

## RESEARCH ARTICLE

# In vitro fermentation of B-GOS: impact on faecal bacterial populations and metabolic activity in autistic and non-autistic children

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One sentence summary: B-GOS affects microbiota of autistic and non-autistic children.

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## ABSTRACT

Children with autism spectrum disorders (ASD) often suffer gastrointestinal problems consistent with imbalances in the gut microbial population. Treatment with antibiotics or pro/prebiotics has been postulated to regulate microbiota and improve gut symptoms, but there is a lack of evidence for such approaches, especially for prebiotics. This study assessed the influence of a prebiotic galactooligosaccharide (B-GOS) on gut microbial ecology and metabolic function using faecal samples from autistic and non-autistic children in an *in vitro* gut model system. Bacteriology was analysed using flow cytometry combined with fluorescence *in situ* hybridization and metabolic activity by HPLC and <sup>1</sup>H-NMR. Consistent with previous studies, the microbiota of children with ASD contained a higher number of *Clostridium* spp. and a lower number of bifidobacteria compared with non-autistic children. B-GOS administration significantly increased bifidobacterial populations in each compartment of the models, both with autistic and non-autistic-derived samples, and lactobacilli in the final vessel of non-autistic models. In addition, changes in other bacterial population have been seen in particular for *Clostridium*, *Rosburia*, *Bacteroides*, *Atopobium*, *Faecalibacterium prausnitzii*, *Sutterella* spp. and Veillonellaceae. Furthermore, the addition of B-GOS to the models significantly altered short-chain fatty acid production in both groups, and increased ethanol and lactate in autistic children.

**Keywords:** autism; gut microbiota; B-GOS; prebiotics; *in vitro* fermentation; SCFAs

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## INTRODUCTION

Autism typically develops in childhood, and it is considered as 'a systemic spectrum disorder with multiple development trajectories with an incidence four times higher in males than in females' (Grossi and Terruzzi 2014). In addition to behavioural traits, GI abnormalities such as diarrhoea, constipation, bloating and abdominal pain are common in autism and they seem to contribute to, and exacerbate, overall behaviour of children (irritability, sleeplessness, posturing) (Van De Sande, Van Buul and Brouns 2014). A crosstalk exists between the gut microbiota and central nervous system (CNS) mediated via a range of different chemical, immunological and signalling interactions that form part of the gut-brain axis. Several studies have demonstrated the role of the gut microbiota in neurodevelopment and mental health (Foster and McVey Neufeld 2013), and there is increasing evidence associating gut microbial dysbiosis with GI problems that might affect autistic children.

Bacteria such as *Clostridium* spp., *Desulfovibrio* spp. and *Streptococcus* spp. are dominant in the guts of children with ASD. Finegold et al. (2002) found nine unique species of clostridia in autistic children compared with controls. Song, Liu and Finegold (2004), using qPCR analysis, found higher levels of *Clostridium bolteae* and *Clostridium* clusters I and XI. Furthermore, Parracho et al. (2005), using FISH analysis, found greater number of species derived from the *C. histolyticum* group (*Clostridium* clusters I and II). *Desulfovibrio* group was found to be 10 times higher in the gut microbiota of autistic children compared with controls (Finegold et al. 2010; Finegold 2011).

High-throughput sequencing has been used in more recent studies to determine bacterial composition of faecal samples from autistic children. The genera *Prevotella*, *Coprococcus* and unclassified Veillonellaceae have been found in lower abundance in autistic individuals (Kang et al. 2013) with high genus *Sutterella* spp. (Williams et al. 2012; Wang et al. 2013). In addition, *Bifidobacterium* species decreased in ASD, comparing with the non-autistic control (De Angelis et al. 2013).

Metabolic associations have also been identified with ASD and may be attributed to gut dysbiosis in autistic individuals. Abnormalities have been reported in tryptophan metabolism where higher amount of indole derivatives in the blood and higher levels of IAG (indolyl-acryloyl-glycine) in the urine of autistic children have been identified. Increased abundance of *Clostridium* spp. in the ASD-associated microbiota may contribute to these metabolic alterations as these organisms can metabolise tryptophan (Bingham 2003). Metabolomic studies also identified alterations in nicotinic acid metabolism (Yap et al. 2010) and amino acid deficiencies in autism with restricted diets, modified gut microbial population and GI symptoms being suggested as potential contributors (Ming et al. 2012).

Modulation of gut microbiota is an interesting potential strategy to reduce presence of harmful microorganisms and their metabolites that might be involved in negative stimulation of CNS and affect behaviour (Shaw, Kassen and Chaves 1995; Sandler et al. 2000). Treating GI disorders in ASD with antibiotics or pro/prebiotics has been postulated to regulate microbiota and improve gut symptoms, but the evidence is scarce, especially for prebiotics.

The bifidogenic properties of B-GOS (Bimuno®, Clasado Biosciences Ltd, Buckinghamshire, UK) have been investigated *in vitro* and in human intervention studies involving healthy volunteers, and conditions that have a purported microbial input such as IBS, travellers' diarrhoea and obesity (Tzortzis et al. 2005; Depeint et al. 2008; Vulevic et al. 2008; Drakoularakou et al. 2009; Silk

et al. 2009; Vulevic et al. 2013). Recently, B-GOS was also shown to reduce cortisol secretion and anxiety in healthy volunteers (Schmidt et al. 2015). Cortisol is a reliable marker of stress and hypothalamic pituitary adrenal axis activity. B-GOS supplementation lowered cortisol reactivity and modulated attention to emotional stimuli compared with a placebo group, supporting the hypothesis that gut microbiota might have a role in behavioural traits (Schmidt et al. 2015).

Our study aimed to assess the effects of B-GOS (65% GOS content) on gut microbial ecology and metabolic end products of microbial fermentation. We used *in vitro*, three-stage, continuous gut model systems, inoculated with faecal samples of autistic and non-autistic children, which simulated different physicochemical characteristics of the proximal, transverse and distal colons.

## MATERIALS AND METHODS

### Substrate

The B-GOS product was supplied by Clasado Biosciences Ltd. The mixture was in syrup format consisting of 65% (w/v) GOS, 10.1% (w/v) lactose, 22% (w/v) glucose and 1.8% (w/v) galactose.

### Faecal inoculation

Faecal samples were obtained from three non-autistic children and three autistic child donors (male, aged 5–10 years old) who were free of any metabolic and gastrointestinal diseases, were not taking probiotic or prebiotic supplements and had not taken antibiotics 6 months before faecal sample donation. Autistic children had formal diagnosis of mild autism. None of the children followed any specific or restricted diet.

All parents were then provided written informed consent for use of their children's faeces in the study. This study was approved by The University of Reading research Ethics Committee (UREC 15/20). Faecal samples were placed in an anaerobic jar (AnaeroJar™ 2.5 L, Oxoid Ltd) including a gas-generating kit (AnaeroGen™, Oxoid). An aliquot of 20 g of samples was diluted in 100 mL anaerobic PBS (0.1 mol/L phosphate buffer solution, pH 7.4, w/w) and homogenised (Stomacher 400, Seward, West Sussex, UK) for 2 min at 240 paddle beats per minute. Samples were added to anaerobic fermenters within 15 minutes of voiding.

### Three-stage continuous culture gut model system

Physicochemical conditions in the colon were replicated in a continuous culture system, comprised of a cascade of three glass fermenters of increasing working volume connected in series. A small-scale version of the validated system described by Macfarlane, Macfarlane and Gibson (1998) was used in this study, with vessels (V) representing the proximal (V1, 80 mL, pH = 5.5), transverse (V2, 100 mL, pH = 6.2) and distal colon (V3, 120 mL, pH = 6.8). The systems were inoculated with 20% (wt:v) faecal homogenate from either non-autistic and autistic children volunteers in a growth medium (Macfarlane, Macfarlane and Gibson 1998). Following inoculation, the colonic model was run as a batch culture for 24 h in order to stabilise bacterial populations prior to the initiation of medium flow. After 24 h (T<sub>0</sub>), the medium flow was initiated and the system ran for at least 8 full volume turnovers to allow for steady state to be achieved (SS1). Short-chain fatty acid (SCFA) profiles (±5%) were assessed before starting B-GOS administration. Taking into account the operating volume (300 mL) and the retention time (48 h, flow rate 6.25 mL/h) of the colonic model system, a syrup containing

GOS (2 g/daily, equivalent to 1 g of GOS) was added daily into V1. The syrup was added to the system for at least a further 8 volume turnovers upon which steady state 2 (SS2) was achieved. Aliquots of 4.5 mL were removed at SS1 and SS2.

### SCFAs analysis by HPLC

The production of SCFAs in the fermentations was determined by HPLC (Merck, NJ) as previously described by Rodriguez-Colinas et al. (2013). Twenty microlitres of each sample was injected with a run time of 45 min. Peaks were integrated using Atlas Lab managing software (Thermo Lab Systems, Mainz, Germany). Quantification of the samples was obtained through calibration curves of lactic, acetic, propionic, butyric and formic acids in concentrations 12.5, 25, 50, 75 and 100 mM, respectively.

### In vitro enumeration of bacterial population by FISH-FCM

Bacterial composition in the gut models was analysed for using fluorescence *in situ* hybridization combined with flow cytometry (FISH-FCM). Seven hundred and fifty microlitres of samples were centrifuged at  $1136 \times g$  for 5 min. Pellets were resuspended in 375  $\mu\text{L}$  of filtered PBS (using a 0.22- $\mu\text{m}$  PVDF membrane) and fixed in 1125  $\mu\text{L}$  of 4% (v/v) paraformaldehyde. Af-

ter 4 h of incubation at 4°C, samples were washed twice using 1 mL of PBS, resuspended in 600  $\mu\text{L}$  PBS-ethanol (1:1, v/v) and stored at -20°C. Permeabilisation steps were performed using 30  $\mu\text{L}$  of the fixed samples added to 500  $\mu\text{L}$  PBS and centrifuged at  $1136 \times g$  for 3 min. Pellets were resuspended using 100  $\mu\text{L}$  of filtered TE-FISH (Tris/HCl 1 M pH 8, EDTA 0.5 M pH 8, distilled H<sub>2</sub>O, 0.22  $\mu\text{m}$  PVDF membrane) containing lysozyme (1 mg/mL of 50 000 U/mg protein) and incubated for 10 min at room temperature. Solutions containing the samples were then vortexed and centrifuged at  $1136 \times g$  for 3 min. Pellets were washed with 500  $\mu\text{L}$  PBS and centrifuged ( $1136 \times g$ , 3 min). Hybridisations were performed by resuspending the pellets in 150  $\mu\text{L}$  of hybridisation buffer (5 M NaCl, 1 M Tris/HCl pH 8, 30% formamide, ddH<sub>2</sub>O, 10% SDS), vortexed and centrifuged ( $1136 \times g$ , 3 min). Pellets were then resuspended in 1 mL of hybridisation buffer and 50  $\mu\text{L}$  aliquoted into Eppendorf tubes. The probes used (Sigma Aldrich Ltd, Poole, Dorset, UK) are reported in Table 1 (Devereux et al. 1992; Wallner, Amann and Beisker 1993; Langendijk et al. 1995; Poulsen et al. 1995; Manz et al. 1996; Franks et al. 1998; Stoffel et al. 1998; Daims et al. 1999; Harmsen et al. 1999, 2000, 2002; Hold et al. 2003; Lay et al. 2005; Walker et al. 2005; Kong et al. 2012). NON EUB338 and EUB338 I-II-III linked at their 5' end either to Alexa488 or Alexa647. Group-specific probes were linked with Alexa647 at their 5' end. Four microlitres of each probe and 4  $\mu\text{L}$  of Eub338 I-II-III (linked to Alexa488)

**Table 1.** Oligonucleotide probes used in this study for FISH-FCM analysis of bacterial populations. +: These probes are used together in equimolar concentration of 50 ng/ $\mu\text{L}$ .

Probe name	Sequence (5' TO 3')	Target group	Reference
Non Eub	ACTCCTACGGGAGGCAGC		Wallner, Amann and Beisker (1993)
Eub338 I +	GCT GCC TCC CGT AGG AGT	Most bacteria	Daims et al. (1999)
Eub338 II +	GCA GCC ACC CGT AGG TGT	Planctomycetales	Daims et al. (1999)
Eub338 III +	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	Daims et al. (1999)
Bif164	CAT CCG GCA TTA CCA CCC	Most <i>Bifidobacterium</i> spp. and <i>Parascardovia denticolens</i>	Langendijk et al. (1995)
Lab158	GGTATTAGCAYCTGTTTCCA	Most <i>Lactobacillus</i> , <i>Leuconostoc</i> and <i>Weissella</i> spp.; <i>Lactococcus lactis</i> ; all <i>Vagococcus</i> , <i>Enterococcus</i> , <i>Melisococcus</i> , <i>Tetragenococcus</i> , <i>Catelicoccus</i> , <i>Pediococcus</i> and <i>Paralactobacillus</i> spp.	Harmsen et al. (1999)
Bac303	CCA ATG TGG GGG ACC TT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	Manz et al. (1996)
Clit135	GTTATCCGTGTGTACAGG	Some of the <i>Clostridium lituseburense</i> group ( <i>Clostridium</i> cluster XI)	Manz et al. (1996)
Erec482	GCT TCT TAG TCA RGT ACGG	Most of the <i>Clostridium coccoides</i> - <i>Eubacterium rectale</i> group ( <i>Clostridium</i> clusters XIVa and XIVb)	Manz et al. (1996)
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the <i>Clostridium histolyticum</i> group ( <i>Clostridium</i> clusters I and II)	Franks et al. (1998)
Rrec584	TCA GAC TTG CCG YAC CGC	Roseburia subcluster	Franks et al. (1998)
Prop853	ATT GCG TTA ACT CCG GCAC	Clostridial cluster IX	Walker et al. (2005)
Ato291	GGT CGG TCT CTC AAC CC	<i>Atopobium</i> , <i>Colinsella</i> , <i>Olsenella</i> and <i>Eggerthella</i> spp.; <i>Cryptobacterium curtum</i> ; <i>Mycoplasma equigenitalium</i> and <i>Mycoplasma elephantis</i>	Harmsen et al. (2000)
Fprau655	CGCCTACCTCTGCACTAC	<i>Faecalibacterium prausnitzii</i> and related sequences	Hold et al. (2003)
DSV687	TAC GGA TTT CAC TCC T	Most <i>Desulfovibrionales</i> (excluding <i>Lawsonia</i> ) and many <i>Desulfuromonales</i>	Devereux et al. (1992)
EC1531	CACCGTAGTGCCTCGTCATCA	<i>Escherichia coli</i> BJ4	Poulsen et al. (1995)
Rbro730 +	TAAAGCCCAGYAGGCCGC	<i>Clostridium sporosphaeroides</i> , <i>Ruminococcus bromii</i> , <i>Clostridium leptum</i>	Harmsen et al. (2002); Lay et al. (2005)
Rfla729 +	AAA GCC CAG TAA GCC GCC	<i>Ruminococcus albus</i> , <i>R. flavefaciens</i>	Harmsen et al. (2002); Lay et al. (2005)
SUBU1237	CCC TCT GTT CCG ACC ATT	<i>Burkholderia</i> spp., <i>Sutterella</i> spp.	Stoffels et al. (1998)
Vei723	ACA CAG TCC AGA AAG GCG	Veillonellaceae	Kong et al. (2012)

were added to the working solution and incubated overnight at 35°C in a heating block. After 12 h of incubation, an aliquot of 150 µL hybridisation buffer was added to the working solution, vortexed and centrifuged (1136 × g, 3 min). One hundred and fifty microlitres of supernatant were removed from each sample and the remaining volume was centrifuged (1136 × g, 3 min). The pellets were washed with 200 µL of washing buffer (5 M NaCl, 1 M Tris/HCl pH 8, 0.5 M EDTA pH 8, ddH<sub>2</sub>O, 10% SDS), homogenised by vortexing and incubated for 20 min at 37°C in a heating block. Afterwards the samples were centrifuged (1136 × g, 3 min) and supernatants were removed. Negative control samples (no probes added) were screened by FCM to detect background before the probe samples were resuspended in an appropriate amount of PBS. Samples were stored at 4°C until determinations. Numbers of specific and total bacteria were determined taking into account dilution factor, calculated from different volumes used in samples preparation steps, and events/µL obtained from NON EUB338 and EUB338 I-II-III probes analysed by FCM.

### Metabolic analysis by <sup>1</sup>H-NMR

Three consecutive days of the three biological replicates for each group (autistic and non-autistic) of all time points (before and after treatment) were analysed by <sup>1</sup>H-NMR (n = 27, each group). Fermentation supernatants were defrosted, vortexed and centrifuged at 599 × g for 5 min. The supernatants were filtered using 0.22 µm low protein binding Durapore polyvinylidene fluoride (PVDF) membranes (Millex; EMD Millipore, Billerica, MA, USA) and 400 µL transferred into fresh Eppendorf tubes. Filtered samples were combined with 200 µL of phosphate buffer (0.2 M (pH 7.4) in D<sub>2</sub>O plus 0.001% TSP), mixed by vortexing, centrifuged at 1136 × g for 10 min and then 550 µL was transferred into 5 mm NMR tubes for analysis. All NMR spectra were acquired on a Bruker Avance DRX 500 MHz NMR spectrometer (Bruker Biopsin, Rheinstetten, Germany) operating at 500 MHz. They were acquired using a standard 1D pulse sequence [recycle delay (RD)-90°-t1-90°-tm-90°-acquire free induction decay (FID)] with water suppression applied during RD of 2 s, a mixing time T<sub>m</sub> of 100 ms and a 90 pulse set at 7.70 µs. For each spectrum, a total of 128 scans were accumulated into 64 k data points with a spectral width of 12 001 ppm. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line broadening.

### Data preprocessing and analysis

All spectra were manually phased, baseline corrected and calibrated to the chemical shift of TSP (3-(trimethylsilyl)-[2,2,3,3-<sup>2</sup>H<sub>4</sub>]-propionic acid, δ 0.00). Spectra were digitised using an in-house MATLAB (version R2014a, The Mathworks, Inc.; Natwick, MA) script. The spectral region containing the water resonance was removed to minimise distortions in the baseline arising from imperfect water saturation. Median fold normalization was performed for both groups: non-autistic and autistic children. Before and after administration of B-GOS, principal components analysis (PCA) using mean-centred data was applied. Orthogonal projection to latent structure discriminant analysis (OPLS-DA) models was constructed using unit variance scaling for pairwise comparisons of the different experimental groups and time points. Correlation coefficients plots were generated from the model outputs by back scaling transformation to display the contribution of each variable (metabolites) to sample classification (e.g. before and after treatment). Colour represents the significance of correlation (R<sup>2</sup>) for each metabolite to

class membership. Predictive strength (Q<sup>2</sup>Y) of the models was obtained using a 7-fold cross-validation method and these were validated using permutation testing (number of permutations = 10 000).

### Statistical analysis

Data from HPLC and FMC-FISH analyses were analysed using paired t-test in order to assess significance of results, comparing the two time points SS1 and SS2, before and after treatment, respectively. Statistical significance was at P < 0.05 for all analyses. Analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

## RESULTS

### Bacterial enumeration

Changes in bacterial compositions in gut model systems are reported in Fig. 1. The data showed lower numbers of bifidobacteria in ASD models compared with non-autistic ones. Significant increases in the *Bifidobacterium* spp., following the addition of B-GOS to models containing both autistic and non-autistic samples, were seen. In autistic models, a significant increase of bifidobacteria occurred from 5.32 to 7.27 log<sub>10</sub> cells/mL (P < 0.01), from 4.81 to 6.79 log<sub>10</sub> CFU/mL (P < 0.001) and from 5.57 to 6.83 log log<sub>10</sub> cells/mL (P < 0.05), in V1, V2 and V3, respectively. A slight but significant increase in *Clostridium* cluster XI in V2 for autistic children was also found, as well as significant decrease in V2 in Veillonellaceae group from 6.06 to 5 log<sub>10</sub> CFU/mL (P < 0.05). In non-autistic models, there was a significant increase in numbers of bifidobacteria in V1, from 5.83 to 7.16 log log<sub>10</sub> cells/mL (P < 0.01), and in V3, from 4.97 to 6.73 log<sub>10</sub> cells/mL (P < 0.001) and in lactic acid bacteria (Lab158) in V3 from 5.13 to 6.01 log<sub>10</sub> cells/mL (P < 0.05). Additionally, B-GOS slightly increased *Roseburia* spp. in V1 and V3 (P < 0.05) and reduced *Atopobium* spp. from 6.06 to 5.28 log<sub>10</sub> cells/mL and *Faecalibacterium prausnitzii* from 6.78 to 5.27 log<sub>10</sub> cells/mL (P < 0.05 for both) in the second vessel, while increasing *Atopobium* spp. from 5 to 5.92 log<sub>10</sub> cells/mL (P < 0.05) in the third vessel of non-autistic models. In these models, numbers of *Clostridium coccoides*-*Eubacterium rectale* were also increased from 6.76 to 7.08 log<sub>10</sub> cells/mL (P < 0.01) in V1 and *Sutterella* spp. significantly decreased in V1 from 7.05 to 6.49 (P < 0.01) and V2 from 7.02 to 6.37 log<sub>10</sub> CFU/mL (P < 0.05) after B-GOS administration. There was a general trend to increase all other bacterial groups analysed in all vessels but this was not significant. Exceptions were seen for *Bacteroides* (V1), Clostridial cluster IX (V1), *F. prausnitzii* (V1), *Escherichia coli* (V3), *Ruminococcus* spp., *Clostridium leptum* (V2), *Sutterella* spp. and Veillonellaceae (all vessels) in autistic models, and for *Clostridium coccoides*-*Eubacterium rectale* (V2), *Atopobium* spp. (V1), Clostridial cluster IX (V2), *Clostridium* cluster XI (V1, V2), *E. coli* (V2), *Sutterella* spp. and Veillonellaceae (all vessels) in non-autistic models that slightly decreased.

### SCFAs production

SCFA concentrations are shown in Fig. 2. Our data show a lower concentration of butyrate and propionate in autistic models, compared with non-autistic models, but no differences in acetate before adding B-GOS into the system. After the administration of B-GOS, acetate and butyrate were the main end products of microbial fermentation. Supplementation of B-GOS to gut models inoculated with faecal samples from autistic children led to a significant increase of acetate and butyrate in V1

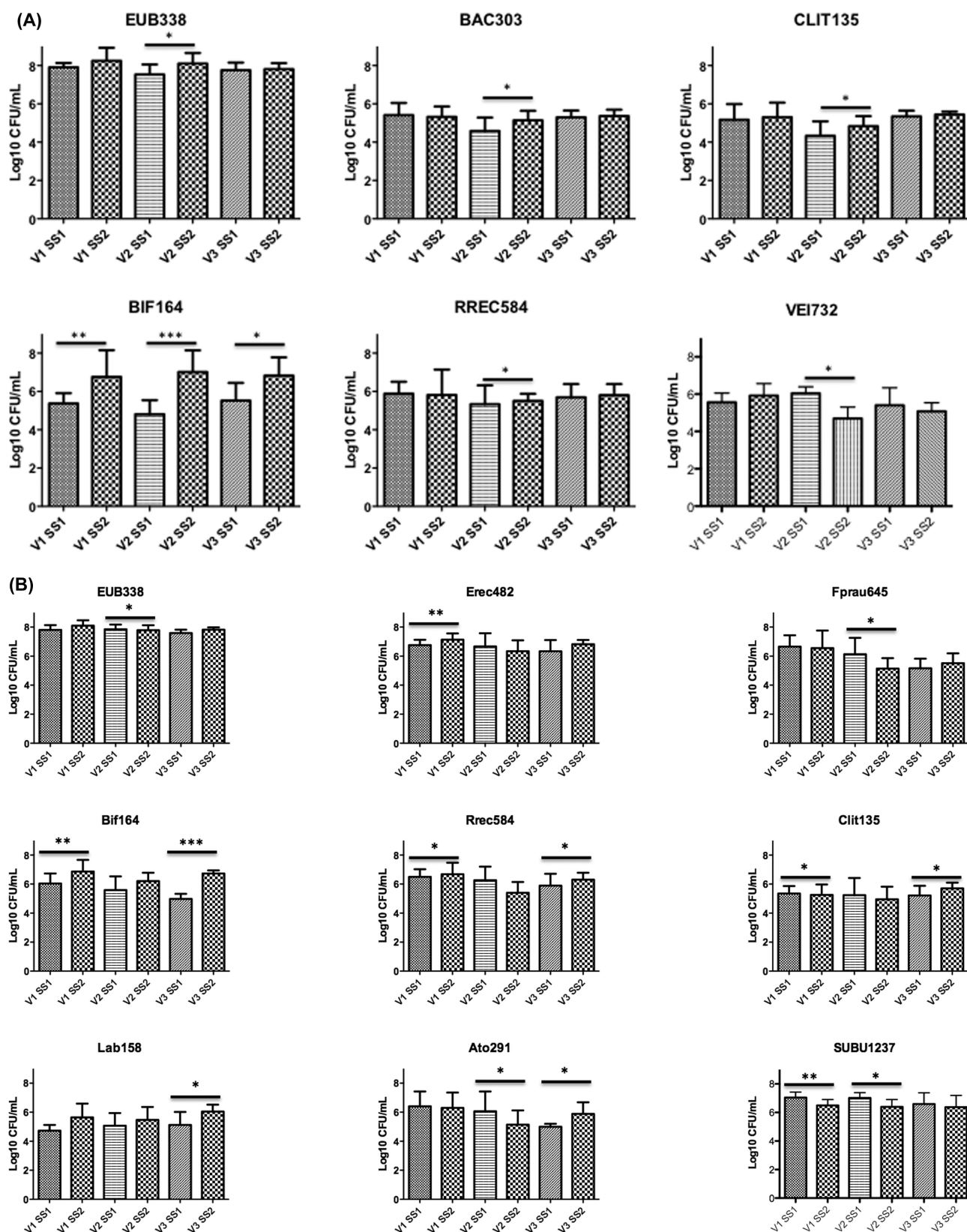
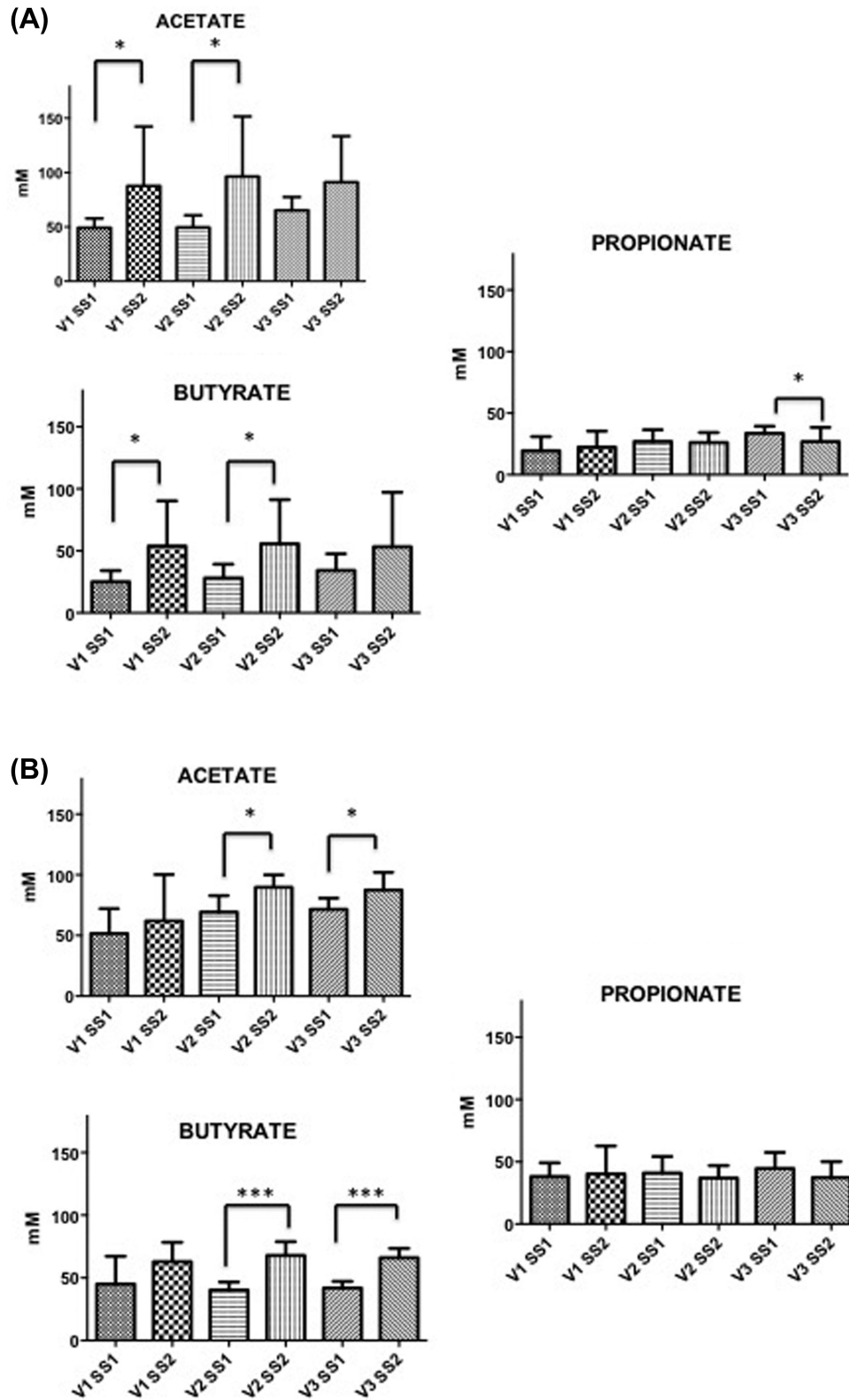
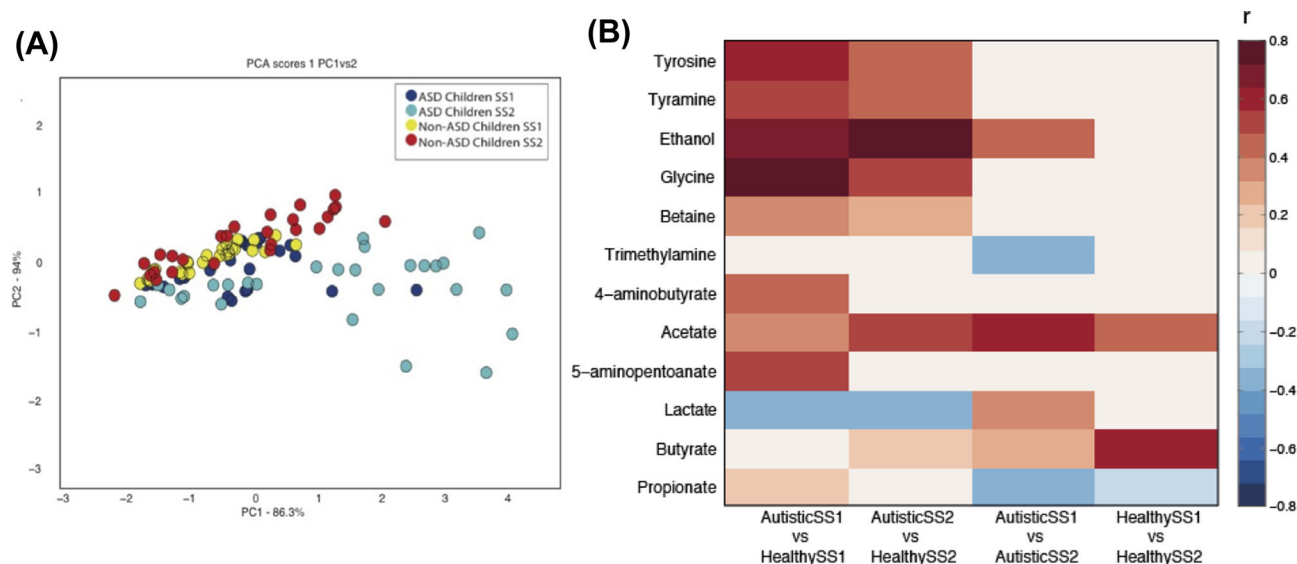


Figure 1. Bacterial groups detected by FISH-FCM (Log<sub>10</sub> CFU/mL) in culture broth recovered from each vessel (V1, V2 and V3) of a colonic model before (SS1) and after (SS2) the daily administration of B-GOS (2 g/d, equivalent to 1 g GOS). Significant difference after the treatment: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ . Probes: total bacteria (Eub338I-II-III), *Bifidobacterium* spp. (Bif164), *Lactobacillus* spp. (Lab158), most Bacteroidaceae and Prevotellaceae (Bac303), *Clostridium coccoides*–*Eubacterium rectale* group (Erec482), *Roseburia* subcluster (Rrec584), *F. prausnitzii* (Fprau655), *Clostridium* cluster XI (Clit135), *Sutterella* spp. (SUBU1237), Veillonellaceae (VEI732), *Atopobium* spp. (Ato291). (A) Autistic children; (B) non-autistic children.



**Figure 2.** HPLC analysis. Acetate, propionate and butyrate concentrations in culture broths recovered from vessels (V1, V2 and V3) of *in vitro* gut model systems before (SS1) and after (SS2) administration of B-GOS (1 g/daily GOS). Results are reported as means (mM) of the data ( $n = 3$ ): (A) autistic children and (B) non-autistic children. Significant difference after the treatment: \*  $P < 0.05$ ; \*\*\* $P < 0.001$ .



**Figure 3.**  $^1\text{H-NMR}$  data analysis. (A) PCA score plot shows a separation between models inoculated with stool samples of non-ASD and ASD children after administration of B-GOS. Dark and light blue dots represent replicates of samples from gut models inoculated with faecal samples of autistic children, before (SS1) and after (SS2) treatment, respectively. Yellow and red dots represent replicates of samples from gut models inoculated with faecal samples of non-autistic children, before (SS1) and after (SS2) treatment, respectively. (B) Correlation coefficients indicating the associations of identified metabolites with autism and their alteration upon B-GOS administration. SS1: before treatment; SS2: after treatment. White cells represent no significant correlations.

and V2, simulating the proximal and transverse colons ( $P < 0.05$ ), respectively, while concentration of propionate was decreased ( $P < 0.05$ ) in V3 mimicking distal colon. In models simulating the colon of non-autistic children, the fermentation of B-GOS mediated significant production of acetate ( $P < 0.05$ ) and butyrate ( $P < 0.001$ ) in V2 and V3, simulating the transverse and distal colon, respectively. There was no effect on propionate.

### $^1\text{H-NMR}$ spectroscopic profiles

PCA was performed on mean-centred data to summarise variance with the dataset. The scores plot (PC1 versus PC2) shown in Fig. 3A showed separation between autistic and non-autistic models after treatment, indicating that B-GOS supplementation contributed to the largest source of variance in the metabolic data. Comparison of the spectra profiles from gut models before and after treatment identified that a number of metabolites changed following B-GOS supplementation to characterise the metabolic variation associated with ASD, B-GOS supplementation and differences in microbial response to B-GOS between the ASD and non-ASD microbiota. The results of these analyses are summarised in Fig. 3B. A significant OPLS-DA model was obtained comparing the metabolic profiles of the autistic and non-autistic models at baseline ( $Q^2Y = 0.07$ ;  $P < 0.05$ ; Fig. SC-I, Supporting Information). Supernatants from the autistic models contained greater amounts of ethanol, glycine, tyrosine, tyramine, 5-aminopentanoate, acetate, 4-aminobutyrate and betaine, and lower amounts of butyrate compared with the non-autistic models. B-GOS supplementation was found to modulate the metabolic profile of the autistic models ( $Q^2Y = 0.08$ ;  $P < 0.05$ ) increasing ethanol, lactate, acetate and butyrate and decreasing propionate and trimethylamine (Fig. SB-I, Supporting Information). Increased butyrate and acetate production was also observed in the non-autistic models following the addition of BGOS ( $Q^2Y = 0.12$ ;  $P < 0.01$ ; Fig. SB-II, Supporting Information). Comparing the metabolic profiles of the

autistic and non-autistic models after B-GOS feeding ( $Q^2Y = 0.17$ ;  $P < 0.01$ ) revealed that some of the metabolic variation was reduced (Fig. SC-II, Supporting Information). There was no longer variation in 4-aminobutyrate between the models; however, the difference in ethanol and acetate between autistic and non-autistic models was increased being higher in the autistic models.

### DISCUSSION

Recent studies have focused on the effect of pre/probiotics on the gut-brain axis (Liu, Cao and Zhang 2015). This study investigated the influence of B-GOS on a small scale, *in vitro*, gut model system inoculated with faeces from autistic and non-autistic children. The results showed a positive modulation of bacterial populations, using an automated FISH method combined with FCM. We also assessed metabolic profiles and key metabolites in both test groups.

Lower concentrations of SCFAs have previously been found in children with ASD by Adams *et al.* suggesting a reduced fermentation capacity by the ASD microbiota. It was hypothesised that this was due to a compromised microbiota characterised by a lower number of bifidobacteria, consistent with microbial signatures observed here (Adams *et al.* 2011). Concomitant with these population changes, functional alterations were also observed in both autistic and non-autistic models with acetate and butyrate, the main end products of microbial fermentation, being increased.

Recent studies have focussed on SCFAs and their effect on the CNS. These fermentation products can cross the blood-brain barrier and might influence early brain development. The synthesis of neuroactive compounds such as dopamine and serotonin can be modulated by SCFA and they are able to produce reversible psychological and physiological changes in rats similar to those found in ASDs (Wang *et al.* 2011). Experimental evidence using intraventricular infusion in rats indicates that

propionic acid can produce brain and behavioural changes similar to ASD (MacFabe et al. 2008).

Recent ASD studies have shown increase in numbers of *Sutterella* spp. and decrease in Veillonellaceae group. In this study, the results did not show any significant differences between ASD and non-ASD group. However, a general decrease in those bacterial groups after treatment was highlighted, suggesting that B-GOS administration might have an impact on the growth of these ASD-associated bacteria.

Following B-GOS feeding, the microbiota of autistic children produced greater amount of ethanol and lactate while the amount of amino acids and the SCFA propionate, present in the model, was reduced. These metabolic alterations were not observed when the faecal microbiota of non-autistic individuals were fed B-GOS. In a healthy colon, lactate production is generally low due to its conversion to other organic acids by many bacteria and because lactate can be used as a substrate for dissimilation of sulphate by some bacteria (e.g. *Desulfovibrio* spp.) (Fite et al. 2004; Marquet et al. 2009; Flint et al. 2014). In children with ASD, the presence of lactate is interesting because its accumulation has been associated with neurological problems, in particular studies show the effect of lactate infusions on anxiety and panic disorders (Cowley et al. 1987; Dillon et al. 1987). Cowley and colleagues in their findings showed that lactate infusion in patients suffering from panic disorder provokes higher panic symptoms reaction compared with controls (Dillon et al. 1987). Dillon et al. have showed similar results in *in vivo*, where panic and anxiety reaction has been measured using Acute Panic Inventory scores. After lactate infusions, the scores were much higher in patients with panic and anxiety disorders compared with normal controls (Cowley et al. 1987).

The lysine degradation product, 5-aminopentanoic acid, was also higher in the autistic compared with the non-autistic models. This metabolite can be produced both endogenously and through the bacterial catabolism of lysine. It is believed to act as a methylene homologue of  $\gamma$ -aminobutyric acid (GABA) and functions as a weak GABA agonist (Callery and Geelhaar 1985). Interestingly, GABA was also higher in the autistic models compared with the non-autistic models pre-treatment but these differences were not present following B-GOS treatment. Certain bacteria, such as lactobacilli, are able to produce molecules that act as neurotransmitters and directly affect the brain (Wall et al. 2014). In our results, its reduction might be due to changes in gut microbiota composition.

Ethanol was found in higher amount in ASD children comparing with non-ASD children. The vast majority of bacteria form ethanol from acetyl-CoA and the glycolytic pathway (Macfarlane and Macfarlane 2003). Microorganisms are able to oxidase ethanol and the impact of bacterial overgrowth on ethanol production has previously been studied (Baraona et al. 1986). Metabolism of ethanol can lead to the production of toxic end products such as acetaldehyde, which may affect the gastrointestinal mucosa. The role of acetaldehyde in ASD has been recently evaluated in particular for its role in oxidative stress and DNA damage. Under healthy conditions, ethanol is converted into acetic acid in the liver by a two-step process involving alcohol dehydrogenase and aldehyde dehydrogenase (ALDH). Mutation of the ALDH gene has been shown to increase the accumulation of acetaldehyde and result in cancers within different regions of the gastrointestinal tract and Alzheimer's disease (Jurnak 2015). The potential role of this toxic compound in neurological disorders, including autism, warrants further exploration.

## CONCLUSIONS

This *in vitro* study showed promising and positive results in that supplementing the microbiota of children with ASD with 65%B-GOS may manipulate the gut bacterial population and alter metabolic activity towards a configuration that might represent a health benefit to the host. However, further work will be required to assess such changes in an *in vivo* human intervention study.

## SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](#) online.

## AUTHORS' CONTRIBUTIONS

RG carried out the experiments and drafted the manuscript. DC helped in experimental work. JRS assisted with NMR analyses. GRG and AC were involved in designing and coordination of the study and revising the manuscript critically for important intellectual content. JV and GT are employed by Clasado Biosciences Ltd, who provided the B-GOS product, marketed as Bimuno(R), used within this research. There are no patents, products in development or other marketed products to declare. This does not alter the authors adherence to all the FEMS policies on sharing data and materials. All the authors reviewed the final version of the manuscript. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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