (Martinez *et al.*, 2009), extensively reported to be increased by GOS feeding (Davis *et al.*, 2011; Vulevic *et al.*, 2013; Walton *et al.*, 2012), we performed additional amplicon pyrosequencing of faecal samples using *Bifidobacterium*-specific primers (Martinez *et al.*, 2010). Sequencing resulted in an average of 3,328±1,319 sequences per sample (149,962 sequences in total), which were assigned to 769 OTUs. 36 OTUs were specifically assigned to the phylum Actinobacteria and 9 to the genus *Bifidobacterium*. We observed an increasing trend in the abundance of Actinobacteria, Bifidobacteriaceae and *Bifidobacterium* associated with GOS90 feeding (Figure 5). A closer examination of samples at day 14 revealed that the effect of GOS90 varied among individual mice. From day 7 to 14, abundance of *Bifidobacterium* increased 2.5- to 15-fold in four out of six GOS90-fed mice while abundance of this group decreased in the other two animals. Following the designation by Davis *et al.* (2011), we denominated the animals that showed increased abundance of *Bifidobacterium* 'responders' and the ones that showed no increase 'non-responders' (Figure 5).

Specific *Bifidobacterium* species affected by prebiotic-fed mice

We next aimed to identify specific *Bifidobacterium* species affected by GOS90. Primers specific for nine species of *Bifidobacterium*, genus *Bifidobacterium*, family Bifidobacteriaceae and phylum Actinobacteria were used to quantify bacterial groups by high-throughput qPCR using the Access Array 192.24 (Fluidigm). We used two primer sets for each taxon, targeting the 16S ribosomal gene and the chaperonin *gro*EL. Positive correlations (Pearson's r>0.5) were observed between the two methods for 16S and *gro*EL gene primer sets (Supplementary Figure S4).

Data showed an increased abundance of Bifidobacteriaceae and *Bifidobacterium* (13- and 4-fold, respectively) after 14 days (Figure 6) as well as a marked increase in the abundance of *Bifidobacterium adolescentis, Bifidobacterium catenulatum, B. lactis and Bifidobacterium gallicum. The B. catenulatum group* (*B. catenulatum and Bifidobacterium pseudocatenulatum*) exhibited the largest increase in the GOS-fed group. A linear fit analysis performed to assess the correlation between 16S rRNA and *gro*EL data for detection of *Bifidobacterium* species showed Pearson's r correlation values >0.70 for *Bifidobacterium longum, B. adolescentis, B. gallicum* and *B. lactis*, while lower correlation indices (Pearson's r<0.70) were observed for *B. catenulatum, Bifidobacterium angulatum, Bifidobacterium dentium, Bifidobacterium bifidum* and *Bifidobacterium breve* (Supplementary Figure S5). Data suggest that all primers targeting the *gro*EL gene were adequate for all *Bifidobacterium* species with the exception of *B. breve*, while primers targeting the 16S rRNA gene were less optimal for *B. angulatum*, *B. longum* and *B. bifidum.*

Predictive analysis of metabolic functions associated with GOS90 feeding

We next used PICRUSt to predict metabolic pathways and enzymes influenced by GOS. We identified a total of 328 pathways in all samples (control and prebiotics). We observed a non-significant (Steel Dwass All Pairs, *P*=0.14) over-representation of the galactose metabolism pathway in the GOS90 group at the end of the trial (Figure 7A). When data were stratified into responders and non-responders, a clear over-representation of this pathway was observed in responders (*P*=0.01), while representation of this pathway in non-responders was similar to the control group (Figure 7A). Similar patterns were observed for the starch and sucrose metabolism pathways (data not shown).

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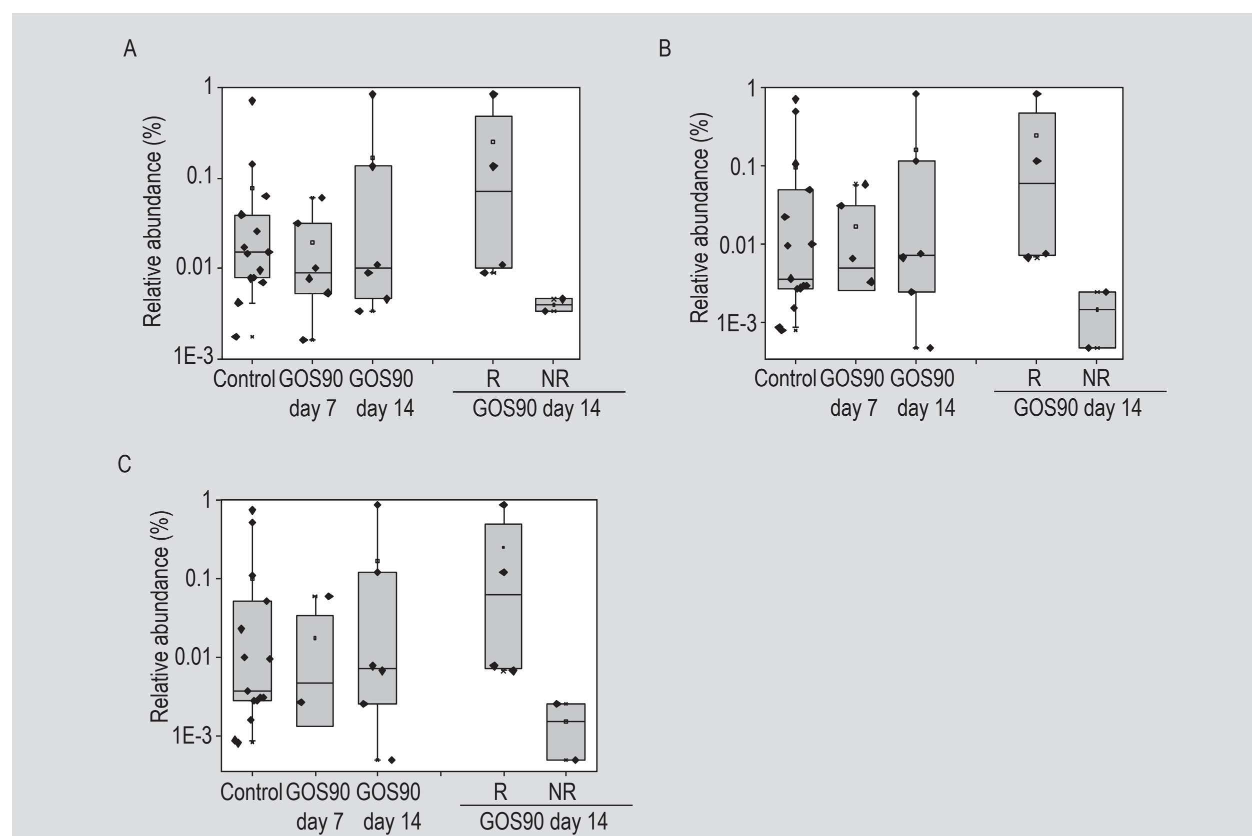

Figure 5. Mean relative abundances of (A) phylum Actinobacteria, (B) family Bifidobacteria and (C) genus *Bifidobacterium* by feeding group at days 7 (n=6) and 14 (n=6). Data was stratified in responders (n=4) showing increased abundance of *Bifidobacterium* in response to prebiotic feeding, and non-responders (n=2), showing no change in response to prebiotic feeding. Error bars indicate the 10th and 90th percentile. Steel-Dwass All Pairs test * *P*≤0.05, §≤0.1.

GOS90 feeding also resulted in the predicted over-representation of a D-lactate dehydrogenase and a lactate transporter from the LctP family (Figure 7B), and one gene involved in the metabolism of galactose, 6-phospho-β-galactosidase [EC 3.2.1.85] (Figure 7C). When data were further divided into responders and non-responders, differences became significant or close to significant for responders (Figures 7B and 7C).

Faecal metabolites from control and prebiotic-fed mice

Intermediate and end metabolites from GOS utilisation (SCFAs, succinate, glycerol, lactate, glucose, aminobutyrate and galactose) were measured in stool samples by chromatography to determine if the prebiotic influenced SCFA generation. Butyrate, lactate, acetate, propanoate, glucose and glycerol were detected at their highest concentration in both groups at day 1 (no dietary modulation). Levels of isobutyrate, valerate, 2-aminobutyrate, succinate and galactose were on average below 1 μmol/g faecal sample (Table 4). We observed a significantly lower abundance of acetate (unpaired t-test, *P*=0.03), and borderline significant decreases of lactate (*P*=0.09), and butyrate (*P*=0.1) in prebiotic-fed mice compared to control mice. The same results were observed when data from responders and non-responders were analysed separately (Table 4).

Inflammatory biomarkers in control and prebiotic-fed mice

Pro-inflammatory cytokines IL-6, IL-12, IL-1β, IFN-γ, TNF-α and the anti-inflammatory cytokine IL-10 were quantified by ELISA in the proximal and distal colon of the control and GOS90 groups. No significant differences were detected between groups; however, we observed different cytokine profiles in the proximal and distal colon in both groups. Specifically, in the distal colon the pro-inflammatory cytokines IL-6 and IL-12 were detected at lower levels while levels of TNF-α were higher. Levels of IL-1β and IFN-γ in both colon sections, and IL-10 in the distal colon, were below the method's detection limits (Table 5).

4. Discussion

The aim of this study was to evaluate the impact of a highly pure GOS formulation (GOS90) (Dagher *et al.*, 2013), composed of GOS (90%) and lactose (10%) on the gut microbiome of wt 129 mice. No significant differences were observed in starting values at day 1. In addition, since

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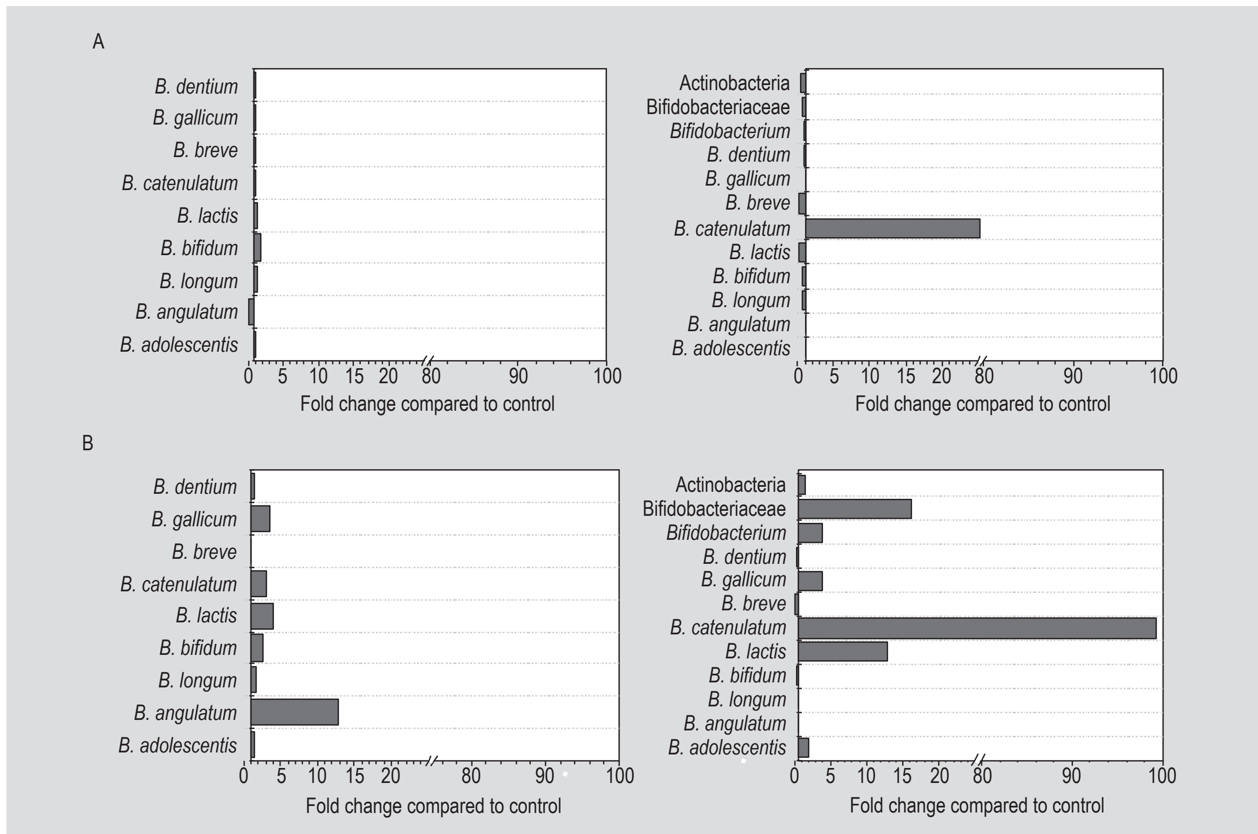

Figure 6. Absolute quantification of *Bifidobacterium* species by high-throughput qPCR. Changes in abundance between GOS90 (n=12) and controls (n=15) are shown at (A) day 7 (n=6) and (B) day 14 (n=6). Left panels show data generated using *gro*EL-specific primers, right panels show data generated using 16S rRNA gene-specific primers.

we intended to focus our analysis on the longitudinal effect of GOS90 rather than cross-sectional differences, we performed most comparisons between the GOS90 group at 7 and 14 days and the overall control. Our data showed that GOS90 feeding for 14 days resulted in an increase of faecal relative abundance of the bifidobacteria *B. adolescentis*, *B. catenulatum*, *B. lactis*, *B. gallicum* and specific OTUs of *Lactobacillus*, Bacteroidetes and Clostridiales. Duration of treatment was chosen based on previously published research in humans and mice, which reported range treatments from 7 days (Ito *et al.*, 1990; Nakayama and Oishi, 2013) to 12 weeks (Davis *et al.*, 2011). Our study showed an specific increase of *B. catenulatum* and a decrease of *Clostridium* at day 7 of the feeding trial in agreement with the study by Nakayama and Oishi (2013),which reported that, in mice, 7 days of GOS treatment were enough to identify changes in the microbiota that resulted in decreases of *Clostridium* and *Escherichia coli* and increases in *Bifidobacterium* abundances.

Our approach used a dose of 0.26 g GOS90/kg weight administered daily over 14 days. Previous human studies reported a prebiotic impact on gut microbiome composition at doses ranging from 4 to 20 g/day with inulin, fructo-oligosaccharides (FOS) and GOS (Tuohy *et al.*, 2005). With FOS or inulin doses higher than 15 g/day (equivalent to 0.21 g/kg weight/day) inducing flatulence or abdominal bloating (Pedersen *et al.*, 1997; Stone-Dorshow and Levitt, 1987). Most of the studies with GOS in humans used a recommended dosage of 8 to 15 g/day (0.11 to 0.21 g/kg weight/day) (Macfarlane *et al.*, 2008), although the bifidogenic effect has been observed at doses as low as 5 g/day (Anthony *et al.*, 2006; Davis *et al.*, 2010, 2011). In animal studies, increased abundance of *Bifidobacterium* and *Lactobacillus* has been reported in mice fed 1 g of GOS/kg weight (Pan *et al.*, 2009) and with diets supplemented with Oligomate at a 1% concentration (Santos *et al.*, 2006). The impact of GOS on the gut microbiome of laboratory rats has not been studied but animals administered between 2.5 and 5 g/kg weight/day of GOS showed no significant adverse toxicological effects attributable to the prebiotic (Anthony *et al.*, 2006).

A fibre-rich diet has been associated with increased microbial diversity (De Filippo *et al.*, 2010), while a low gut microbial diversity has been correlated with recurrent *Clostridium difficile* infection, Crohn's disease, and obesity (Chang *et al.*, 2008; Manichanh *et al.*, 2006). In this study, we observed a marginal, statistically insignificant increase in diversity and species richness in GOS90-fed mice. These

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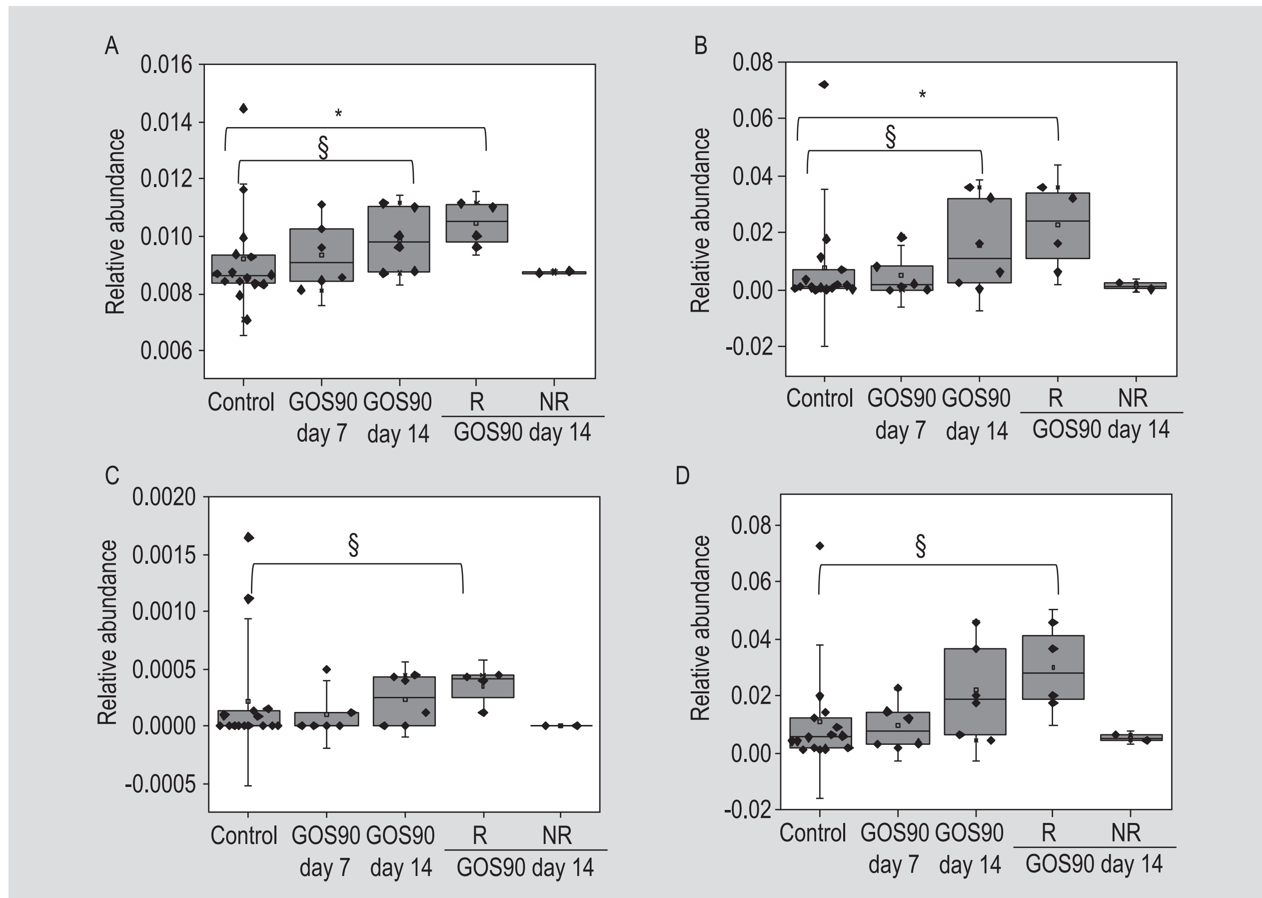

Figure 7. Relative abundances of specific KEGG pathways and enzymes impacted by GOS90 feeding. (A) Galactose metabolism pathway; (B) 6-phospho-beta-galactosidase; (C) D-lactate dehydrogenase; and (D) lactate transporter. Control (n=15), GOS90 at day 7 (n=6), GOS90 at day 14 (n=6), responders (R) to GOS90 at day 14 (n=4) and non-responders (NR) to GOS90 at day 14 (n=2). Boxes represent data between 25th and 75th percentile. Error bars indicate the 10th and 90th percentile. Steel-Dwass All Pairs tests § *P*≤0.1, * *P*≤0.05.

Table 4. Concentration of faecal metabolites from control and GOS90-fed mice at the end of the feeding trial.[1]

Metabolite	Control (n=15)	GOS90 (n=6)	GOS90 (responders) (n=4)	*P*-value (control vs GOS90)	*P*-value (control vs GOS90 responders)
Acetate	**6.93±4.02**	**4.17±1.65**	**3.52±1.52**	**0.03**	**0.02**
Propanoate	6.12±5.39	3.19±2.38	3.26±2.90	0.17	0.16
Isobutyrate	**0.24±0.28**	**0.15±0.19**	**0.08±0.09**	**0.28**	**0.10**
Butyrate	**16.13±8.99**	**10.41±5.64**	**9.30±5.41**	**0.10**	**0.06**
Valerate	**0.14±0.16**	**0.11±0.09**	**0.04±0.02**	**0.58**	**0.05**
Lactate	7.36±6.90	2.22±3.83	2.73±4.54	0.09	0.11
2-aminobutyrate	0.14±0.06	0.13±0.10	0.13±0.13	0.90	0.99
4-aminobutyrate	**2.15±0.93**	**1.61±0.85**	**1.33±0.86**	**0.40**	**0.08**
Glycerol	3.62±3.07	2.55±2.69	3.26±2.97	0.71	0.81
Succinate	0.12±0.10	0.15±0.08	0.16±0.09	0.36	0.39
Galactose	0.90±0.71	0.64±0.57	0.79±0.63	0.62	0.74
Glucose	4.64±3.22	3.34±3.12	4.00±3.57	0.57	0.71

[1] Significant differences (unpaired t-tests *P*<0.1) between controls and prebiotic-fed mice and between controls and prebiotic responders are bold.

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Table 5. Cytokine levels of the proximal and distal colon of control (n=3) and GOS90-fed female mice at day 14 (n=6).

Cytokine[1]	Proximal colon		Distal colon	
	Control	GOS90	Control	GOS90
IL-12	19.37±14.57	16.70±10.94	6.50±3.38	6.00±14.95
IL-6	14.71±10.98	34.38±20.91	9.40±6.17	14.09±14.95
TNF-α	20.80±8.46	22.34±6.01	47.92±6.39	47.24±9.63
IL-10	10.74±14.71	4.83±7.44	<LOD	<LOD
IFN-γ	<LOD[2]	<LOD	<LOD	<LOD
IL-1β	<LOD	<LOD	<LOD	<LOD

[1] IL = interleukin; TNF = tumour necrosis factor; INF = interferon. Quantification was performed by ELISA. Values are expressed as pg/mg protein.
[2] LOD = limit of detection.

results suggest that longer periods of prebiotic feeding could result in a higher bacterial diversity.

Interestingly, we observed gender-specific differences in the faecal microbiome of control mice, with lower bacterial diversity in males and an over-representation of the butyrate producers *Roseburia* and *Butyricicoccus* in female mice. A study by Bernbom *et al.* (2006) showed that the faecal microbiota of SPF rats and human microbiota-associated rats clustered according to the gender of the host animal (Bernbom *et al.*, 2006). Our results are consistent also with the observations by Markle *et al.* (2013) who suggested that sex hormones could influence microbial populations. Moreover, a type 1 diabetes study in non-obese diabetic mice showed a hormone-supported increase of selected microbial taxa, which may work as a positive-feedback mechanism contributing to the sexual dimorphism of autoimmune diseases (Yurkovetskiy *et al.*, 2013). More recently, Bolnick *et al.* (2014) analysed associations between gut microbiota and sex×diet, and showed that microbial responses to diet depended on sex in laboratory mice fed either a control diet or a high fat diet. These and our data warrant more studies to elucidate the mechanisms driving differences in the gut microbiota associated with sex×diet.

Impact of GOS90 on the composition of the faecal microbiome varied between individual mice. Moreover, since in our study control mice did not receive any carrier solution, it is possible that stress had an additional impact on GOS90 fed mice. Specifically, four out of six treated mice (responders) showed an increase of up to 15-fold in *Bifidobacterium* abundance while two animals (non-responders) showed no response. The selective response to prebiotics has been previously reported in humans (Davis *et al.*, 2010, 2011). Also, the enrichment of bifidobacteria at the expense of other groups, such as *Bacteroides* has been reported in humans fed GOS (Davis *et al.*, 2011; Vulevic *et al.*, 2013). In addition, in our study we detected a reduced representation of other Bacteroidetes taxa including unclassified Bacteroidales and *Parabacteroides*. Species of these taxa are often associated with opportunistic infections, mainly intra-abdominal and systemic, as well as harbouring some antimicrobial resistance traits that could be transferred to other strains (Boente *et al.*, 2010).

Since 16S rRNA amplicon sequencing methods do not have enough sensitivity to identify bacterial taxa at the species level, we developed and optimised a high-throughput real-time qPCR assay using the Biomark instrument by Fluidigm to quantify bifidobacterial species. Although we observed an overall positive correlation between primers targeting the 16S rRNA and *gro*EL genes for detection of *Bifidobacterium* species, our analysis suggests that primers targeting the *gro*EL gene efficiently detected and quantified the species selected in our study with the exception of *B. lactis.* Conversely, primers targeting the 16S rRNA gene were less optimal for *B. angulatum, B. longum* and *B. bifidum.* The *gro*EL gene has been reported to be more discriminative for the detection and quantification of *Bifidobacterium* species (Junick and Blaut, 2012), in part because it is present in one copy per genome. We observed striking differences in the levels of detection of the *B. catenulatum* group with the two different primer sets, which are explained by the different specificity of each set. The *gro*EL primer is specific for *B. catenulatum*, while the oligonucleotide targeting the 16S ribosomal gene can detect both *B. catenulatum* and *B. pseudocatenulatum*. This result indicates a more pronounced expansion of *B. pseudocatenulatum* in response to the prebiotic.

Data obtained with this new platform confirmed pyrosequencing results and showed an increased abundance of *B. pseudocatenulatum*, *B. gallicum*, *B. lactis* and *B. angulatum* in prebiotic-fed mice at day 14. The most dramatic effect was observed in the *B. catenulatum* group (*B. catenulatum* and *B. pseudocatenulatum*) with

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a 100-fold expansion. Significant GOS-induced increases of this group have been also reported in humans (Davis *et al.*, 2011). In fact, a study that tested the growth of *Bifidobacterium* species with different prebiotics showed that, of those tested, GOS was the only prebiotic to support growth of *B. pseudocatenulatum* (Scott *et al.*, 2014). An increase of 13-fold was also observed in *B. angulatum*; β-galactosidases extracted from *B. angulatum* have been used for the utilisation and generation of GOS with high concentrations of lactose (Rabiu *et al.*, 2001). Finally, we also observed proliferation of *B. lactis*, a species widely used as a probiotic capable of using GOS (Gopal *et al.*, 2001; Milani *et al.*, 2013). In addition, synbiotics – where this species and GOS act synergistically – have been used in different clinical studies proving their beneficial effect on health (Martinez *et al.,* 2011; Prasad *et al.*, 2013).

In this study, we observed a decreased abundance of *Helicobacter* and *Clostridium* in GOS90-fed mice. Previous studies have reported a reduction of colitis severity in *Smad3*-deficient mice treated with the pathogen *Helicobacter* as well as reduction of *Clostridium* by GOS *in vitro* and in mice (Gopalakrishnan *et al.*, 2012; Hopkins and Macfarlane, 2003; Morishita *et al.*, 2002). In addition to their impact on gut bacterial taxa, prebiotics have been shown to enhance host health by directly inhibiting adherence of pathogens to the host epithelial cell surface (Kunz *et al.*, 2000) and modulating the colon microenvironment, which results in a reduction of intestinal infections (Arslanoglu *et al.*, 2008; Shoaf *et al.*, 2006). The role of *Helicobacter* in gastric cancer is well-documented (Plummer *et al.*, 2014; Polk and Peek, 2010) and recent reports suggest a potential role of this bacterium in IBD (Hansen, 2009; Laharie *et al.*, 2009; Whary *et al.*, 2006). Likewise, *Clostridium* has been correlated with increased incidence and growth of colonic tumours in animal studies (Horie *et al.*, 1999; Onoue *et al.*, 1997). Further studies are needed to elucidate the role and potential mechanisms involved in reduction of pathogenic species by the prebiotic used in this study.

SCFAs, especially butyrate, have an important role in host health since they function as energy source for host tissues, have antimicrobial activity and have anti-inflammatory and anti-proliferative properties (Vinolo *et al.,* 2011; Waldecker *et al.*, 2008). In the present study, we observed a decrease in the faecal concentration of acetate, lactate, and butyrate in prebiotic-fed mice. SCFAs' faecal concentration reflects the amount remaining after colonic absorption. Although an inhibited microbial production of these SCFAs cannot be excluded, the reduction of these metabolites could reflect an increase in colonic absorption and reduced excretion. In fact, Vogt and Wolever (2003) reported a negative correlation between acetate faecal concentration and absorption suggesting that faecal SCFA concentrations may better reflect colonic SCFA absorption than production. Undoubtedly, these results warrant further experiments to elucidate generation and fate of SCFAs in the colon of GOS90-fed animals.

We used PICRUSt to infer metagenome functional content from 16S rRNA amplicon sequencing input data. Since GOS are composed of galactose and glucose molecules joined in a β-configuration, we specifically looked at pathways and enzymatic functions involved in galactose metabolism. We observed an enrichment of the galactose metabolism pathways in the prebiotic group that became more evident when only responders were included in the analysis. We also observed an over-representation of pathways involved in the metabolism of starch and sucrose, which suggest that GOS-metabolising bacteria could also have the ability to use other prebiotics such as resistant starch. In fact, studies have reported increases in the relative abundance of *Bifidobacterium* and *Lactobacillus* when starch was used as prebiotic (Senevirathne *et al.*, 2009; Silvi *et al.*, 1999). A lactate dehydrogenase, an enzyme that catalyses the conversion from pyruvate to lactate, and a lactate transporter from the LctP family were also over-represented in the prebiotic group. We also observed a significant over-representation of the 6-phospho-beta-galactosidase [EC 3.2.1.85], an enzyme involved in the utilisation of GOS that hydrolyses the 6-phospho-β-D-galactosides previously phosphorylated and translocated by PTS transporters. Otherwise, we did not observe significant changes in β-galactosidases that are more commonly used by some lactic acid bacteria or by *Bifidobacterium* (Schwab and Ganzle, 2011). This could be explained by the GOS90 fermentation by other species whose abundance was higher than *Bifidobacterium* and that use mainly the phosphoenolpyruvate:carbohydrate phospho–transferase system to ferment galactose, species such as *Streptococcus*, *Lactococcus* and some species of *Lactobacillus*, like *L. casei* and *L. acidophilus* (Chassy and Thompson, 1983; Kanatani *et al.*, 1992).

Studies have shown that prebiotics can attenuate the pro-inflammatory response in intestinal epithelial cells in animals model of disease (Gourbeyre *et al.*, 2013; Komiyama *et al.*, 2011; Tanabe and Hochi, 2010). Modulation of inflammatory biomarkers by GOS90 was not observed in prebiotic fed mice due most probably to the fact that this study was conducted in healthy wt mice with no inflammatory conditions. Overall, in this study we demonstrated that GOS90 has the potential to modulate the gut microbiota and its metabolites, specifically enriching for *Bifidobacterium* and *Lactobacillus* and promoting a reduction in Bacteroidales and potential pathogens such as *Clostridium* and *Helicobacter* in mice. Future studies will evaluate this prebiotic alone or in conjunction with probiotics in animal models of inflammation and colon cancer to evaluate its potential role in prevention and treatment.

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Supplementary material

Supplementary material can be found online at http://dx.doi.org/10.3920/BM2015.0114.

Figure S1. Bacterial composition over time at phylum level in control and GOS90-fed mice.

Figure S2. Bacterial OTUs significantly impacted by GOS90 feeding at days 7 and 14 of study.

Figure S3. Pearson's correlation of the C_q values obtained by conventional qPCR and high-throughput qPCR in BioMark.

Figure S4. Pearson's correlations between 16S rRNA and *gro*EL gene primers for detection of *Bifidobacterium* species using high-throughput qPCR.

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