University of Reading

Evaluating the effect of a prebiotic B-GOS in Autism Spectrum Disorders (ASDs)

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Thesis submitted for the degree of Doctor of Philosophy

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June 2017

Declaration

I declare that this thesis is my own work and the contribution of others has been properly and fully acknowledged.

Roberta Grimaldi, 2017

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Acknowledgement

I would like to express my deepest gratitude to my supervisors Prof. Glenn R Gibson and Dr. Adele Costabile for their guidance and constant support during the course of my PhD.
I would like to thank Clasado Biosciences Ltd. for the sponsorship and my supervisor Dr. Jelena Vulevic for providing support and advice.

I thank to all my friends in the department who kept me smiling even during the hardest periods.

Special thank goes to Luke, for all his patience and love.

Last but not least, I thank my family for always be there for me.

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Abstract

Autism is a neurodevelopment disorder characterised by problems in communication and social interaction skills. In addition to psychological comorbidities, autistic individuals might suffer of GI problems that have been previously associated with gut bacteria, altered dietary intake and barrier function problems. In order to improve these aspects, exclusion diets, such as gluten and casein free diets (GFCF), and/or use of food supplements have been suggested in ASD, but little is known about their impact on gut microbiota composition and metabolic activity, especially for prebiotics. The aim of this work was to investigate the effect of a prebiotic B-GOS on different aspects of autism, in *in vitro* and in *in vivo*. B-GOS (65%GOS content) was tested in dose response batch culture experiments inoculated with faecal samples from healthy donors, compared to the commercial B-GOS (50%GOS content). It showed an increase in bifidobacteria and modulation of SCFAs production. 65% B-GOS was then tested on faecal samples from autistic and non-autistic children using a three-stage continuous fermentation system that mimicked various anatomical regions of the colon. Results showed that B-GOS administration significantly increased bifidobacterial populations in the models inoculated with both autistic and non-autistic samples and influences changes in other bacterial groups, such as *Clostridium*, *Roseburia*, *Bacteroides*, *Atopobium*, *Faecalibacterium prausnitzii*, *Sutterella* spp. and Veillonellaceae. In addition, B-GOS modulated SCFA production in both groups, and increased ethanol and lactate inocula from autistic children. Next, a parallel, double blind, randomised study was designed in order to confirm these results in *in vivo*. Baseline samples from 30 autistic children aged 4-11 years old, were analysed to understand the effect of the exclusion diets on the gut microbiota and metabolome. In addition, parents were asked to fill in GI symptoms and food diaries. Results showed ASD children following GFCF diet had significant reduction in abdominal pain and bowel movement, compared to the un-restricted dietary group, and reduced abundance of *Bifidobacterium* spp. and Veillonellaceae family, with increased *F. prausnitzii* and *Bacteroides* spp. In both groups, no significant differences were found in urine metabolome, but significant correlations were found between bacterial populations and amino acids in faeces. After 6 weeks B-GOS intervention, no effect was reported on GI issues, but there were improvements in sleep habits and anti-social behaviour. B-GOS significantly modulated bifidobacteria and Lachnospiraceae family, as well as the metabolome in children whose diet was not restricted. Butyrate and valerate were the main SFCAs produced and reduced amino acid excretion was detected

in faecal samples of the intervention group. Urine samples were dominated by citrate, creatine, creatinine, DMA (dimethylamine), DMG (dimethylglycine), malonate, carnitine, TMAO (trimethylamine-N-oxide), and α-hydroxybutyrate, comparing to the control group, where PAG (phenylacetiyglycine), phenylalanine and β-hydroxybutyrate were detected. Overall, the results presented in this study demonstrated, for the first time, that a prebiotic B-GOS is able to modulate different aspects of autism and be considered as potential dietary therapeutic approach for ASD individuals.

LIST OF ABBREVIATIONS

°C	Degree Celsius
1D	One Dimensional
^{1}H-NMR	proton nuclear magnetic resonance
AA	amino acid
ACC	average cell count
ADH	alcohol dehydrogenase
ADHD	Attention Deficit Hyperactivity Disorder
ALDH	aldehyde dehydrogenase
ANS	autonomic nervous system
API	Acute Panic Inventory
AQ	Autism spectrum Quotient
ASD	Autism Spectrum Disorders
ATEC	Autism Treatment Evaluation Checklist
AV	average
B-GOS	Bimuno
BBB	blood-brain barrier
BGI	Gut Bacteria Index
bp	base pair
CD	Crohn's disease
CFU	colony forming unit
CNS	central nervous system
D2O	deuterium oxide
DA	D-arabinitol
DA/LA	D-/L-arabinitol
DF	dilution factor
DMA	dimethylamine
DMG	dimethylglycine
DNA	Deoxyribonucleic acid
DP	degree of polymerisation
DSM	Diagnostic and Statistical Manual of Mental Disorders
ECs	enterochromaffin cells
EDTA	Ethylenediaminetetraacetic acid
ENS	enteric nervous system
EPEC	enteropatogenic *E. coli*
EQ-SQ	Empathy and Systemizing Quotient
EtOH	Ethanol
FAO	Food and Agriculture Organization of the United Nations
FID	free induction decay
FIS	US Foundation for Innovation in Medicine
FISH	florescence in situ hybridisation
FISH-FCM	fluorescence *in situ* hybridisation combined with flow cytometry
FOS	fructo-oligosaccharides
xg	relative centrifugal force
GABA	gamma aminobutyric acid
Gal	Galactose
GALT	Gut Associated Lymphoid Tissue
GC	gas cromatography
GF	gluten free
GFCF	gluten free casein free

GFD	gluten-free diet
GI	gastrointestinal
Glc	glucose
GLP-1	Glucagon-like peptide
GOS	galacto-oligosaccharides
GPCRs	G protein-coupled receptors
GRAS	Generally Recognized As Safe
h	hours
H_2O	water
HCl	cloridic acid
HMO	human milk oligosaccarides
HPA	hypothalamic–pituitary–adrenal axis
HPLC	high performance liquid cromatography
IAG	indolyl-acryuloyl-glycine
IAG	indolyl-acryuloyl-glycine
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IL	interlukine
iNOS	nitric oxide
l	litre
LAB	lactic acid producing bacteria
LPS	lipopolysaccharides
M	molar
MAPK	mitogen-activated protein kinase
MCTs	monocarboxylate transporters
mg	milli grams
MHz	mega hertz
Min	minutes
mL	milli litre
mm	milli metre
mM	milli molar
mOATs	multispecific organic anion transporters
ms	milli second
Na+	sodium
NaCl	sodium chloride
NaOH	sodium hydroxide
NC	negative control
ND	nano drop
ng	nano grams
NGS	next generation sequencing
NK	natural killer
nmol	nano mole
Olfr78	olfactory receptor
OPLSA-DA	Orthogonal projection to latent structure discriminant analysis
OR51E2	olfactory receptor family 51 subfamily E member 2
OTU	operational taxonomic units
PAG	phenylacetilglycine
PBS	phosphate buffer solution
PCA	Principal component analysis
PCoA	Principal Coordinate analysis
PDD	Pervasive Development Disorder

PEP	posphonenolpyruvate
pH	potential of hydrogen
ppm	parts per million
PVDF	Polyvinylidene difluoride
PYY	peptide YY
qPCR	quantitative polimerase chain reaction
RD	recycle delay
RDA	redundancy analysis
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
s	second
SAH	S-adenosylhomocysteine
SCAS-P	Spence's Children Anxiety Scale-Parent version
SCFA	short chain fatty acid
SD	standard deviation
SI	Selectivity Index
sIgA	secretory Immunoglobulin A
SLC5A8	sodium-coupled monocarboxylate transporter
SS	steady state
TeNT	tetanus neurotoxin
TH	tyrosine hydroxylase
TMA	trimethylamine
TMAO	trimethylamine-N-oxide
TNF-α	tumor necrosis factor alpha
Tris/HCl	Tris hydrochloride
TSP	3-(trimethylsilyl)-[2,2,3,3,-2H4]-propionic acid
U	unit
UC	ulcerative colitis
UREC	University of Reading Research Ethics Committee
V	vessels
V-GOS	Vivinal GOS
WHO	World Health Organization
µL	micro litre
µm	micro metre
µm	micro second

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Grimaldi R, Swann RJ, Vulevic V, *et al*. Fermentation properties and potential prebiotic activity of B-GOS (65% GOS content) on *in vitro* gut microbiota parameters. *Br J Nutr* 2016;**116**:480–6.

Grimaldi R, Cela D, Swann JR, *et al. In vitro* fermentation of B-GOS: impact on faecal bacterial populations and metabolic activity in autistic and non-autistic children. *FEMS Microbiol Ecol* 2017;**93**:1–10.

Grimaldi R, Baldini J, Todisco I, *et al.* Impact of exclusion diet on gut microbiota composition and metabolite production in autistic children. *In prep.*

Grimaldi R, Baldini J, Todisco I, *et al.* A double-blind, placebo-controlled, parallel-designed, prebiotic B-GOS intervention study in Autism Spectrum disorders (ASDs). *In prep*

CHAPTER 1

1.1 Gut microbiota

1.1.1 Health and disease

The human gut is an extremely complex ecosystem whereby microbiota, nutrients, and host cells interact. The relationship between microorganisms and the host involves important aspects such as metabolism, barrier effect, and trophic functions. Consequently, dysbiosis, or bacterial imbalance, can be considered a cause or consequence of several gastrointestinal disorders (Butel et al. 2014). Microorganisms colonise the gastrointestinal tract from the mouth to the large intestine, where they reach a maximum estimated concentration of 10^{12} bacteria per gram of gut contents (Sender et al. 2016). Therein, fermentative metabolism of undigested food and host-derived products creates a microbial metabolome that impacts on the host. The major end products of carbohydrate fermentation are short chain fatty acids (SCFAs) such as acetate, propionate and butyrate (Cummings 1981). They are produced in particular via glycolytic and pentose phosphate pathways that are briefly summarised in Figure 1.1.

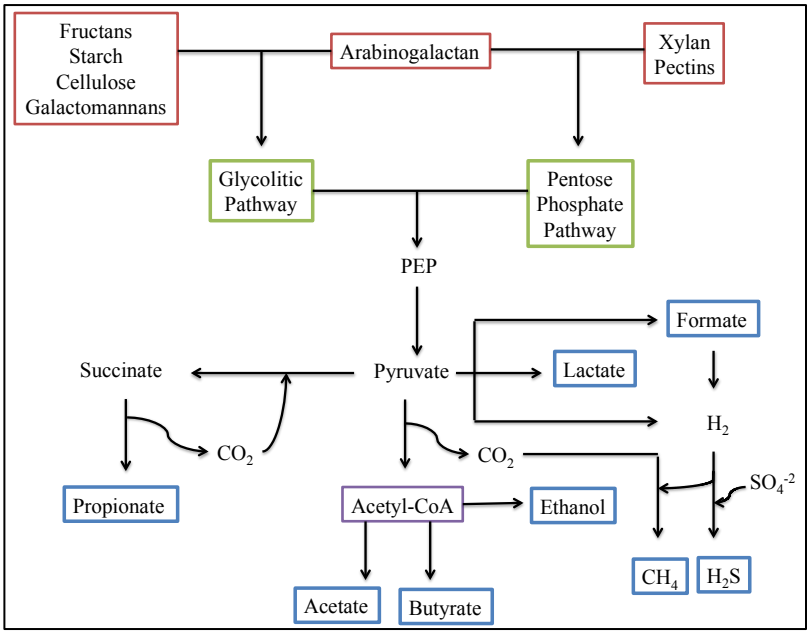

Figure 1.1. Diagram of non-digestible carbohydrate breakdown in the colon and main pathways. PEP: posphonenolpyruvate (adapted from Macfarlane & Macfarlane 2003).

SCFAs are sources of energy for the host tissues but may also have anti-apoptotic and anti-inflammatory effects. In addition, it seems that they may have effects on lipogenesis and their interaction with host receptors seems linked to the regulation of hormones affecting the satiety. In addition, SCFAs have an important role in keeping the gut environment stable, influencing pH, gut transit, nutrient uptake and microbial composition (Macfarlane and Macfarlane 2011) (Table 1.1).

Table1.1: Role of SCFAs in gut environment (Saulnier et al. 2009)

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

SCFAs are transported from the large intestine to the blood stream via three main transporters: MCTs, monocarboxylate transporters; mOATs, multispecific organic anion transporters; and SLC5A8, a sodium-coupled monocarboxylate transporter.

The most well–studied trasporter is SLC5A8, expressed in the apical membranes of the colon, which leads to SCFA absoption in association with increased Na^+ intake (ratio 1:3). The other two transporters are considered as potential candidates. MCT1 is associated with lactate and butyrate absoption, but there is still conflicting evidence in the literature so its role needs to be fully elucidated (Natarajan et al. 2014).

Recent studies have shown how G protein-coupled receptors (GPCRs) may play a role in microbial communication with the host. Four SCFAs receptors have been identified: Gpr41, Gpr43, Gpr109a and Olfr78. Gpr41 and Gpr43 mainly respond to propionate but they can be stimulated also by formate, acetate, butyrate, iso-butyrate and acetate, butyrate, respectively.

Gpr41 is expressed in different tissues and cell types and it seems to be involved in inhibition of cell proliferation and apoptosis via the activation of p53 and MAPK. Gpr43 is expressed on lymphocytes, neurotrophils, monocytes and peripheral blood mononuclear cells, and its functionally regulates inflammatory responses, activating cytokines and chemokines (Brown et al. 2003; Samuel et al. 2008). Gpr109a responds only to butyrate and it is located on epithelial cells. Its activation has been associated with carcinogenesis suppression (Singh et al. 2014). Olfr78 (human ortholog OR51E2) responds to propionate and acetate and is located on enteroendocrine cells in the murine colon, but not in small intestine, and it seems to be involved in the regulation of PYY hormone (Fleischer et al. 2015).

Composition of the human gut microbiota is very complex and studies using 16S RNA sequencing have shown Firmicutes, Bacteroidetes and Actinobacteria as dominant phyla in healthy adult, with *Bacteroides, Faecalibacterium, Bifidobacterium, Roseburia, Alistipes, Collinsella, Ruminococcus, Prevotella and Akkermansia* as main genera (Arumugam et al. 2011). In addition, Arumugam and colleagues were able to identify three main enterotypes, persistent in all samples from European individuals, based on variations in genus level and activity, such as *Bacteroides* (carbohydrates and proteins fermenters); *Prevotella* and *Desulfovibrio* (respectively mucin-degraders and involved in mucin desulphation); *Ruminococcus* and *Akkermansia* (able to degrade mucin and sugars; Arumugam et al. 2011).

Beneficial bacteria in the human gut include bifidobateria and lactobacilli. They are therefore main targets for the modulation of the gut microbiota through diet. Bifidobacteria have several positive effects such as inhibition of pathogens (Tejero-Sariñena et al. 2012), modulation of the immune system (Dong et al. 2010) and restoration of the intestinal microflora after antibiotic therapy (Madden et al. 2005). Beneficial effects of lactobacilli involve in particular the production of antimicrobial compounds that help in controlling proliferation of potential harmful microorganisms (Saulnier et al. 2009). An imbalance in composition of the gut microbiota can lead to changes in interactions between the intestinal microflora and host, which are related to different gastrointestinal diseases such as infectious and non-infectious disorders.

Infectious disorders include pathogens such as *Salmonella* or *Escherichia coli*, principal microorganisms responsible for Traveller's diarrhoea and *Clostridium difficile* that can lead to complications associated with mortality. The success of recent protocols for faecal transpantation as potential therapy for *C. difficile* infection have shown an important role of

the gut microbiota (Fischer et al. 2015).

Non-infectious disorders may be caused by factors such as genetics, environment and lifestyle and they involve obesity, Inflammatory Bowel Disease (IBD) or Irritable Bowel Syndrome (IBS). Obesity is a physiological state in which the ratio between the two main dominant phyla in the gut seems to be shifted in favour of *Firmicutes* instead of *Bacteroidetes*. The microbiota, may regulate appetite, producing hormones like PYY and GPL-1 that stimulate satiety, reduce permeability of the gastrointestinal trait and reduce inflammatory markers (Rastall and Gibson 2015). Inflammatory Bowel Diseases such as Crohn's Disease, Ulcerative colitis and pouchitis are a range of recurrent inflammatory disorders of the colon with a complex undefined aetiology. Environmental factors may play a role and genetically susceptible hosts seem to be more affected, but the possibility that microbes could be involved has been suggested. In CD patients, there is an increase in *Proteobacteria* and *Bacteroidetes*, in comparison to UC patients who no displayed significant differences compared to healthy volunteers (Gophna et al. 2006). This similarity could be explained by the fact that the cause of UC symptoms may be an increase of bacterial activity, especially *Desulfovibrio* spp. Metabolomic studies, indeed, confirm this hypothesis showing different production of metabolic compounds between remission and active phase of disease pathology (Balasubramanian et al. 2009, Bjerrum et al. 2010).

IBS is a disorder that affects 10-20% of the population in developed countries and it is characterised by abdominal pain, bloating and changes in stool frequency and consistency. The pathogenesis is multifactorial and includes the intestinal microbiota, with a higher risk of gastroenteritis and gas production (Blatchford et al. 2013).

1.1.2 Infant microbiota

Colonisation of the gastrointestinal (GI) tract starts immediately following delivery (natural or caesarean birth). Several research groups have recently started questioning this dogma focusing on the hyphothesis that gut microbial colonisation might initiate *in utero*.

Collado and collegues analysed amniotic fluid, placenta, colostrum, maternal and infant faeces and they showed unique microbial communities characterised by low abundance, low richness and low diversity (Collado et al. 2016). Proteobacteria was the most predominant phylum in amniotic fluid and placenta with *Enterobacter*, *E. coli* and *Shigella* as main types also in colostrum, meconium and infant faeces, but in lower numbers. The second predominant

genus was *Propionibacterium,* found in placenta parenchyma during the second trimester of pregnancy and meconium, confirming data previously published (Onderdonk 2008). Since meconium is formed during foetal life, their findings support the hypothesis that meconium and colostrum microbiota share a common maternal source and that the foetus might not be sterile but colonised by microbes in the placenta and amniotic fluid (Collado 2016). These results match findings from two pioneer studies by Ardissone *et al.* (2014) and Jimenez *et al.* (2008) who showed, respectively, correlation between bacterial populations in meconium and amniotic fluid in a human study (Ardissone 2014) and in a mouse trial (Jimenez 2008). Labelling *Enterococcus faecium* and orally inoculating pregnant mice, this strain was detected in the meconium of inoculated mice but not in the control group (Jimenez 2008).

After birth, several factors might alter bacterial composition, such as mode of delivery, diet, environment, genetics and antibiotic treatment from early life to adulthood (Figure 1.2).

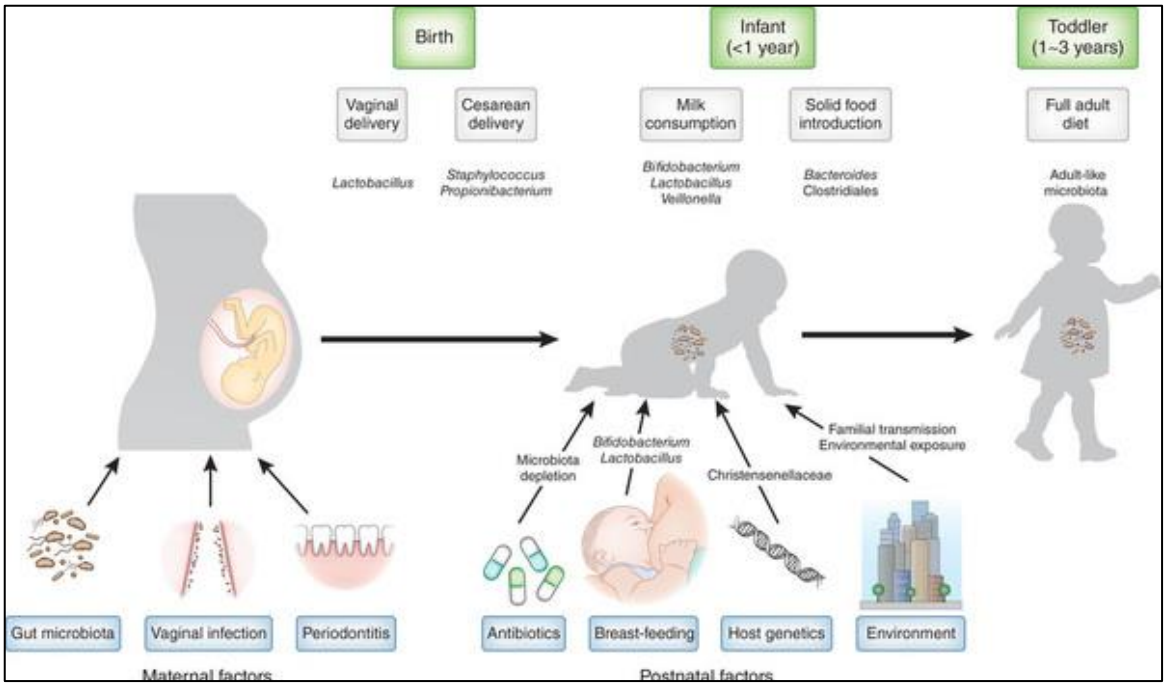

Figure 1.2: Factors influence gut microbiota during early life (Tamburini 2016).

Vaginally delivered infants are initially enriched in *Lactobacillus* spp. which resembles the maternal vaginal microbiota. In contrast children delivered by C-section were instead colonised by common skin and environmental microbes, such as *Staphylococcus*, *Streptococcus* or *Propionibacterium* spp. (Tamburini 2016).

Due to its unique composition, human milk modulates the gut bacterial population of breast fed infants by promoting the growth of beneficial bacterial, such as bifidobacteria and lactobacilli. Rich in oligosaccharides (human milk oligosaccharides, HMO), other nutrients and characterised by a specific microbial ecosystem, human milk has a strong impact on the immune system and gut function (Gomez-Gallego 2016). Breast and formula-fed infants have different gut microbiota composition with the latter one characterised by high diversity and dominated by *Clostridium* spp., *Bacteroides* and *E. coli* (Hascoet et al. 2011).

Antibiotic treatments in early life are another important factor with a strong impact on diversity and stability. Recent studies have shown that their effects are more pronounced during the first year of life, causing delays in gut microbiota maturation and gene resistance (Bokulich 2016, Yassour 2016). Alterations of gut microbial ecology may have a long-term impact on adult phenotype, modulating susceptibility to allergy, infection, obesity or diabetes (Kirjavainen 2002, Sjögren 2009, Abrahamsson 2012).

The infant GI microbiota is more variable in composition and less stable over time, compared to the adult one. The first 3 years of life are the most critical in terms of dietary intervention and introduction of solid food, but between 3 and 5 years of age, the microbiota of infants resembles that of the adult.

1.2 Gut-Brain axis

In the last 20 years, the gut microbiota and its interaction with neurological disorders has had a raised profile. The field of microbial endocrinology is expressly devoted to understanding the mechanisms by which the microbiota (bacteria within the microbiome) interact with the host. Microbial endocrinology was founded by Lyte in 1993 who focused on neuro-active compounds produced by the microbiota that can modulate the brain and behaviour within the microbiota-gut-brain axis (Lyte 1993).

Psychobiotics is a new definition of gut bacteria involved in the production of active compounds associated with mammalian neurotransmission. They have been defined by Dinan *et al*. 2013 as an “emerging class of probiotics that, when ingested in adequate amounts, produces a health benefit in patients suffering from psychiatric illness” (Dinan 2013).

It is well-known that certain bacteria are able to produce molecules that may act as neurotransmitters and directly affect the brain. Wall and colleagues reported some of the main neurochemicals that have been isolated from gut bacteria and the impact that they have on the

central nervous system, brain function and behaviour (Wall 2014) (Table 1.2).

Table 1.2. List of neurochemicals isolated from gut bacteria. BBB=blood-brain barrier (adapted from Wall 2014)

Bacterial genus	Neurochemicals	Functions
Lactobacillus, Bifidobacterium	GABA	Inhibitor of different physiological and psychological processes
Streptococcus, Escherichia, Enterococcus, Lactococcus, Lactobacillus	Serotonin	Metabolite from tryptophan and it has an important role in mood regulation
Escherichia, Bacillus	Norepinephrine	Major neurotransmitters that mediate motor control, cognition, memory processing, emotion and endocrine regulation
Streptococcus, Escherichia, Bacillus, Lactococcus, Lactobacillus	Dopamine	
Lactobacillus, Bacillus	Acetylcholine	Neurotransmitter important in memory and learning
Lactobacillus, Lactococcus, Streptococcus, Enterococcus	Histamine	Important role in the maintenance of wakefulness
Gut microbiota	SCFAs	They can cross BBB and work as energy source during early brain development; play a role in cell signaling and neurotransmitter synthesis and release

Interaction between intestinal microbiota and the brain includes the central nervous system (CNS), neuroendocrine and neuroimmune systems, sympathetic and parasympathetic arms of the autonomic nervous system (ANS) and enteric nervous system (ENS). The vagus nerve is the major nerve of ANS and mouse studies showed the impact of probiotics such as *L. rhamnosus* and *Bifidobacterium* spp. on stress, depression and anxiety via this path and alteration in GABA receptors (Bercik 2011, Bravo 2011).

In addition to SCFAs and neurotransmitter production, other routes where the gut microbiota is involved include tryptophan metabolism and kynurenine pathway, previously shown to be altered in autism (Boccuto 2013), the hypothalamic-pituitary-adrenal axis (HPA) and cytokine production.

Tryptophan is an essential amino acid and once in the bloodstream, it can cross the blood-brain barrier (BBB) and participate in serotonin synthesis in the CNS. However, serotonin, mainly located in the gut, is synthesised from tryptophan in enterochromaffin cells (ECs) of the gastrointestinal tract and from certain bacterial strains that produce indole from tryptophan (O'Mahony 2015). Yano and colleagues showed that spore-forming bacteria had an effect on serotonin in mice, by producing metabolites directly involved in signalling between gut microbiota and ECs (Yano 2015).

The HPA is a major neuroendocrine system that regulates different processes, including stress response. Cortisol is an important biomarker of stress and both excessive and deficient cortisol responses have been associated with dysregulation of the HPA axis. Acute stress seems to be involved in mast cell activation and can cause increases in GI barrier permeability allowing gut bacterial metabolites, toxins and lipopolysaccharides (LPS) to enter the bloodstream and reach the CNS (Santos 2001). Mouse experiments showed how *Bacteroides* spp. and *Clostridium* spp. respectively decrease and increase after chronic stress exposure, and this led to immune system activation (Bailey 2011).

There is bi-directional communication between the HPA axis and the immune system. A number of cytokines, such as IL-1, IL-6, IL-10 and TNF-α can activate the HPA axis. During an immune response, pro-inflammatory cytokines are released into the peripheral circulation system and can pass through the BBB where they can interact with the brain and activate the HPA axis. Interactions between pro-inflammatory cytokines and the brain can alter the metabolic activity of neurotransmitters and cause symptoms such as fatigue, depression, and mood changes (Padgett and Glaser 2003).

Interaction with gut bacterial populations has a strong impact in health and well being of the host from early life, including brain development and function. Changes in early life nutrition influence gut microbiota composition and metabolism, resulting in depressive-like behaviour and metabolic phenotype in non-weaned rats (Farshim 2016).

Psychiatric co-morbidities, such as anxiety and depression are common in patients with chronic bowel disorders, including IBS and IBD. Typical symptoms in IBS such as sleep difficulties, anxiety, depression, headache, fatigue, in particular abdominal pain, seem to get worse during periods of stress (Lacker 2010). Stress has a big impact on the gastrointestinal tract, altering intestinal motility, barrier function, and mucosal transport (Hyland 2014). Consequently, behavioural changes have been noticed in relation to modification of gut microbiota composition and it has been postulated that stress might have a long-term effect on gut bacterial populations (Collins 2012).

The GI symptoms associated with IBS seem to be similar to those of autism spectrum disorders (ASD) and to date there are no biological biomarkers for both conditions. Based on this, it may be relevant to note the potential therapeutic effects of pre- and probiotics in the treatment of certain neurological conditions (O'Mahony 2005, Whorwell 2006). Recent rodent studies have also shown increased expression of neurotransmitters and neuromodulators in the

hippocampus, after FOS and GOS supplementation (Savignac 2016, De Vadder 2014, Williams 2016).

1.2.1 Autism spectrum disorders (ASD)

"Autism typically develops early in childhood, and is considered as a systemic spectrum disorder with multiple development trajectories with an incidence four times higher in males than in females" (Grossi 2014).

Around 700,000 people may be autistic, or more than 1 in 100 of the population. The latest prevalence studies of autism indicate that 1.1% of the population in the UK may have autism. This prevalence rate is based on two relatively recent studies, one on children in the South Thames area (Baird 2006) and the other on adults (Brugha 2011).

Autism is currently diagnosed using behavioural criteria from the Diagnostic and Statistical Manual of Mental Disorders (DSM). ASDs is a term used to include and replace all subtypes of autism, including autistic disorder, Aspergers syndrome, childhood disintegrative disorder, and pervasive developmental disorder (not otherwise specified). The DSM-V (fifth edition), published in May 2013, eliminated the four sub-types listed above, by dissolving them into one diagnosis called Autism Spectrum Disorders (American Phychiatric Association 2013).

In this new edition ASD is defined as:

1. Persistent deficits in social communication and social interaction across contexts, not accounted for by general developmental delays
2. Restricted, repetitive patterns of behavior, interests, or activities
3. Symptoms must be present in early childhood (but may not become fully manifest until social demands exceed limited capacities)
4. Symptoms together limit and impair everyday functioning.

Autism was first considered as a distinct clinical disorder by Kanner (1943) but its exact cause is still unknown. It is a complex condition and may occur as a result of genetic predisposition and/or environmental factors.

There is clearly a genetic component and this has been established through twin and family studies (Ronald and Hoekstra 2011, Robinson 2016). Genetic aspects have involved parental age, male sex (gender bias with a ratio of 4:1), autism in the family, deficiency in

gene transcription and it is known that autism disorder is commonly found in genetic syndromes such as Fragile X and tuberous sclerosis (Liu 2001).

Despite evidence of a genetic component, genetics does not explain the whole picture. Environmental factors, such as dysbiosis in gut microbiota, diet or antibiotic therapy in early age, seem to have a huge impact on several aspects of autism. Correlation between medical history, GI symptoms and antibiotic treatments in 99 children with and without ASD has been investigated (Niehus and Lord 2006). ASD children seem to have significantly more ear infections than typically developing children, with consequently signficant higher use of antibiotics before the age of 2 years. As mention above, early life history of antibiotic use have a big impact on gut microbiota composition and dysbiosis seen in ASD might be due to such therapy (Niehus and Lord 2006).

1.2.2 Gut microbiota in ASD

Apart from the cognitive aspects, a significant majority of ASD sufferers also have associated GI issues. A recent detailed survey compared children with ASD to their non-ASD siblings and ASD was associated with diarrhoea, constipation, smelling stools, gaseousness, abdominal bloating and discomfort and food regurgitation. Moreover, 76% of children with ASD had at least one GI symptom compared to only 30% of a non-ASD siblings (Horvath 2002); with most children with ASD having two or more GI symptoms (64%). Several studies reported a prevalence of GI symptoms as high as 70% in children with ASD, although it was recently suggested that prospective studies be performed to determine the actual prevalence of GI disorders in ASD and their biological basis (Buie 2010).

Bolte hypothesized that autistic symptoms may be based on colonisation of *Clostridium tetani*. It was thought that the toxin produced from this spore-forming species, tetanus neurotoxin (TeNT), could impact on autistic symptomatology in different ways. In fact, autistic children do not show typical effects of tetanus infection, such as spasms, due to the presence of proteolytic enzymes in the human digestive tract. Basically these enzymes break down the toxin in fragments, reducing its effect but, unfortunately, they can still exert an influence (Bolte 1998).

To date, more recent studies have confirmed the role of *Clostridium* spp. in the gut of autistic children and how antimicrobial and antifungal therapies could help in relieving the symptoms. Finegold *et al.* (2002) found nine unique species of clostridia in autistic children

compared to healthy controls (Finegold 2002). Song *et al.* (2004), using qPCR analysis, found higher levels of *C. boltea* and *Clostridium* cluster I and XI (Song 2004). Furthermore, Parracho and co-authors, using FISH analysis, found greater number of those species derived from the *Clostridium histolyticum* group (*Clostridium* clusters I and II) (Parracho 2005). Comparing the microbiota of autistic and non-autistic children, Kang and colleagues, using recent pyrosequencing techniques, demonstrated that autistic children suffering GI problems have a distinct and less diverse gut bacterial population, characterised by lower levels of *Prevotella*, *Coprococcus*, and unclassified Veillonellaceae; and higher presence of *Akkermansia* genus (Kang 2013).

The genus *Sutterella* has been identified in intestinal biopsy and faecal samples from individuals with gastrointestinal disorders such as Crohn's disease and ulcerative colitis. On this basis, Williams and colleagues investigated its presence in biopsy samples from autistic children with gastrointestinal problems and non-autistic controls with only gastrointestinal dysfunction. In these results, they showed higher presence of this bacterial group only in autistic individuals but not in the control (Williams 2012). These data were confirmed by Wang *et al.* who analysed faecal samples of autistic children and showed increase number of *Sutterella* spp. and *Ruminococcus torque* (Wang 2013).

Shaw *et al.* found high levels of tartaric acid in the urine of two autistic siblings which was suggested to be due to overgrowth of *Candida albicans.* Potential toxins released may cause an effect on the central nervous system and subsequently affect behaviour (Shaw 1995). Antifungal treatments seem to be a potential strategy to reduce presence of the yeast (Shaw 2000). In another study, antibiotic Vancomycin therapy, in an uncontrolled trial (n=11) with regressive autistic children, showed a short-term improvement in behavioural parameters, which waned following discontinuation of antibiotic treatment (Sandler 2000).

Another interesting aspect of the effect of *Clostridium* spp. involves tryptophan metabolism. Normally, this amino acid is catabolised with indole pyruvate and indole acetate being detected in the urine of healthy people as well the end product of their metabolic pathway, IAG (indolyl-acryuloyl-glycine). IAG seems to be a detoxified version of an acidic precursor that affects permeability of membranes. It has been shown that in autistic children, higher amounts of indole derivates are present in the blood and higher levels of IAG have been identified in urine. Increased numbers in *Clostridium* spp. can be, again, an explanation. *Clostridium* can metabolise tryptophan and contribute to an abnormal metabolic state

(Bingham 2003).

Recently, a model of the autism pathology has been proposed based on three main pathways: oxidative stress, gut bacterial dysbiosis and "leaky gut" syndrome. Taking into account different studies on autism pathogenesis, Heberling and colleagues suggested that overgrowth of some bacterial populations, in particular *Clostridium* spp. and *Desulfovibrio* spp., can lead to an inflammatory state that might cause increased gut permeability. This can cause inappropriate passage of molecules to the bloodstream and BBB affecting the central nervous system (Heberling 2013). Gram-negative bacteria, that possess LPS on the cell wall surface, may be involved in ASD. LPS activates the innate immune system (Trent 2006), is involved in tissue damage and it has been suggested to induce the nitric oxide (iNOS) gene, causing release of nitric oxide, which is a neurotransmitter (Berdeaux 1993). It also damages the BBB, making it porous and allowing LPS to cross (Gaillard 2001, Xaio 2001). Moreover, LPS seems to work with other toxins, notably heavy metal ions, to increase the extent of cellular damage (Rumbeiha 2000). In addition, oxidative stress seems to have an impact on sulphate metabolism in cysteine and methionine pathways and consequences related to that, alteration in DNA, RNA, protein, lipids methylation and gene expression (Heberling 2013). James *et al.* in an *in vivo* human intervention study, involving 20 ASD children and 33 controls, showed imbalances in the methionine cycle mainly due to a "bottle-neck" at S-adenosylhomocysteine (SAH) and adenosine. Supplement of folinic acid, betaine and methylcobalamin have shown stabilisation in metabolite concentrations within the methionine cycle into the normal range (James 2004).

Based on Heberling hypothesis and taking into account the main bacterial populations involved in autism dysbiosis, Weston and his research group developed a model which simulates direct and indirect effects of the interaction between bifidobacteria, clostridia and *Desulfovibrio* spp. (Figure 1.3) (Weston 2015).

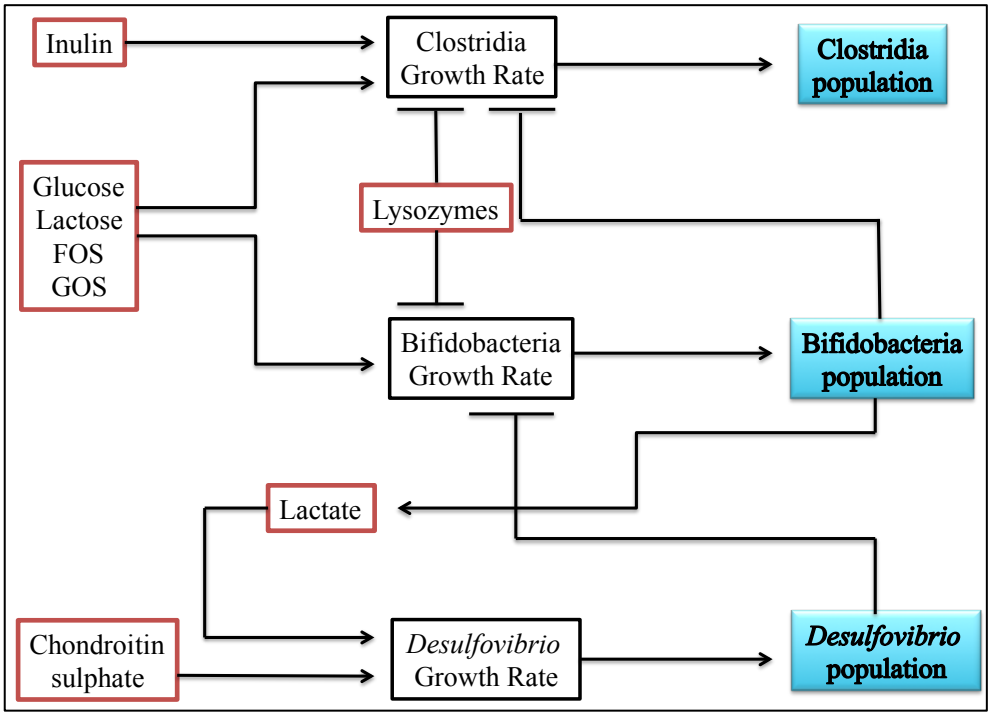

Figure 1.3: Model of bifidobacteria, clostridia and *Desulfovibrio* interaction. Arrows represent positive interaction; Flat head negative interaction (adapted from Weston et al. 2015).

In addition the authors proposed a new quantitative measure in order to valuate the risk to develop autism based on bacterial shift from a steady state:

$$\text{Gut Bacteria Index (GBI)} = (B / Bss) / [(C + D) / (Css + Dss)] - 1$$

where B, C and D are bifidobacteria, clostridia and desulfovibrio respectively and SS is the steady state, before any treatments. If GBI is 0, this means that gut bacteria are similar to the control, if it is positive or negative it means that there is lower or higher risk to develop autism, respectively. Basically in their computational model, the authors introduced step-by-step variables that have been seen in previous studies to affect gut bacterial populations and improvement of ASD symptoms, in order to see how GBI changes. Variables involve use of pre- or probiotics, since they can enhance bifidobacterial growth; or use of antibiotics as treatments for ear infection; or use of lysozyme since it is present in breast milk (Weston 2015). Even though it is an intriguing model, the Gut Bacteria Index has not been validated and it has limitations. Autism is a wide spectrum disorder and three variables are not enough to explain the all picture.

Autism is a wide-ranging disorder and complex pathogenesis, involving both environmental and genetic factors. Differences in data between studies suggest that there may

be more than one possible cause. Potential treatments may need to be personalised according to individual needs and, perhaps, a combination of treatments would result in a greater improvement of symptoms.

1.2.3 Potential treatments and their influence on gut microbiota activity

Symptoms reported in ASD people can be considered individual and heterogeneous, but many research groups are looking at dietary intervention as potential therapy for GI issues in autism in order to modulate the intestinal microbiota and alleviate gut symptoms.

The term nutraceuticals is defined ‘‘any substance that is food or a part of food and provides medical or health benefits, including the prevention and treatment of disease’’ and was introduced for the first time in 1989 by the US Foundation for Innovation in Medicine (FIS) (Alanazi 2013). This approach is interesting and promising in autistic disorders since food selectivity is a common characteristic in children with ASD (Schreck and Williams 2006) and might be associated with nutrient inadequacies (Bandini 2010) (Cermak 2010). Kral *et al.* in recent review paper reported some of the main studies involving food supplements and their impact on different aspects of autism. Omega-3, multivitamins, minerals, L-carnitine, ascorbic acid, vitamin B12 and B6, folinic acid, dietary fatty acid, folate, creatine and probiotics are some examples of nutraceticals that have been investigated so far, with interesting results in improving ASD behavioural symptoms and gut dysfunction, but there are still limitations in the trial designs and lack of strong evidence to support efficacy (Kral 2013).

Gluten and casein-free (GFCF) diets are common as individuals with ASD have been shown to have an impaired epithelial barrier function. Gliadin (glycoprotein present in wheat) activates zonulin signalling, a protein that modulates the permeability of tight junctions between cells wall of the digestive tract, leading to increased intestinal permeability to macromolecules (Fasano 2011, Lammers 2008). Opioid peptides released from gluten and casein can pass through the BBB and have an impact on the CNS (Lázaro 2016). Studies have been conducted to evaluate the efficacy of gluten and casein-free diets (Mulloy 2010). Some results suggested positive effects on behaviour and GI symptoms (Pennesi and Klein 2012, Harris and Card 2012); other findings did not show any improvement in the intervention group (Hyman 2015, Elder *et al.* 2006), suggesting that more studies are required in order to assess efficacy of the GFCF diet.

Unfortunately none of those studies took into account the impact of these particular

diets on gut microbiota composition and its correlation with potential amelioration in GI problems. A recent metagenomic study aimed to compare the gut bacterial populations of samples from healthy adults after 4 weeks gluten-free diet (GFD) to those collected during normal diet. They noticed, in GFD, significant changes in alpha diversity and reduction of *Veillonellaceae* family, *Ruminococcus bromii* and *Roseburia faecis*. While families *Victivallaceae*, *Clostridiaceae* and *Coriobacteriaceae*, and genus *Slackia* significant increase in abundance. No significant modifications have been seen in normal dietary groups (Bonder 2016). *Veillonellaceae* is considered to be a pro-inflammatory family of bacteria and its high abundance was consistently found in IBD or IBS (Gevers 2014, Shukla 2015). Decrease in this bacterial family might be correlated to the beneficial effects of GF diets observed in patients with gluten-related disorders, including potentially autism.

Prebiotics and probiotics have been widely investigated and have been shown to modulate the intestinal microflora. Therefore, they could be a useful novel therapeutic approach in order to potentially ameliorate behavioural symptoms and GI discomforts associated with some children with ASD (Critchfield 2011).

A recent survey conducted on ASD children taking Delpro® (*Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbruecki, Bifidobacterium longum* and *Bifidobacterium bifidum*) for 6 months, showed improvements in GI symptoms (diarrhoea or constipation), stool frequency and ASD symptoms. These data, even lacking of a controlled clinical trial, suggest that probiotics have an impact on GI distress and ATEC signs and symptoms among autistic population (West 2013).

Parracho *et al.* reported in children with ASD a significant behavioural improvement when administered *Lactobacillus plantarum* WCSF1 compared to ASD treated with placebo (Parracho 2010). Adams *et al.* compared gut microbiota composition and inflammatory markers from stool samples of 58 children with ASD and 39 healthy typical children of similar ages, and found reduced levels of total SCFAs in the ASD group with a higher difference in children with autism taking probiotics. In addition, they showed a strong correlation between GI symptoms and severity of autism (Adams 2011). Kaluzna-Czaplinska administered oral supplementation of *Lactobacillus acidophilus* twice a day for 2 months to a group of ASD children and found significant modifications in the level of D-arabinitol (DA) and the ratio of D-/L-arabinitol (DA/LA), and an improvement in the ability to carry out instructions and concentration (Kaluzna-Czaplińska 2012). Recently, Tomova and colleagues confirmed the

results from Adams *et al.*, revealing a strong positive correlation of autism severity with severity of GI dysfunction. In their study, 4 months of probiotic mixture diet supplementation normalised gut bacterial populations of ASD children. However, no behavioural traits were investigated (Tomova 2015). Furthermore, probiotic supplement has been demonstrated to reduce the concentration of myeloperoxidase, a marker for inflammation and oxidation, in autistic children compared to non-autistic controls (Russo 2015).

Considering previous study limitations, Santocchi and colleagues suggested a protocol in order to improve probiotic study outcomes. A six month parallel, randomised, probiotic feeding study will aim to assess changes GI symptoms, autism severity, affective and behavioural comorbid symptoms, plasmatic, urinary and faecal biomarkers related to abnormal intestinal function and neurophysiological patterns. However, potential limitations might include the length of the study and kind of samples requested (blood, urine and faecal samples) and lack of control group (non-ASD children) in order to compare biomarkers levels (Santocchi 2016).

Little is known about prebiotic supplementation and its impact on autism. The prebiotic FOS (fructo-oligosaccharides) has been tested in order to determine effects on end products of trytophan metabolism, known to be altered in ASD individuals, from gut microbiota using a mixed bacterial population from the large intestines of pigs. The results showed indole-3 acetic acid (a microbial precursor of IAG) decreased with the addition of FOS (Xu 2002).

The positive effects of pre- and probiotics on wellbeing of the host are known and confirmed in healthy people and different clinical conditions. Even if the causes of the GI symptoms in the autism are still unknown, further studies are necessary to better understand correlation with dysbiosis in the gut microbiota and the potential of pre- and probiotics in this neurological disorder.

1.3 Probiotics

One definition of probiotic has been proposed by the World Health Organization as "live microorganisms which, when administrated in adequate amounts, confer a health benefit to the host" (FAO/WHO 2002). They are usually strains of lactic acid producing bacteria (LAB), in particular members of *Lactobacillus* and *Bifidobacterium* genera. Probiotic bacteria,

in order to exert their activity, have to survive the gastrointestinal transit (Gibson 2005). Their positive effects on the human health may include:

1) Improvement of tolerance to lactose, thanks to the presence of enzymatic lactase activity
2) Competition with pathogens for colonisation and nutrients
3) Vitamin synthesis and improvement of mineral absorption
4) Stimulation of the immune system
5) Improvement digestion and gut function

To be known as a probiotic, a candidate microorganism has to be regarded as GRAS (Generally Recognized As Safe). This means it should not be pathogenic and should not be able to transfer antibiotic resistance genes, as well as be able to maintain genetic stability.

Although the beneficial effect of probiotics have been widely tested, these food supplements do have limitations. In order to be effective, probiotics must remain viable and stable along the gastrointestinal tract, and the host should gain beneficial impacts. However, such live microorganisms may reach the lower gut in a compromised state due to bile and stomach acid secretions. For the microorganisms to be fully effective, they will then have to compete with an established colonic microbiota for nutritional sources (Walton 2013).

1.4 Prebiotics

Prebiotics are defined as "substrates that are selectively utilized by host micro-organisms conferring a health benefit" (Gibson 2017).

All prebiotics are dietary carbohydrates but not all of them can be considered prebiotics. In order to be classified as prebiotic, there are certain criteria:

1) Resistance to the low gastric pH, hydrolysis by intestinal enzymes and gastrointestinal absorption
2) Be selective substrates for certain bacteria
3) Stimulate the growth and the activity of the beneficial bacteria that contribute to the well being of the host

Gibson *et al.* (2004) in a review paper revisited the concept of prebiotic and updated the list of the candidate prebiotic according to criteria mentioned above (Gibson 2004).

Inulin and Fructo-oligosaccharides (FOS), are non-digestible oligosaccharides present in plants, fruits and vegetables such as bananas, onions, chicory root, garlic, asparagus, artichoke, and leeks. FOS can be obtained by partial enzymatic hydrolysis of inulin using an endo-inulinase, and by enzymatic synthesis using β-fructosidase from *Aspergillus niger*, but the end products have different degree of polymerisation (DP). Orafti NV, have developed an inulin, known as Synergy1, which consists of 1:1 inulin and oligosaccharide. The chicory-derived inulin has a DP of 10 to 60 (average DP of 25). The oligofructose is produced by partial enzymatic hydrolysis of chicory derived inulin and has a DP ranging between 3 and 7 (average DP of 4; Gibson 2004).

Several *in vitro* and *in vivo* studies have been carried out using both inulin and FOS. Kaplan and Hutkins demonstrated the selectivity properties of FOS for bifidobacteria and lactobacilli. Studying 28 strains, their results showed *L. acidophilus*, *L. casei*, *L. plantarum, B. adolescentis, B. infantis, B. breve* and *B. longum* as FOS-fermentors and *L. lactis, L. bulgaricus, L. rhamnosus GG, Streptococcus thermophilus* and *B. bifidum* as non FOS-fermentors (Hutkins 2000). Batch fermentation experiments, inoculated with human faecal samples, showed fructans were consumed accordingly to their DP and that different gut bacteria were also able to grow on these carbon sources. In particular, Rossi and colleagues demonstrated that bifidobacteria grew by cross-feeding on mono- and oligosaccharides produced by primary inulin intestinal degraders (Rossi 2005). According to the different human intervention studies reported by Kolida & Gibson, 5–8 g/d of inulin intake is an adequate dose in order to modulate the gut microbiota. One potential side effect is gas production that might be explained by bacteria other than the target organisms being involved in the fermentation, since bifidobacteria and lactobacilli do not produce gas, or high doses of prebiotic (Kolida and Gibson 2007).

These criteria have been fulfilled also for lactulose. It is produced by isomerisation of lactose to generate the disaccharide galactosyl β-(1-4) fructose and different human studies show how it can be selective fermented by bifidobacteria and lactobacilli and reduce the growth of potential harmful bacteria (Tuohy 2002, Bouhnik 2004).

Galacto-oligosaccharides (GOS) are defined as "a mixture of those substances produced from lactose which comprise between two and eight saccharide units, with one of these units being a terminal glucose and the remaining saccharide units being galactose, and disaccharides comprising two units of galactose" (Tzortzis 2011) (Table 1.3).

Table 1.3: Different definitions of GOS (Roberfroid 2010)

GALACTANS	
Mixture of β(1-6); β(1-3); β(1-4) galactosyl-galactose	Galacto-oligosaccharides,
GOS	*trans*-galacto-oligosaccharides
Galn-Gal and/or Galn-Glc; DP 2-8	(enzymatic transgalactosylsation of lactose)

The structural size of GOS plays a role in targeting the bifidobacterial genus, however this relationship has not been extensively determined at species level (Matsuki 2004, Rodriguez-Colinas 2013).

Differently from FOS, GOS is not a food component but it is a synthetic product and to date, the main companies involved in the GOS market include Yakult Honsha (Oligomate 55N and 55NP and TOS-100), Nissin Sugar Manufacturing (Cup-Oligo P and H-70) and Purimmune from Japan; Floraid GOS from the United States; Friesland Food Domo (Vivinal GOS), Nestle GOS and Clasado (B-GOS) from Europe. They produce usually GOS in syrup or powder format that, in both cases, contain oligosaccharides of different DP, non-reacted lactose, glucose and galactose. Table 1.4 summarises characteristics of commercially GOS available (Torres et al. 2010).

Table 1.4: Characteristics of commercially GOS (adapted from Torres et al. 2010)

	GOS								
	Glucose	**Galactose**	**Lactose**	**DP2**	**DP3**	**DP4**	**DP>4**	**Total GOS content**	**Enzyme source**
CUP-oligo		25 to 30		-	-	-	-	70	*Cryptococcus laurentii*
Oligomate55		18 to 39	10 to 22	15 to 17	18 to 24	10 to 16	2 to 5.4	50 to 60	*A.oryzae* and *S. thermophilus*
TOS-100		0	0 to 1	0	55	33 to 35	12 to 14	99 to 100	*Bacillus circulans*
Vivinal GOS	19 to 22	0.8 to 1.3	10 to 23	19 to 27	22 to 23	11	6 to 7.6	57 to 59	*Bacillus circulans*
B-GOS	18	12	22	25 to 29	12 to 14	6.7 to 7.7	3.8 to 4.4	48 to 55	*Bifidobacterium bifidum*
Purimune	0 to 1	0 to 0.5	7 to 10	16 to 21	38 to 51	25 to 29		90 to 92	*Bacillus circulans*
Floraid GOS	21	8.5	18	-	-	-	-	20-28	*A.oryzae*
Nestle GOS	10	5	20 to 40	-	-	-	-	46	*A.oryzae*

DP: degree of polymerisation;
DP and total GOS content expressed in %;
- : data not available

1.4.1 Galacto-oligosaccharides as prebiotics

GOS is thought to remain largely undigested through the human gut making it is a prebiotic. Not only is it suitable for administration, its functional properties enable it to be easily manufactured. A low pH tolerance enables GOS to withstand acidic conditions in the stomach but also offers potential for use in fruit juices (Sangwan 2011). At present, GOS has been associated with numerous health benefits. In fact, the two main mechanisms by which this is achieved are through the production of SCFAs from its fermentation and the selective enhancement of beneficial gut organisms, thus modulating the microbiota (Sangwan 2011).

Acetate, butyrate and propionate are the most common SCFA in the colon. SCFAs reduce the luminal pH increasing mineral absorption, suppressing the growth of pathogens and influencing intestinal motility. In addition, they may be absorbed by the colonic mucosa and provide an energy supply (mainly butyrate). Acetate can be metabolised in human muscle, kidney, heart and brain. Propionate is metabolised by the liver and as a glucogenic substrate affects the hepatic control of lipids, cholesterol production and adipose tissue deposition. Butyrate is an energy substrate for cell growth and differentiation, it also reduces the risk of colon cancer stimulating apoptosis (Roberfroid 2010). There has been a range of studies demonstrating the efficacy of GOS. It should be noted that bifidobacteria mainly generate acetate and lactate, but not propionate or butyrate.

Another important factor could be the ability of prebiotics to act as anti-adhesive agents against pathogens. Studies have revealed that GOS was more effective than inulin and FOS at preventing the attachment of EPEC to gut a cell line *in vitro* (Tzortzis 2005, Shoaf 2006). GOS has also been seen to reduce the severity of pathogen infection and prevent colonization in mice e.g. of *S. typhimurium* (Searle 2009).

In most cases, GOS has been proven to elicit a bifidogenic effect increasing levels of these potentially beneficial organisms. This positive ability is supported by the presence of specific membrane mechanisms of transport like diffusion, cation symport and proton symport, and the presence of extracellular β-galactosidases that hydrolyse GOS, internalise the products and degrade them in the cell, avoiding competition with other bacteria (Boehm 2005, Fanaro 2009). Recently, GOS and inulin has become a standard component in infant formulae, attempting to generate similar compositions of the gut microbiota between breast fed infants and supplemented formula fed infants i.e. higher concentrations of bifidobacteria (Scholtens 2008, Ben 2008).

Another important factor is immune-modulatory activities related to the GALT system (Gut Associated Lymphoid Tissue) that contain 60% of lymphocytes and sIgA that play a role

against pathogenic invasion. These effects are due to direct interaction with the immune, mucosal and epithelial cells or indirect by selective modulation of the microbiota.

1.4.2 B-GOS

B-GOS as manufactured by Clasado Biosciences Ltd., is synthesised from food grade lactose using the transgalactosidic activity of a microbial derived β-galactosidase enzyme preparation which catalyses lactose to form a mixture of β-linked disaccharide, trisaccharide, tetrasaccharide and pentasaccharide chains in configurations depicted in Table 1.5.

Table 1.5: Dimeric and oligomeric saccharides present in B-GOS. Gal= Galactose; Glc = glucose. (Clasado Inc.)

Carbohydrate	Configuration of the Saccharide Linkages in Bimuno
Disaccharides	Gal-β-(1,3)-Glc Gal-β-(1,3)-Gal Gal-β-(1,6)-Glc Gal-α-(1,6)-Gal
Trisaccharides	Gal-β-(1,3)-Gal-β-(1,6)-Gal Gal-β-(1,6)-Gal-β-(1,4)-Glc Gal-β-(1,6)-Gal-β-(1,6)-Gal Gal-β-(1,3)-Gal-β-(1,4)-Glc
Tetrasaccharides	Gal-β-(1,6)-Gal-β-(1,6)-Gal-β-(1,4)-Glc Gal-β-(1,3)-Gal-β-(1,6)-Gal-β-(1,6)-Gal Gal-β-(1,6)-Gal-β-(1,6)-Gal-β-(1,6)-Gal
Pentasaccharides	Gal-β-(1,6)-Gal-β-(1,6)-Gal-β-(1,6)-Gal-β-(1,4)-Glc Gal-β-(1,3)-Gal-β-(1,6)-Gal-β-(1,6)-Gal-β-(1,4)-Glc

The β-galactosidase activity of *Bifidobacterium bifidum* NCIMB 41171 has been used to produce the B-GOS mixture (Table 1.6):

Table 1.6: B-GOS composition-in dry matter. DP, degree of polymerisation (Tzortzis 2009)

Ingredient	g/100 g
Monosaccharide	<15.3
Lactose	<28
GOS	<50
DP2	52%
DP3	26%
DP4	14%
DP5	8%
Minerals	<5
Protein	<1
Fat	0.5

Tzortzis *et al.* (2005) showed that *B. bifidum*, isolated from faecal samples of a healthy human volunteer could convert 55% of lactose to a novel mixture of GOS at the optimum activity condition (pH: 6.9 and temperature: 40°C). The bifidogenic activity of B-GOS was higher than inulin and a GOS mixture generated from enzymes of a *Bacillus* strain. The prebiotic potential of this mixture has been tested also *in vivo* in a pig-feeding trial (Tzortzis 2005b). Four β-

galactosidase enzymes, three β- and one α-galactosidase, involved in the reaction have been cloned, sequenced and characterised by Goulas *et al.* to better understand their physiological and biotechnological properties, including selectivity, interaction with pathogens and immune system (Goulas 2009).

B-GOS has been tested further in human studies. Depeint *et al.* showed the bifidogenic effects of B-GOS in healthy volunteers comparing to a GOS mixture produced by an industrial β-galactosidase from *Bacillus circulans* ATCC 4516 (V-GOS). Using a B-GOS mixture in three different doses (0 g, 3.6 g, 7 g), each for 7 days, with an equivalent washout period between each treatment, there was a positive effect in a dose-response relation in terms of increasing *Bifidobacterium* group. There was a significant increase in *B. bifidum* and *B. longum* compared to V-GOS (Depeint 2008).

Furthermore, the positive effects of B-GOS have been also assessed by Vulevic *et al.*, in elderly healthy volunteers. To date, several studies have shown negative modifications of elderly intestinal microflora composition compared to younger persons. One possible explanation is related to the aging process that is associated with a reduced activity of the immune system and an increase of pro-inflammatory cytokines. Forty-four volunteers were randomly assigned into 2 groups: one consuming placebo and B-GOS (both powder form, 5.5g) for 2 treatment periods of 10 weeks each, separating by a 4 weeks washout period. The results showed how, after 5 weeks, the microbiota was similar to younger counterparts, reducing the presence of putrefactive bacteria. The study highlighted significant changes, enhanced at 10 weeks, in production of IL-10, reduced production of pro-inflammatory cytokines like IL-1 β, TNF-α and IL-6 and increased NK cell activity. This is a key point that shows how B-GOS can influence immune senescence, decline of immune functions, which promotes hypo responsiveness to vaccination and predisposition to infectious and non-infectious diseases (Vulevic 2008). These potential immune modulatory properties of B-GOS have been shown also *in vitro* by Dubert-Ferrandon *et al.* (2008) using adult human colonic NCM 460 cells showing a positive effect on TNF-α mediated inflammation in human intestinal epithelial cells.

Another interesting area, in which the B-GOS mixture has been tested, was related to functional GI disorders, in particular IBS. Silk *et al.* investigated the efficacy of B-GOS in changing the colonic microflora and improving symptoms in IBS sufferers. In this clinical trial, patients were randomised to receive either 3.5 g⁄d B-GOS, 7 g⁄d B-GOS or 7 g⁄d equivalent placebo. Both doses were well tolerated and no adverse side effects were reported by any of the patients. Moreover, this study highlighted that B-GOS has potential as a therapeutic agent effective in alleviating symptoms and in increasing bifidobacteria numbers (Silk 2009).

The efficacy of B-GOS against pathogens has been shown by Searle *et al.* using *in vitro* and *in vivo* models where 2mM of B-GOS significantly reduced colonisation and pathology associated with *Salmonella enterica* typhimurium, but the mechanism of action is still to be elucidated (Searle 2009). Drakoularakou *et al.* focused on the severity and/or incidence of travellers' diarrhoea, in healthy volunteers who travel and stay in a high-risk destination (Asia, Middle East, Africa, Mexico, Central and South America). The volunteers were consuming B-GOS (5.5g/d) for 1 week before the holiday and for the 2 weeks holiday. Results showed reduced diarrhoeal episodes and decreased symptoms such as abdominal pain, vomiting, fever, anorexia, headache and dizziness (Drakoularakou 2009).

Another study on the potential activity of B-GOS focused on its effect in reducing risk factors of metabolic syndrome. Two groups of volunteers started the treatment respectively with placebo and B-GOS (powder 5.5g/d) for 12 weeks, followed by 4 weeks washout before swapping the treatment for other 12 weeks. The results showed that B-GOS had a significant prebiotic effect on aspects of insulin resistance (Vulevic 2013).

B-GOS was also shown to reduce cortisol secretion and anxiety in healthy volunteers. Schmidt and colleagues, in their human intervention study, showed that B-GOS supplementation lowered cortisol reactivity and modulate attention to emotional stimuli compared to a placebo group and FOS (Schmidt 2015).

Aims and Objectives

To date, studies focussing on the interactions between gut microbiota dysbiosis and ASD show common and consistent results. Some bacterial groups, such as *Clostridium* spp., *Desulfovibrio* spp. and *Sutterella* spp. seem to be present in higher numbers in autistic children compared to non-autistic ones, and beneficial bacteria such as bifidobacteria and lactobacilli have been found to be in lower amounts in ASD individuals. In addition, strong alterations in some metabolic pathways have also been seen. Modifications in gut microbiota composition might be associated to GI discomforts observed in ASD and have an impact on CNS and consequently on mood and behaviour. Therefore, dietary intervention therapies have been postulated to improve these aspects. The potential of prebiotics to alleviate gut symptoms and modulate the GI microbiota has been widely investigated in healthy individuals and different bowel disorders but there is a lack of knowledge about their impact on autism and gut-brain axis.

The aims and objectives of this research project were therefore (Figure 1.4):

- To investigate the impact of a purified prebiotic B-GOS (65%GOS content) on overall diversity of the anaerobic faecal bacterial population using *in vitro* batch culture fermentation systems;
- To determine the effect of B-GOS (65%GOS content) on the gut microbiota composition and metabolites production using a three-stage continuous system models inoculated with faecal samples from autistic and non-autistic children;
- To design and perform a human intervention study investigating the effects of B-GOS (83%GOS content) on different aspects of autism:
  - The first aim was to investigate the effect of a 6-weeks prebiotic supplementation on gut microbiota and its metabolic by-products in children with ASD.
  - The second aim was to assess the impact of this treatment period on GI symptoms (including adequate relief of symptoms, severity and incidence of symptoms, stool consistency and frequency), sleep, mood and behaviour.

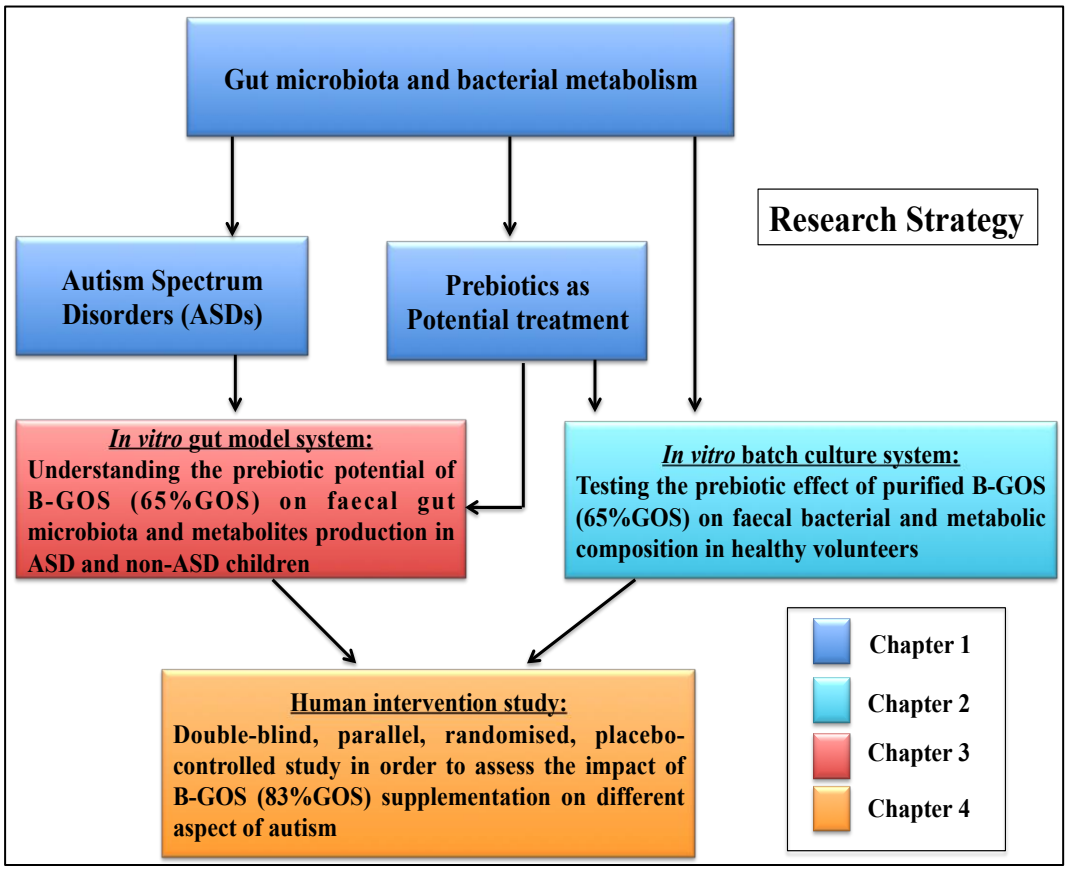

Figure 1.4: Summary of the research strategies used to achive principal aims of the study

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CHAPTER 2

Fermentation properties and potential prebiotic activity of Bimuno® galacto-oligosaccharide (65% galacto-oligosaccharide content) on *in vitro* gut microbiota parameters.

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Published in the ***British Journal of Nutrition 2016, 116:480-486***

Abstract

Prebiotic oligosaccharides have the ability to generate important changes in the gut microbiota composition that may confer health benefits to the host. Reducing impurities in prebiotic mixtures could expand their application in food industries and improve selectivity and prebiotic effects on potential beneficial bacteria such as bifidobacteria and lactobacilli. This study aimed to determine the *in vitro* potential fermentation properties of a 65 % galacto-oligosaccharide (GOS) content, Bimuno® GOS (B-GOS), on gut microbiota composition and metabolites. Fermentation of 65 % B-GOS was compared with 52 % B-GOS in pH- and volume-controlled dose–response anaerobic batch culture experiments. In total, three different doses (1, 0·5 and 0·33 g equivalent to 0·1, 0·05 and 0·033 active GOS g/l) were tested. Changes in the gut microbiota during a time course were identified by fluorescence *in situ* hybridisation, whereas small molecular weight metabolomics profiles and SCFA were determined by ^{1}H-NMR analysis and GC, respectively. The 65% B-GOS showed positive modulation of the microbiota composition during the first 8 h of fermentation with all doses. Administration of the specific doses of B-GOS induced a significant increase in acetate as the major SCFA synthesised compared with propionate and butyrate concentrations, but there were no significant differences between substrates. The 65% B-GOS in syrup format seems to have, in all the analysis, an efficient prebiotic effect. However, the applicability of such changes remains to be shown in an *in vivo* trial.

2.1 Introduction

In vitro and *in vivo* studies involving prebiotic oligosaccharides have been carried out using inulin and its fructo-oligosaccharide (FOS) derivatives, as well as various galacto-oligosaccharides (GOS). It has been shown that these food ingredients have the ability to selectively improve the growth of bifidobacteria, and consequently lead to important changes in the gut microbiota composition that may confer health benefits to the host. To date, GOS has been associated with numerous health benefits such as low energy content, insulin-independent metabolism and stimulation of growth and metabolism of specific colonic microbiota. The two main mechanisms by which this is achieved are through the production of SCFA from its fermentation and selective enhancement of beneficial gut microorganisms (Sangwan 2011).

GOS can be defined as a mixture of the end products of lactose breakdown by β-galactosidases, containing two to eight saccharide units, with a terminal glucose unit (Tzortzis 2009). These mixtures can be complex and their structures are often imperfectly characterised. They tend to be mixtures of β-1,3, β-1,4 and β-1,6 linkages with degrees of polymerisation ranging from two to five. A characteristic of GOS is that the set of structures present depends on source of the enzyme used to bring about synthesis. Structural and functional relationships of GOS play a role in targeting the *Bifidobacterium* genus (Matsuki 2004). Another important aspect is the presence of impurities such as monosaccharides, disaccharides or metabolic products from purification steps. Removing these compounds can lead to a mixture with a GOS content as high as possible that can be better used to study fermentation and structural properties of novel prebiotics in *in vitro* experiments.

In addition, purified Bimuno® GOS (B-GOS) mixture might have significantly increased interest in production and application in various food and pharmaceutical processes, especially if the prebiotic is incorporated directly into food such as diabetic or low-energy foods. Reducing monosaccharides and disaccharides such as glucose, galactose and lactose from the mixture might help minimise their impact on consumers, particularly by taking into account lactose intolerance. Several techniques have been suggested in order to obtain high recovery of GOS, but all of them have some limitations (Hernandez 2009). B-GOS (Bimuno® 52% GOS content; Clasado Biosciences Ltd) refers to prebiotic GOS compounds that have multiple biological health activities within the colonic environment. It is produced from the activity of galactosidase enzymes isolated from *Bifidobacterium bifidum* NCIMB 41171 (Tzortzis 2009). The method for

B-GOS purification has been studied by Goulas *et al.* (2007) in order to reduce the amount of free glucose and galactose produced during its synthesis, and it led to the removal of 92% of glucose by fermentation with *Saccharomyces cerevisiae*.

The bifidogenic properties of B-GOS have been investigated *in vitro* and *in vivo*. Tzortzis *et al.* (2005) showed *in vitro* and in a pig trial the prebiotic potential of GOS. In this study, it was established that B-GOS prebiotic activity was relevant in terms of increase in bifidobacteria numbers, SCFA production and decreased pH, compared with other prebiotics such as inulin and other GOS types (Tzortzis 2005). B-GOS has also been tested in healthy volunteers, clinical conditions that have a purported microbial aetiology such as Irritable Bowel Syndrome, traveller's diarrhoea and obesity, and on cognitive functions (Schmidt 2015; Depeint 2008; Drakoularakou 2009; Silk 2009; Vulevic 2008; Vulevic 2013).

This study aimed to determine the potential prebiotic activity of a purified 65% GOS content B-GOS, compared with 52% GOS content B-GOS, used as positive control in *in vitro* dose–response batch cultures.

2.2 Methods

2.2.1 Substrates

The two B-GOS products, supplied by Clasado BioSciences Ltd, used in this study were produced from the activity of galactosyltransferases from *B. bifidum* NCIMB 41171 on lactose (Tzortzis 2005). Both were used in syrup form. The B-GOS mixtures consisted of (w/w) 52 % GOS, 8 % lactose, 22 % glucose, 16·5 % galactose; and 65% GOS, 10·1% lactose, 22% glucose, 1·8% galactose, respectively.

2.2.2 Faecal inoculation

Experiments were carried out using fresh faecal samples from three healthy donors (one female aged 26 years and two males aged 25 and 31 years, respectively) who were free of any metabolic and gastrointestinal diseases, were not taking probiotic or prebiotic supplements and had not taken antibiotics for 6 months before faecal sample donation. All donors then provided written informed consent, and filled in a standard questionnaire to collect information regarding health status, drug use, clinical anamnesis and lifestyle factors. This study was approved by The University of Reading Research Ethics Committee (UREC 15/20). Faecal samples were placed in an anaerobic jar (AnaeroJarTM 2.5L; Oxoid Ltd) including a gas- generating kit (AnaeroGenTM; Oxoid).

Samples were diluted 1/10 w/w in anaerobic PBS (0·1 mol/l phosphate buffer solution, pH 7·4) and homogenised (Stomacher 400; Seward) for 2min at 240 paddle beats/min. Samples were added to anaerobic fermenters within 15 min of voiding.

2.2.3 *In vitro* batch culture fermentation

This method was previously described by Rodriguez-Colinas *et al.* (2013). B-GOS were added at concentrations shown in Table 2.1 before adding faecal slurry.

Table 2.1: Doses of the B-GOS syrups tested (equivalent to 0.1, 0.05 and 0.033 g/L) in 100mL working volume vessels during 24 hours fermentation in pH and volume controlled batch fermentation experiments.

Vessel	Treatment	Syrup (g)	DM (g)	GOS (g) in 100mL
1	B-GOS (52% GOS content)	2.66	2	1
2	B-GOS (52% GOS content)	1.33	1	0.5
3	B-GOS (52% GOS content)	0.88	0.66	0.33
4	B-GOS (65% GOS content)	2.05	1.54	1
5	B-GOS (65% GOS content)	1.02	0.77	0.5
6	B-GOS (65% GOS content)	0.68	0.51	0.33
7	Negative control (only faeces)			

DM: dry matter.

An extra vessel with no added carbohydrate source was also included as a negative control. Culture pH was maintained in the range of 6·7–6·9 via automatic controllers (Fermac 260; Electrolab) and adjusted by adding 0·5mM-NaOH and HCl to the vessels when required. The pH and temperature mimicked the conditions of the distal region of the human large intestine. Batch culture fermentations were run for 24 h, and samples (3·5 ml from each vessel) were collected at 0, 4, 8 and 24 h for analysis of bacterial populations and metabolite production. Fermentation experiments were performed in triplicate.

2.2.4 Bacterial enumerations by fluorescence *in situ* hybridisation

Differences in bacterial populations were assessed by fluorescence *in situ* hybridisation (FISH) with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA, as previously described (Costabile 2010). The probes were commercially synthesised and labelled at the 5′ end with the fluorescent dye Cy3 (Sigma-Aldrich) as reported in Table 2.2 (Langendijk 1995; Manz 1996; Harmsen 1999; Franks 1998; Daims 1999; Walker 2005).

Table 2.2: Oligonucleotide probes used in the study for FISH analysis of bacterial populations

Probe name and Sequence (5' to 3')	Target species	Hybridization-Washing Temperature (C)	References
Bif164 CATCCGGCATTACCACCC	Most *Bifidobacterium* spp. and *Parascardovia denticolens*	50-50	Langendijk 1995
Bac303 CCAATGTGGGGGACCTT	Most *Bacteroides sensu stricto* and *Prevotella* spp.; *all Parabacteroides*;*Barnesiella viscericola and Odoribacter splanchnicus*	46-48	Manz 1996
Lab158 GGTATTAGCAYCTGTTTCCA	Most *Lactobacillus, Leuconostoc* and *Weissella* spp.; *Lactococcus lactis*; all *Vagococcus, Enterococcus, Melisococcus, Tetragenococcus, Catellicoccus, Pediococcus* and *Paralactobacillus* spp.	50-50	Harmsen 1999
Chris150 TTATGCGGTATTAATCTYCCTTT	Most members of *Clostridium cluster I*; all members of *Clostridium cluster II;Clostridium tyrobutyricum; Adhaeribacter aquaticus* and *Flexibactercanadensis* (family *Flexibacteriaceae*)*;* [Eubacterium] *combesii* (family *Propionibacteriaceae*)	50-50	Franks 1998
Erec 482 GCTTCTTAGTCARGTACCG	Most members of *Clostridium cluster XIVa; Syntrophococcus sucromutans*, [Bacteroides] *galacturonicus* and [Bacteroides] *xylanolyticus, Lachnospira pectinschiza* and *Clostridium saccharolyticum*	50-50	Franks 1998
EUB338 I GCTGCCTCCCGTAGGAGT	Total bacteria	46-48	Daims 1999
EUB338 II GCAGCCACCCGTAGGTGT	Total bacteria	46-48	Daims 1999
EUB338 III GCTGCCACCCGTAGGTGT	Total bacteria	46-48	Daims 1999
Rrec584 TCAGACTTGCCGYACCGC	*Roseburia - Eubacterium rectale* (a component of cluster XIVa)	50-50	Walker 2005
Prop853 ATTGCGTTAACTCCGGCAC	Clostridium cluster IX	50-50	Walker 2005

Numbers of specific and total bacteria were determined using the following equation: DF×ACC×6732·42×50×DF sample, where DF is the dilution factor (300/375 = 0·8), ACC is the average cell count of fifteen fields of view and DF sample refers to the dilution of sample used with a particular probe or stain. The figure 6732·42 refers to the area of the well divided by the area of the field of view and the factor 50 takes the cell count back to per millilitre of sample.

2.2.5 SCFA analysis

Production of SCFA was determined using GC as previously described (Massot-Cladera 2015). Peaks were integrated using Agilent ChemStation software (Agilent Technologies), and SCFA content was quantified by single-point internal standard method. Peak identity and internal response factors were determined using a 20 mM calibration cocktail including acetic, propionic, iso-butyric, butyric, iso-valeric, valeric, caproic and caprylic acids.

2.2.6 Metabolite analysis by ^{1}H-NMR

Fermentation supernatants from all time points were defrosted, vortexed and centrifuged at 599 xg for 5 min. Supernatants were filtered using 0·22-µm low protein binding durapore polyvinylidene fluoride membrane (Millex; EMD Millipore) and 400µl was transferred into fresh eppendorf tubes. Filtered samples were then combined with 200 µl of phosphate buffer (0·2M (pH 7·4) in D2O plus 0·001% (w/v) trimethylsilyl propionate (TSP)). The mixture was vortexed and centrifuged at 1136 x g for 10 min and then 550 µl was transferred into 5-mm NMR tubes for analysis. All NMR spectra were acquired on Bruker Avance DRX 500MHz NMR Spectrometer (Bruker BioSpin) operating at 500 MHz. They were acquired using a standard 1-dimensional (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 tm 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 parts per million. The FID were multiplied by an exponential function corresponding to 0·3 Hz line broadening. All spectra were manually phased, baseline corrected and calibrated to the chemical shift of TSP (δ 0·00). Spectra were digitised using an in-house MATLAB (version R2014a; The Mathworks Inc.) script. The spectral region containing the water resonance was removed to minimise distortions in the baseline arising from imperfect water saturation. Principal component

analysis (PCA) was performed with Pareto scaling using scripts provided by Korrigan Sciences Ltd.

2.2.7 Statistical analysis

All statistical tests were performed using GraphPad Prism (version 5.0; Graph-Pad Software). A two-way ANOVA test was used to compare dose, substrates and time-dependent effects. When there was no significant effect, one-way ANOVA tests and paired T-tests, including post hoc tests appropriate for the individual data sets (Bonferroni post-test with significance set at $P<0{\cdot}05$), were used for bacterial counts and organic acid concentrations.

2.3 Results

2.3.1 SCFA analysis

Results showed acetate as the dominant SCFA produced for both substrates with significant differences between 4 and 8 h of fermentation using 65 % B-GOS at 0·033 g/l ($P<0{\cdot}01$) (Table 2.3). In particular, there was a clear dose–response effect during the first 4h of fermentation. However, no significant differences were observed between the two substrates at the same dose. The dose–response effect was also confirmed by ^{1}H-NMR data. PCA revealed a clear trajectory over time, showing a clear separation between 0 and 4 h with acetate as main component influencing variation through time (Figure 2.1). Significant decreases in propionate concentration throughout fermentation were observed, mainly between 8 and 24h of fermentation ($P<0{\cdot}01$). None of the substrates generated major changes in butyrate production (Table 2.3). Two-way ANOVA data analyses did not show significant differences on SCFA production between the two substrates.

Table 2.3: SCFAs production measured by Gas Chromatography (GC) in the pH controlled and volume controlled batch cultures at 0, 4, 8 and 24 hour fermentatior Results reported as average (AV) of the data expressed in millimolar (mM) ± standard deviation (SD). Branched SCFAs: Iso-butyric acid and iso-valeric acid.

SUBSTATE	Time [h]	ACETATE [AV (mM) ± SD]	PROPIONATE [AV (mM) ± SD]	BUTYRATE [AV (mM) ± SD]	BRANCHED [AV (mM) ± SD]	TOTAL [AV (mM) ± SD]
	0	26.77 ± 22.13	6.50 ± 7.25	4.63 ± 5.11	15.60 ± 17.26	53.49 ± 51.53
52%B-GOS 1g	4	30.96 ± 18.64	6.43 ± 8.32	4.44 ± 6.92	16.08 ± 11.36	57.92 ± 56.63
	8	28.39 ± 6.91	2.59 ± 0.76	0.95 ± 0.47	3.13 ± 1.42	35.06 ± 6.89
	24	17.14 ± 0.1	1.06± 0.1	0.55± 0.1	0.22± 0.1	18.98± 0.1
	0	17.27 ± 8.37	3.63 ± 3.42	2.57 ± 2.61	8.81 ± 9.28	32.28 ± 23.49
52%B-GOS 0.5g	4	28.33 ± 19.27	4.67 ± 6.09	2.29 ± 3.41	7.71 ± 11.36	43.01 ± 40.13
	8	31.25 ± 15.30	2.11 ± 1.65	0.80 ± 0.51	1.37 ± 0.98	35.52 ± 18.34
	24	20.3 ± 0.1	0.91± 0.1	0.39± 0.1	0.23± 0.1	21.84± 0.1
	0	11.41 ± 8.94	2.26 ± 1.92	1.63 ± 1.52	5.74 ± 5.89	21.04 ± 16.22
52%B-GOS 0.33g	4	17.43 ± 19.16	4.56 ± 6.62	1.75 ± 2. 63	4.62 ± 7.01	28.36 ± 35.33
	8	26.54 ± 26.11	4.47 ± 5.63	1.24 ± 1.57	1.13 ± 0.92	33.37 ± 35.22
	24	26.24± 0.1	0.75± 0.1	0.24± 0.1	0.17± 0.1	27.39± 0.1
	0	18.29 ± 10.59	2.33 ± 2.31	1.32 ± 1.39	4.69 ± 4.87	26.63 ± 19.12
65%B-GOS 1g	4	38.42 ± 2217	4.65 ± 4.45	1.42 ± 1.91	4.47 ± 5.65	48.97 ± 34.11
	8	43.37 ± 28.55	6.81 ± 5.19	1.21 ± 0.99	1.47 ± 1.15	52.82 ± 33.81
	24	6.78± 0.1	0.73± 0.1**	0.4± 0.1	0.39± 0.1	8.31± 0.1
	0	17.94 ± 7.95	2.01 ± 1.94	0.92 ± 0.97	3.42 ± 3.61	24.29 ± 14.33
65%B-GOS 0.5g	4	30.61 ± 20.92	3.97 ± 3.69	1.27 ± 1.59	3.19 ± 3.95	39.03 ± 30.10
	8	24.43 ± 11.06	3.32 ± 2.47	0.72 ± 0.56	1.18 ± 0.81	29.65 ± 13.96
	24	21.51± 0.1	0.61± 0.1**	0.50± 0.1	0.28± 0.1	22.89± 0.1
	0	12.29 ± 5.37	1.33 ± 1.14	0.58 ± 0.6	2.1 ± 2.31	16.3 ± 9.21
65%B-GOS 0.33g	4	24.68 ± 15.65	3.39 ± 3.19	0.84 ± 1.03	2.14 ± 2.65	31.05 ± 22.49
	8	27.18 ± 12.41**	3.98 ± 2.72	0.71 ± 0.48	1.25 ± 1.02	33.12 ± 15.48
	24	20.88± 0.1	1.18± 0.1*	0.97± 0.1	0.77± 0.1	23.79± 0.1
	0	5.28 ± 5.69	0.71 ± 0.51	0.36 ± 0.32	1.15 ± 1.25	7.5 ± 5.34
NEG.CONTROL	4	8.68 ± 5.25	1.68 ± 0.97	0.61 ± 0.47	1.3 ± 1.66	12.31 ± 8.28
	8	18.82 ± 8.74	2.71 ± 1.47	0.67± 0.12	0.97 ± 0.84	23.17 ± 10.64
	24	18.43± 0.1	2.37± 0.1	1.47± 0.1	0.49± 0.1	22.76± 0.1

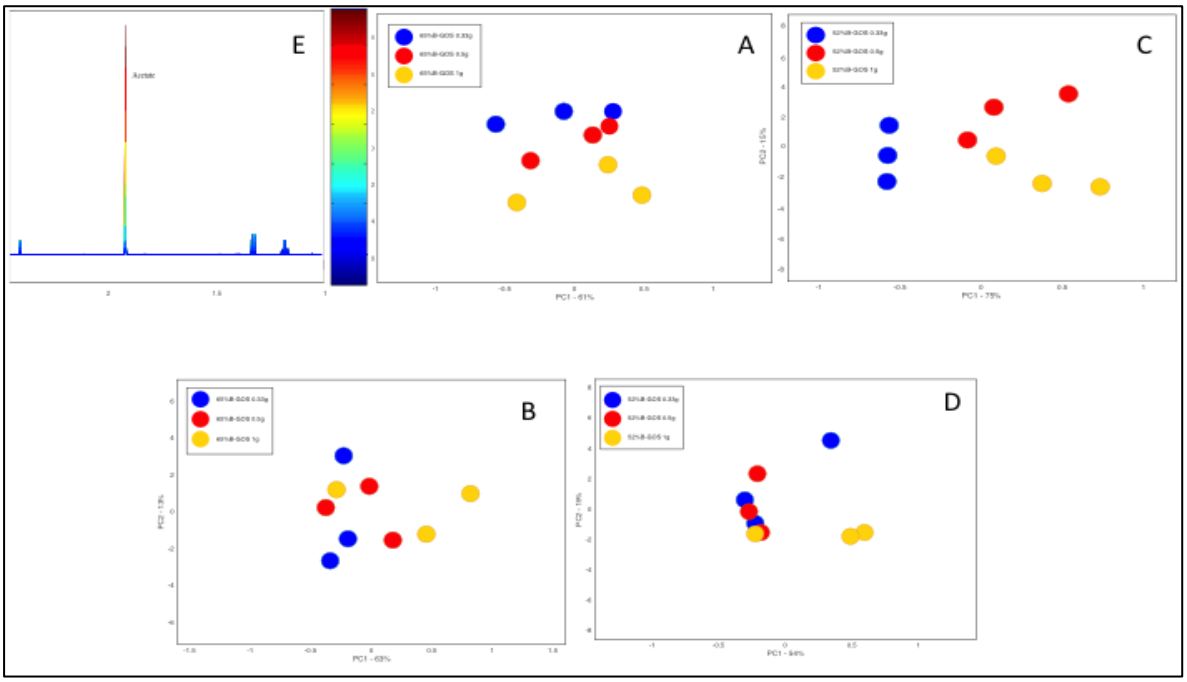

Figure 2.1: ^{1}H-NMR data analysis. (A-C) PCA score plot B-GOS 65% - T4 and PCA score plot B-GOS 52% - T4, respectively, show a clear separation during the first 4 hours fermentation due to dose response effect. (B-D) PCA score plot B-GOS 65% - T8 and PCA score plot B-GOS 52% - T8, respectively, show how dose effect is lost after for 4 h fermentation. (E) Colour plot illustrates the main compound, acetate, influencing the separation. The 65%B-GOS and 52%B-GOS were tested at 1g, 0.5g and 0.33 g equivalent to 0.1g/L, 0.05 g/L and 0.033 g/L.

2.3.2 Changes in bacterial populations

A significant increase in bifidobacteria was observed with 52 %B-GOS 0·5 and 0·33 g (equivalent to 0·05 and 0·033 g/l) at same time 0 and 24 h and with 65 % B-GOS doses at all time points tested ($P < 0·05$; Table 2.4). Lactobacilli significantly increased after addition of 52 % B-GOS and 65 % B-GOS 1 and 0·33 g (equivalent to 0·1 and 0·033 g/l) ($P < 0·01$) and 52 % B-GOS 0·5 g (equivalent to 0·05 g/l) ($P < 0·05$) between 8 and 24 h. An overall decrease in the *Bacteroides–Provotella* group with 52% B-GOS at doses of 1 and 0·33 g (equivalent to 0·1 and 0·033 g/l) was observed between 0 and 24h ($P<0·05$). With 65% B-GOS at 1 and 0·5g (equivalent to 0·1 and 0·05 g/l), there was a significant decrease between 4 and 24 and 0 and 4 h, respectively ($P < 0·05$). Two- way ANOVA data analyses did not show significant differences in microbiota composition between the two substrates.

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Table 2.4: Bacterial groups detected by FISH in the pH controlled and volume controlled batch cultures at 0, 4, 8 and 24 hours fermentation Results are reported as mean of the data of three experiments (n=3) and expressed as Log10 cells/mL ± standard deviation (SD). The 65%B-GOS and 52%B-GOS were tested at 1g, 0.5g and 0.33 g (equivalent to 0.1g/L, 0.05 g/L and 0.033 g/L). [a] Significant difference from 0 h with P<0.05; [b] Significant difference from 4 h with P<0.05; [c] Significant difference from 8 h with P<0.05; In italics type: significant differences with P<0.01; In bold type: significant differences with P<0.001 (T-test).

Probes	Time point (h)	Bacterial population (log10 cells/ml)						
		52%B-GOS 1g	52%B-GOS 0.5g	52%B-GOS 0.33g	65%B-GOS 1g	65%B-GOS 0.5g	65%B-GOS 0.33g	NC
Eub338 I-II-III	0	9.24 ± 0.09	9.13 ± 0.11	9.22± 0.11	9.24 ±0.11	9.27 ±0.12	9.23 ±0.1	9.19 ±0.08
	4	9.11 ± 0.17	9.15 ± 0.18	9.14 ± 0.44	9.39 ±0.14	8.9 ± 0.14	9.35 ±0.07	9.04 ±0.31
	8	9.16 ± 0.13	9.49 ± 0.23	9.05 ± 0.41	9.52 ± 0.1	8.92 ± 0.1	9.46 ±0.04	9.2 ± 0.59
	24	9.14 ± 0.01	9.33 ± 0.03	9.1 ± 0.05	8.94 ±0.07[a,c]	9.21 ±0.01	9.56 ±0.04[a]	8.95 ±0.02[a]
Lab158	0	7.63 ± 0.09	7.48 ± 0.11	7.55 ± 0.05	7.52 ±0.07	7.54 ±0.15	7.47 ±0.06	7.48 ±0.06
	4	7.57 ± 0.05	7.64 ± 0.16	7.77 ± 0.27	7.78 ±0.14	7.73 ±0.12	7.31 ±0.29	7.54 ± 0.2
	8	7.74 ± 0.1	8.05 ± 0.11	7.6 ± 0.36	7.98 ±0.27	8.21 ±0.56	7.66 ±0.12	7.55 ± 0.4
	24	8.39 ±0.02[*a,***b**,*c*]	7.7 ± 0.01[c]	7.94 ± 0.07[*a*]	7.94 ±0.09[*a*]	7.68 ±0.06	7.85 ±0.05[*a*]	7.29±0.08[a]
Bif164	0	8.61 ± 0.28	8.7 ± 0.17	8.58 ± 0.26	8.45 ±0.35	8.63 ±0.21	8.47 ± 0.3	8.68 ±0.21
	4	8.73 ± 0.33	9.1 ± 0.22	8.79 ± 0.67	8.86 ± 0.2[b]	8.75 ±0.19	8.47 ± 0.4	8.45 ±0.31
	8	8.93 ± 0.29	8.87 ± 0.61	8.95 ± 0.63	9.12 ±0.21	9.25 ±0.04[a,b]	9.31 ± 0.1[a,b]	8.53 ±0.13
	24	9.2 ± 0.06	9.31 ± 0.03[a]	9.19 ± 0.08[a]	9.12 ±0.21	9.2 ± 0.07[a,b]	9.24 ±0.04[a]	8.25 ±0.07
Bac303	0	8.26 ± 0.16	8.23 ± 0.35	8.27 ± 0.33	8.33 ±0.25	8.33 ± 0.3	8.2 ± 0.19	8.26 ±0.29
	4	8.07 ± 0.55	8.41 ± 0.53	8.09 ± 0.86	8.49 ±0.15	7.94 ±0.26[a]	8.39 ±0.12	7.75 ±0.83
	8	7.33 ± 064	7.83 ± 0.82	8.11 ± 0.66	8.31 ±0.46	7.88 ±0.32	8.63 ±0.19	6.71 ±0.37[a]
	24	7.78 ± 0.05[a]	8.39 ± 0.15	7.06 ± 0.2[a]	7.94 ±0.03[b]	8.41 ±0.06	8.29 ±0.04	8.39±0.2[a,c]
Erec482	0	8.35 ± 0.16	8.34 ± 0.13	8.42 ± 0.03	8.34 ±0.06	8.38 ±0.09	8.3 ± 0.04	8.32 ±0.05
	4	8.29 ± 0.12	8.39 ± 0.15	8.17 ± 0.31	8.4 ± 0.21	8.36 ± 0.2	8.24 ±0.34	8.08 ±0.43
	8	7.85 ± 1.21	7.92 ± 1.42	7.88 ± 1.22	7.91 ±1.36	8.45 ±0.08	8.38 ±0.38	7.98 ±0.76
	24	8.85 ± 0.37[a,b]	8.65 ± 0.06[a]	7.94 ± 0.04[**a**]	8.58 ±0.01[a]	8.59 ±0.01[a]	8.56 ±0.04[*a*]	8.06±0.05[*a*]
Rrec584	0	8.34 ± 0.25	8.24 ± 0.08	8.06 ± 0.54	8.15 ± 0.3	8.17 ±0.17	8.28 ±0.09	8.34 ±0.04
	4	7.54 ± 1.04	8.13 ± 0.3	7.85 ± 0.59	8.2 ± 0.41	7.29 ±0.86	7.77 ±0.62	7.78 ±0.69
	8	7.06 ± 0.87	7.47 ± 0.74	7.37 ± 0.78[a]	7.71 ±0.98	7.21 ±0.78	7.6 ± 0. 56	7.66±0.21[a]
	24	7.2 ± 0.02	7.14 ± 0.05[**a**,b]	6.92 ± 0.01	7.78 ±0.14	7.23 ±0.06[*a*]	8.51 ±0.03[a]	7.54 ± 0.2[**a**]
Chis150	0	5.83 ± 0.2	5.73 ± 1.1	5,83 ± 0.2	5,73 ± 1.1	5.93 ± 0.3	5.73 ± 1.1	5.73 ± 1.1
	4	5.86 ±0.04	6.34 ± 0.5	5.79 ± 0.1	6.56 ± 0.6	6.11± 0.2	6.2 ± 0.3	5.9 ± 0.1
	8	6.03 ± 0.1	6.74 ± 1	6.53 ± 1	6.80 ± 1.1	6.92 ± 1.3	7.24 ± 0.8	5.87 ± 0.2
	24	5.73 ± 0.02	5.73 ± 0.05	7.45 ± 0.01	5.73 ± 0.2	6.43 ± 0.03	5.73 ± 0.01	5.73 ±0.01
Prop853	0	7.98 ± 0.18	7.97 ± 0.27	8.05 ± 0.22	8.18 ±0.26	8.16 ±0.22	8.08 ±0.22	8.04 ±0.06
	4	8.04 ± 0.28	7.97 ± 0.02	7.81 ± 0.47	8.02 ±0.09	8.02 ±0.05	8.11 ±0.09	7.99 ±0.19
	8	7.84 ± 0.37	7.74 ± 0.48	7.83 ± 0.37	7.85 ±0.68[*a*]	7.67 ±1.03[*a*]	8.16 ± 0.3[a]	7.76±0.08[a]
	24	8.19 ± 0.01[b,c]	7.99 ± 0.01	7.95 ± 0.04[*a,***b**]	6.53 ± 0.02[**b**]	6.15 ± 0.06[**b**]	8.72 ± 0.01[*b,* c]	8.1 ±0.07[c]

2.4 Discussion

The present study was carried out in order to evaluate the *in vitro* fermentation properties of B-GOS (65 % GOS content) in pH- and volume-controlled batch culture fermentation. The fermentability and selectivity of GOS have been previously evaluated *in vitro* by several comparative studies. Rycroft *et al*. (2001) compared the efficacy of different prebiotics including FOS and GOS in 24-h batch culture experiments, and the results showed how GOS induced the largest significant increases in bifidobacteria, lactobacilli and total bacterial numbers during fermentation (Rycroft 2001). In our study, administration of B-GOS showed the same trend, especially considering bifidobacteria and lactobacilli populations. There was also a significant decrease in *Bacteroidetes* numbers, except for 52 % B-GOS at 0·05 g/l and 65 % B-GOS at 0·033 g/l. The 65 % B-GOS also had a strong influence in the production of SCFA, compared with B-GOS that is commercially available (52% GOS content). Our results showed a double increase in acetate production at all doses using 65 % B-GOS, but were not significant, except for 65 % B-GOS at 0·033 g/l, probably due to the high standard deviation.

The fermentation of all different doses induced the production of acetate, which correlated with an increase in *Bifidobacterium* populations (Macfarlane 2008). Palframan *et al.* (2002) in a study comparing the effect of the pH and dose on batch culture fermentation of five commercial prebiotics have shown similar results. FISH analysis showed how highest bacterial numbers were obtained with GOS at pH 6 and 1% (w/v) (Palframan 2002). The global effect on the bacterial population of 52% B-GOS has been tested in a previous fermentation study where the commercial B-GOS mixture was compared with different purified GOS. Using Selectivity Index (SI) as an estimate for the growth of beneficial bacteria, Rodriguez-Colinas *et al.* (2013) have shown that 52 % B-GOS had the highest SI, and consequently a strong degree of selectivity for *Bifidobacterium* population.

Our results highlighted that 65 % GOS had an effective prebiotic activity, in terms of increasing numbers of bifidobacteria and metabolite production. This was especially seen for acetate, probably due to the lower content of monosaccharides and disaccharides in the mixture that might have affected *in vitro* fermentation experiments overall. However, a previous study of Costabile *et al.* (2014) demonstrated that the carbohydrates that remained following *in vitro* pre-digestion processes did not have any selective properties to invoke a bifidogenic effect, which perhaps would not persist *in vivo* (Costabile 2015).

In our study, major changes in other bacterial populations were seen, which might be due to the presence of these sugars. Different concentrations of SCFA have been identified at time 0, but it may be explained by the high inter-individual variability among each individual. Significant differences were observed between the time points in all analyses but not between the two substrates.

The effects of B-GOS 52 % as a potential modulator of the gut microflora and the immune system have been extensively investigated in several human intervention studies. The 65% B-GOS has shown a significant modulation of health-promoting beneficial bacteria, and our findings proved that reducing impurities in the prebiotic mixture might improve the selectivity of prebiotics in *in vitro* experiments. However, the comparison between the effect of 65 % B-GOS and 52 % B-GOS has shown similar bifidogenic effects (data not published). The applicability of such changes remains to be investigated in *in vivo* human intervention studies.

Author's contribution

RG carried out the experiments and drafted the manuscript. 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 assisted in designing the study and is employed by Clasado Biosciences Ltd, who provided the B-GOS product, marketed as Bimuno®, used within this research.

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CHAPTER 3

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

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Published in ***FEMS Microbial Ecology 2017, 93:1-10***

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* hybridisation and metabolic activity by HPLC and ^{1}H-NMR. Consistent with previous studies, the microbiota of ASD children contained a higher number of *Clostridium* spp. and a lower number of bifidobacteria compared to 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 bacteria were seen in particular for *Clostridium*, *Roseburia*, *Bacteroides*, *Atopobium*, *F. 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 inocula.

3.1 Introduction

Autism typically develops in childhood, and is considered as "a systemic spectrum disorder with multiple development trajectories with an incidence four times higher in males than in females" (Grossi 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 2014). A cross talk 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 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 ASD children. Finegold *et al.* found nine unique species of clostridia in autistic children compared to controls (Finegold 2002). Song *et al*., using qPCR analysis, found higher levels of *C. boltea* and *Clostridium* cluster I and XI (Song 2004). Furthermore, Parracho and co-authors, using FISH analysis, found greater number of species derived from the *C. histolyticum* group (*Clostridium* clusters I and II) (Parracho 2005). *Desulfovibrio* group was found to be ten times higher in the gut microbiota of autistic children compared to controls (Finegold 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 2013) with high genus *Sutterella* spp. (Wang 2013; William 2012). In addition, *Bifidobacterium* species decreased in ASD, comparing to non-autistic control (De Angelis 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 derivates in the blood and higher levels of IAG (indolyl-acryuloyl-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 microorganisms can metabolise tryptophan (Bingham 2003).

Metabonomic studies also identified alterations in nicotinic acid metabolism (Yap 2010) and amino acid deficiencies in autism with restricted diets, modified gut microbial population and GI symptoms being suggested as potential contributors (Ming 2012).

Modulation of the gut microbiota is an interesting potential strategy to reduce the presence of harmful microorganisms and their metabolites that might be involved in negative stimulation of CNS and affect behaviour (Shaw 1995; Sandler 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 2005; Depeint 2008; Vulevic 2008; Silk 2009; Drakoularakou 2009; Vulevic 2013). Recently, B-GOS was also shown to reduce cortisol secretion and anxiety in healthy volunteers (Schmidt 2015). Cortisol is a reliable marker of stress and hypothalamic pituitary adrenal (HPA) axis activity. B-GOS supplementation lowered cortisol reactivity and modulated attention to emotional stimuli compared to a placebo group, supporting the hypothesis that the gut microbiota might have a role in behavioural traits (Schmidt 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, that simulated different physicochemical characteristics of the proximal, transverse and distal colons.

3.2 Materials and methods

3.2.1 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, 1.8% (w/v) galactose.

3.2.2 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 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 (AnaeroJarTM 2.5 L, Oxoid Ltd) including a gas-generating kit (AnaeroGenTM, Oxoid). Aliquots of 20 g of samples were 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 minutes at 240 paddle beats per minute. Samples were added to anaerobic fermenters within 15 minutes of voiding.

3.2.3 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 *et al*. (1998) was used in this study, with vessels (V) representing the proximal (V1, 80ml, pH=5.5), transverse (V2, 100ml, pH=6.2), and distal colon (V3, 120ml, pH=6.8). The systems were inoculated with 20% (w/v) faecal homogenate from either non-autistic and autistic children volunteers in a growth medium (Macfarlane 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 (T0), 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). SCFA profiles (+/-5%) were assessed before starting B-GOS administration. Taking into account the operating volume (300 mL) and retention time (48 h, flow rate 6.25 mL/h) of the colonic model system, a syrup containing GOS (2g/daily, equivalent to 1g 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.

3.2.4 Short chain fatty acids (SCFAs) analysis by HPLC

The production of SCFAs in the fermentations was determined by HPLC (Merck, NJ) as previously described by Rodriguez-Colina *et al.* 2013. Twenty µL 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 of 12.5, 25, 50, 75 and 100 mM.

3.2.5 *In vitro* enumeration of bacterial population by FISH-FCM

Bacterial composition in the gut models was analysed for using fluorescence *in situ* hybridisation combined with flow cytometry (FISH-FCM). 750 µl of samples were centrifuged at 1136 x g for 5 min. Pellets were re-suspended in 375 µL of filtered PBS (using a 0.22 µm PVDF membrane) and fixed in 1125 µL of 4% (v/v) paraformaldehyde. After 4 hours incubation at 4 °C, samples were washed twice using 1 mL of PBS, re-suspended in 600 µL PBS-ethanol (1:1, v/v) and stored at -20 °C. Permeabilisation steps were performed using 30 µL of the fixed samples added to 500 µL PBS and centrifuged at 1136 x g for 3 min. Pellets were re-suspended using 100 µL of filtered TE-FISH (Tris/HCl 1 M pH 8, EDTA 0.5 M pH 8, distilled H_2O, 0.22 µm 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 x g for 3 min. Pellets were washed with 500 µL PBS and centrifuged (1136 x g, 3 min). Hybridisations were performed by re-suspending the pellets in 150 µL of hybridisation buffer (5M NaCl, 1M Tris/HCl pH8, 30% formamide, ddH_2O, 10% SDS), vortexed and centrifuged (1136 x g, 3 min). Pellets were then re-suspended in 1 mL of hybridisation buffer and 50 µL aliquoted into Eppendorf tubes. The probes used (Sigma Aldrich Ltd., Poole, Dorset, UK) are reported in Table 3.1 (Wallner 1993; Daims 1999; Langendijk 1995; Harmsen 1999; Manz 1996; Franks 1998; Walker 2005; Harmsen 2000; Hold 2003; Devereux 1992; Poulsen 1995; Harmsen 2002; Lay 2005; Stoffels 1998; Kong 2012). NON EUB338 and EUB338 I-II-III linked at their 5' end either to Alexa488 and Alexa647. Group specific probes were linked with Alexa647 at their 5' end. 4µL of each probe and 4 µL of Eub338 I-II-III (linked to Alexa488) were added to the working solution and incubated overnight at 35°C in a heating block. After 12 hours incubation, an aliquot of 150 µL hybridisation buffer was added to the

working solution, vortexed and centrifuged (1136 x *g*, 3 min). 150 µL of supernatant was removed from each sample and the remaining volume centrifuged (1136 x *g*, 3 min). The pellets were washed with 200 µL of washing buffer (5M NaCl, 1M Tris/HCl pH8, 0.5 M EDTA pH8, ddH_2O, 10% SDS), homogenised by vortexing and incubated for 20 min at 37 °C in a heating block. Afterwards the samples were centrifuged (1136 x *g*, 3 min) and supernatants removed. Negative control samples (no probes added) were screened by flow cytometry to detect background before the probe samples were re-suspended 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 (DF), calculate from different volumes used in samples preparation steps, and events/µl obtained from NON EUB338 and EUB338 I-II-III probes analysed by flow cytomotry.

Table 3.1. Oligonucleotide probes used in this study for FISH-FCM analysis of bacterial populations. +: These probes are used together in equimolar concetration of 50 ng/µl.

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

3.2.6 Metabolic analysis by ^{1}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 ^{1}H-NMR (n=27, each group). Fermentation supernatants were defrosted, vortexed and centrifuged at 599 x *g* for 5 minutes. The supernatants were filtered using 0.22µm low protein binding Durapore polyvianylidene 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 D2O plus 0.001% TSP), mixed by vortexing, centrifuged at 1136 x *g* for 10 minutes 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 one-dimensional (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 Tm 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.

3.2.7 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,-$^{2}H_4$]-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 normalisation 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 were 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^2) for each metabolite to class membership. Predictive strength (Q^2Y) of the models was obtained using a seven-fold cross validation method and these were validated using permutation testing (number of permutations=10,000).

3.2.8 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).

3.3 Results

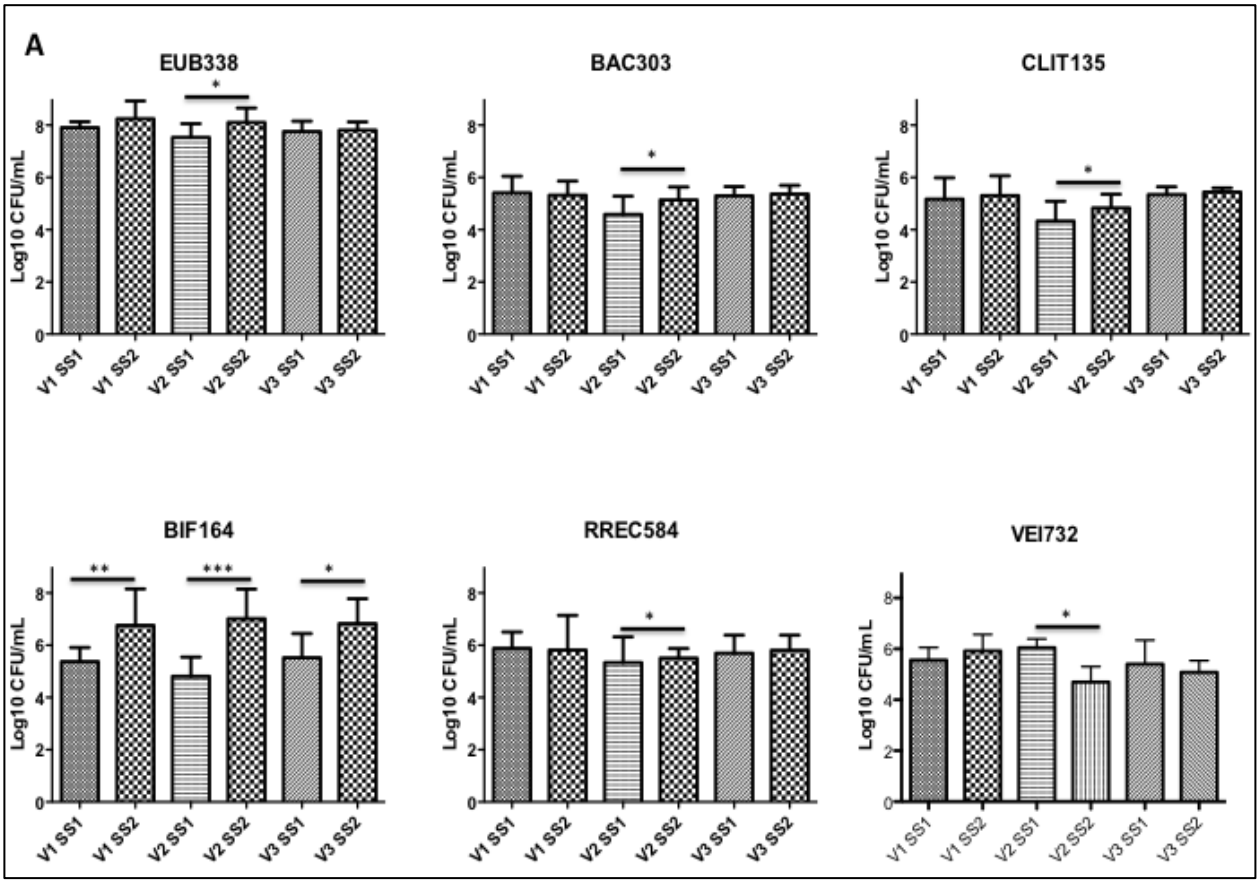
3.3.1 Bacterial enumeration

Changes in bacterial compositions in gut model systems are reported in Figure 3.1. The data showed lower numbers of bifidobacteria in ASD models compared to non-autistic. Significant increases in *Bifidobacterium* spp, following 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 $\log_{10}$ cells/ml ($P<0.01$), from 4.81 to 6.79 $\log_{10}$ CFU/ml ($P<0.001$) and from 5.57 to 6.83 log $\log_{10}$ 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 $\log_{10}$ 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 $\log_{10}$ cells/ml ($P<0.01$), and in V3, from 4.97 to 6.73 $\log_{10}$ cells/ml ($P<0.001$) and in lactic acid bacteria (Lab158) in V3 from 5.13 to 6.01 $\log_{10}$ cells/ml ($P<0.05$). Additionally, B-GOS slightly increased *Roseburia* spp. in V1 and 3 ($P<0.05$) and reduced *Atopobium* spp. from 6.06 to 5.28 $\log_{10}$ cells/ml and *F. prausnitzii* from 6.78 to 5.27 $\log_{10}$ cells/ml ($P<0.05$ for both) in the second vessel, while increasing *Atopobium* spp. from 5 to 5.92 $\log_{10}$ 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 $\log_{10}$ 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 $\log_{10}$ 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), *E. 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.

3.3.2 Short chain fatty acids production

SCFAs concentrations are presented in Figure 3.2. Data show a lower concentration of butyrate and propionate in autistic models, compared to non-autistic models, but no differences in acetate before adding B-GOS into the system. After 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 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, 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 colons respectively. There was no effect on propionate.


A
EUB338
BAC303
CLIT135
BIF164
RREC584
VEI732
Log10 CFU/mL
V1 SS1
V1 SS2
V2 SS1
V2 SS2
V3 SS1
V3 SS2

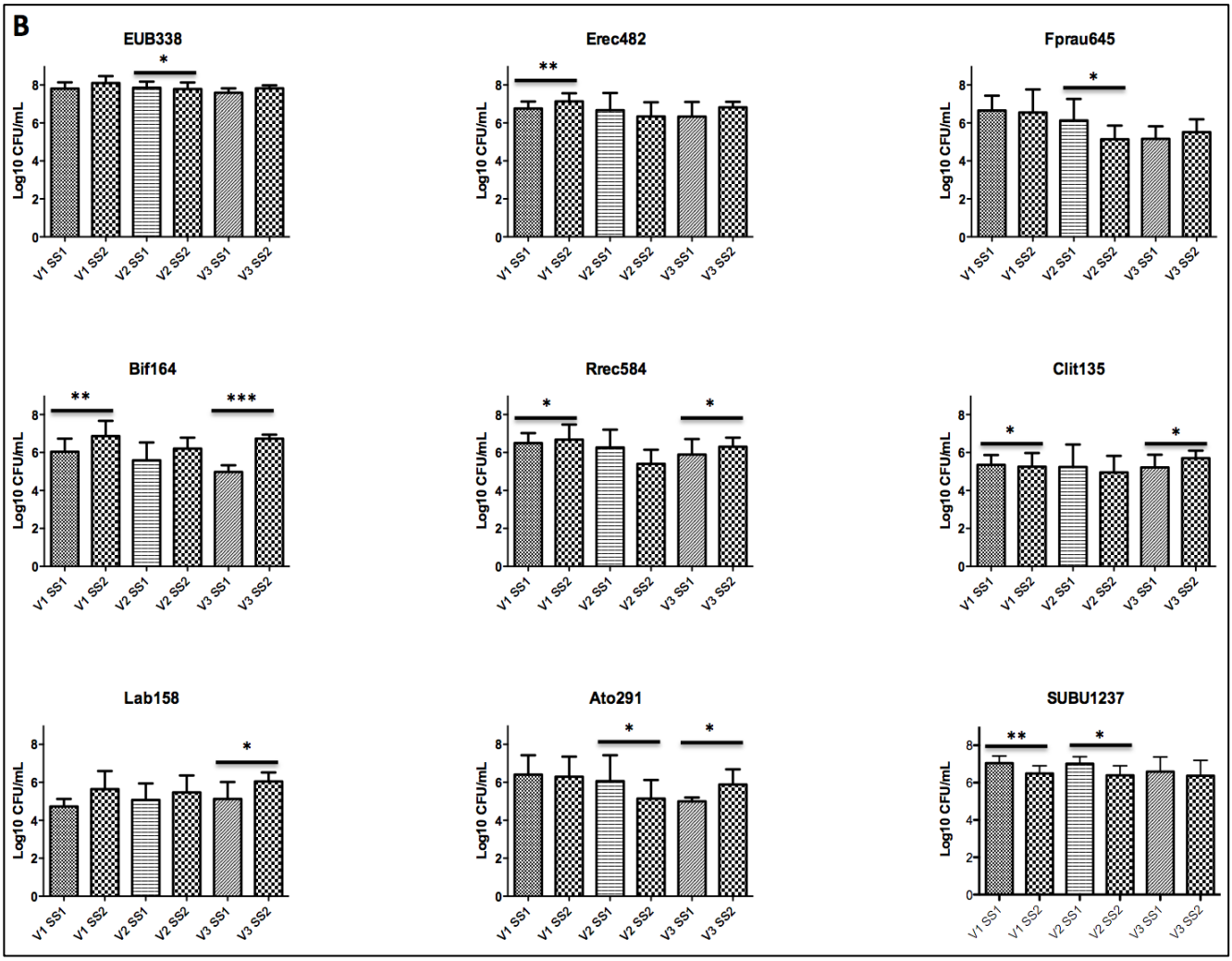


Figure 3.1: Bacterial groups detected by FISH-FCM (Log10 CFU/ml) in culture broth recovered from each vessel (V1, V2 and V3) of a colonic model before (SS1) and after (SS2) the daily administration of B-GOS (2g/d, equivalent to 1g GOS). Significant difference after the treatment: * P<0.05; **P<0.01; ***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* sub cluster (Rrec584), *F. prausnitzii* (Fprau655), *Atopobium* spp. (Ato291), *Clostridium* cluster XI (Clit135), *Sutterella* spp. (SUBU1237), Veillonellaceae (VEI732). (A) autistic children; (B) non-autistic children.

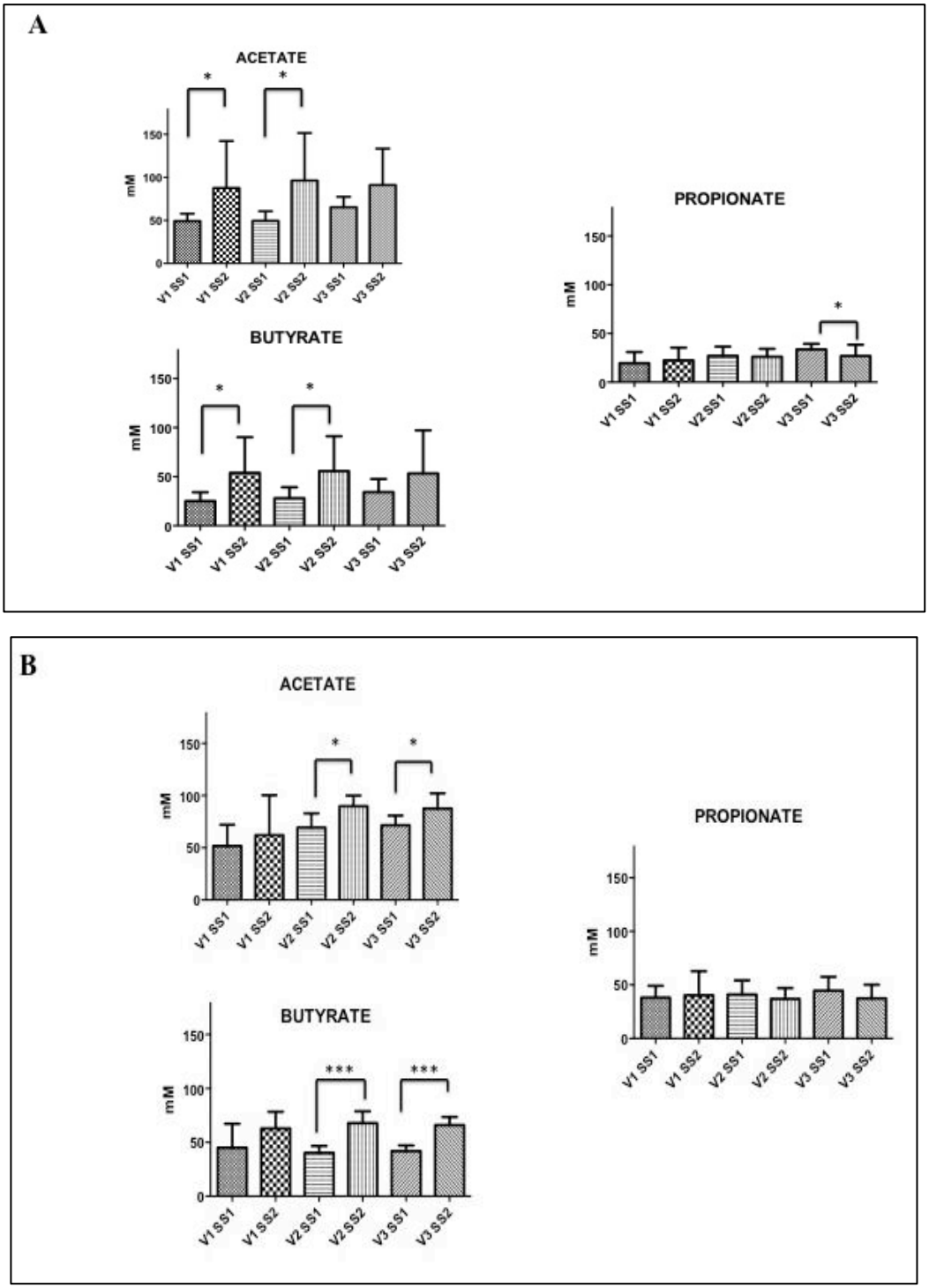


Figure 3.2: HPLC analysis. Acetate, propionate and butyrate concentrations in culture broths recovered from vessels (V1, V2 and V3) of *in vitro* gut model systems before (SS1) and after (SS2) administration of B-GOS (1g/daily GOS). Results are reported as means (mM) of the data (n=3): A) autistic children and B) non-autistic children. Significant difference after the treatment: * P<0.05; ***P<0.001.

3.3.3 ^{1}H-NMR Spectroscopic Profiles

PCA analysis was performed on mean-centred data to summarise variance with the dataset. The scores plot (PC1 versus PC2) shown in Figure 3.3A, 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 analysis are summarised in Figure 3.3B. A significant OPLS-DA model was obtained comparing metabolic profiles of autistic and non-autistic models at baseline ($Q^2Y = 0.07$; $P < 0.05$; Figure 3.4C-I). Supernatants from the autistic models contained greater amounts of ethanol, glycine, tyrosine, tyramine, 5-aminopentoanate, acetate, 4-aminobutyrate and betaine, compared to the non-autistic models and lower amounts of butyrate. B-GOS supplementation was found to modulate metabolic profile of the autistic models ($Q^2Y = 0.08$; $P < 0.05$) increasing ethanol, lactate, acetate and butyrate and decreasing propionate and trimethylamine (Figure 3.4B-I). Increased butyrate and acetate production was also observed in the non-autistic models following the addition of B-GOS ($Q^2Y = 0.12$; $P < 0.01$; Figure 3.4B-II). Comparing metabolic profiles of the autistic and non-autistic models after B-GOS feeding ($Q^2Y = 0.17$; $P < 0.01$) revealed that metabolic variation was reduced (Figure 3.4C-II). 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.

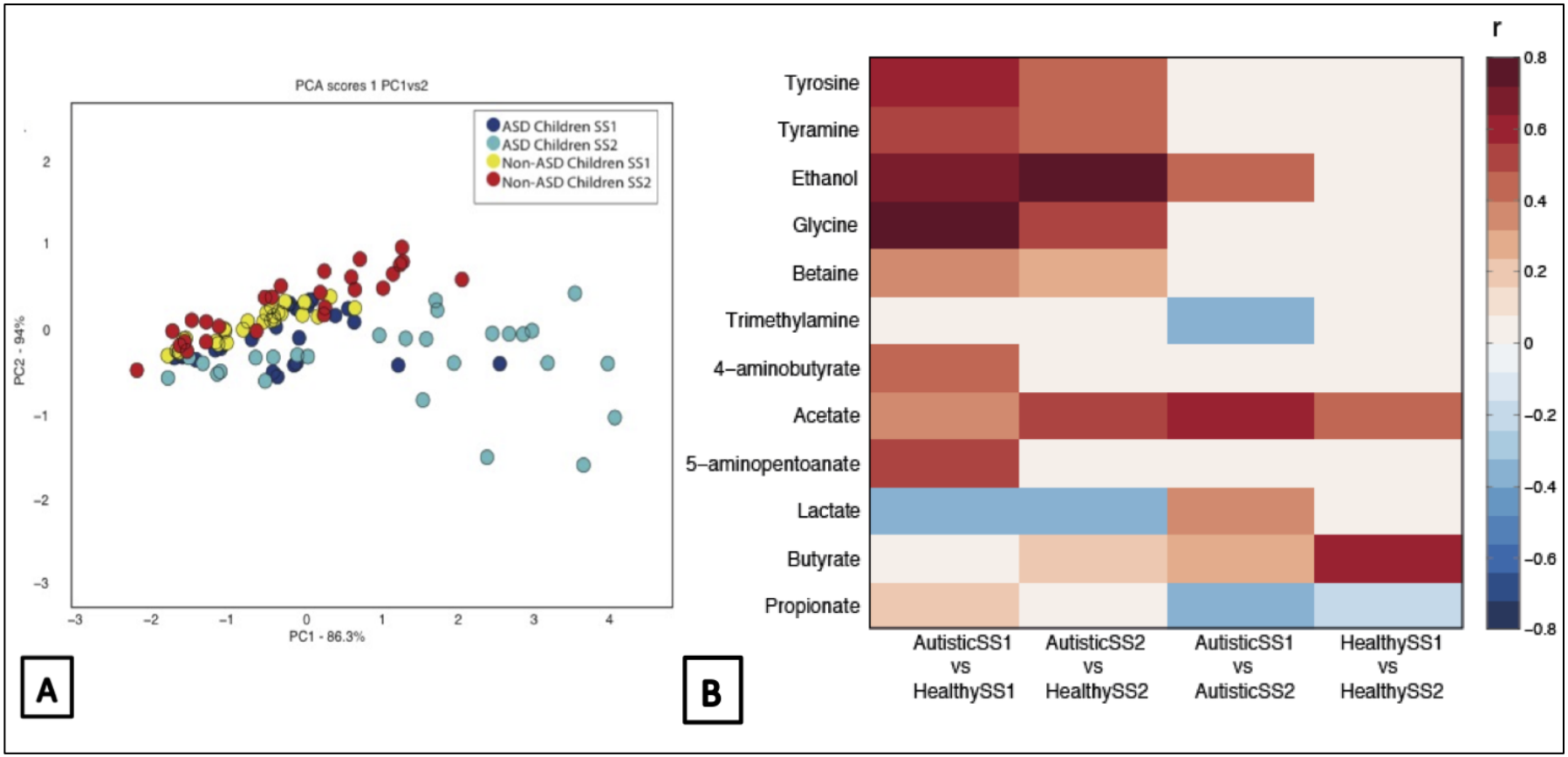


Figure 3.3: ^{1}H-NMR data analysis. (A) PCA score plot show a separation between models inoculated with stool samples of non-ASD and ASD children after administration of B-GOS. Dark and light blue dots represent replicates of samples from gut models inoculated with faecal samples of autistic children, 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.

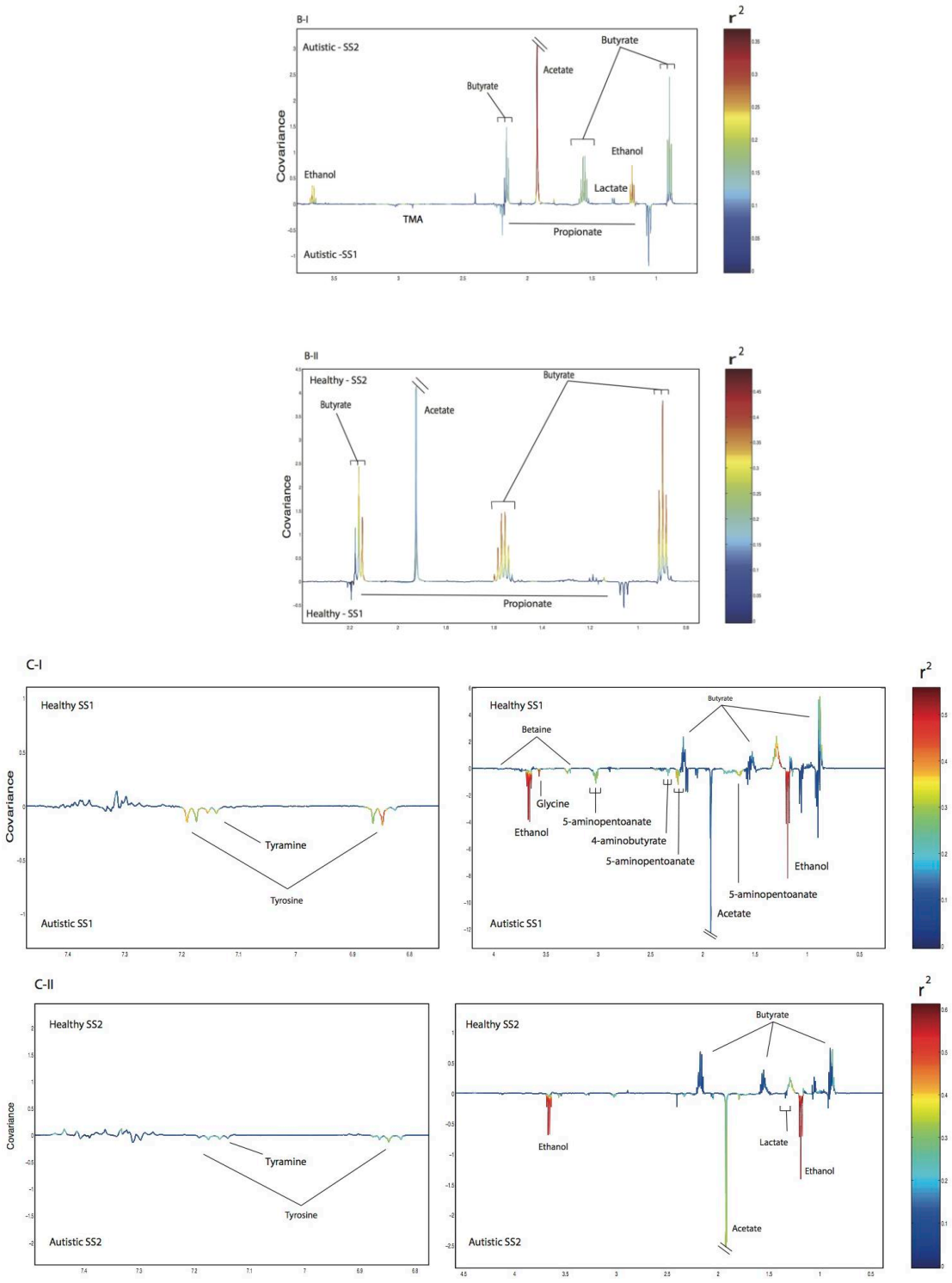


Figure 3.4: (B-C) OPLS-DA model constructed from NMR spectra of the culture broth recovered from each vessel (V1, V2 and V3) of the colonic model discriminating between before (SS1) and after (SS2) the daily administration of B-GOS (1g/daily GOS) in autistic and non-autistic model models. B shows the comparison between before and after treatment in autistic (I) and non-autistic children (II). C shows the comparison between the combined data of autistic and non-autistic models before (I) and after (II) B-GOS supplementation. Compounds identified: Acetate, butyrate, propionate, lactate, ethanol, glycine, betaine, 5-aminopentoanate, 4-aminobutyrate, tyrosine, tyramine, trimethylamine (TMA).

3.4 Discussion

Recent studies have focused on influences of pre/probiotics on the gut-brain axis (Liu 2015). This study investigated 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 flow cytometry. We also assessed metabolic profiles and key metabolites in both test groups.

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

Recent studies have focused on SCFAs and their effect on the CNS. These fermentation products can cross the blood-brain barrier and might influence early brain development. 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 2011). Experimental evidence using intraventricular infusion in rats indicates that propionic acid can produce brain and behavioural changes similar to ASD (MacFabe 2008).

Recent ASD studies have shown increase in numbers of *Sutterella* spp. and decrease in Veillonellaceae group. In this study, 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 ASD-associated bacteria.

Following B-GOS feeding, the microbiota of autistic children produced greater amounts 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 (e.g. by *Desulfovibrio* spp.) (Flint 2014; Marquet 2009; Fite 2004). In ASD children, presence of lactate is interesting because its accumulation has been associated with neurological problems, in

particular studies show the effect of lactate infusion on anxiety and panic disorders (Dillon 1987; Cowley 1987). Cowley and colleagues, in their findings, showed that lactate infusion in patients suffering from panic disorder, provoked higher panic symptom reactions compared to controls (Dillon 1987). Dillon *et al.* have showed similar results in *in vivo*, where panic and anxiety reaction has been measured using Acute Panic Inventory (API) scores. After lactate infusions scores were much higher in patients with panic and anxiety disorders compared to normal controls (Cowley 1987).

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

Ethanol was found in higher amount in ASD children comparing to non-ASD. The vast majority of bacteria form ethanol from acetyl-CoA and the glycolytic pathway (Macfarlane 2003). Microorganisms are able to oxidase ethanol and the impact of bacterial overgrowth on ethanol production has previously been studied (Baraona 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 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 (ADH) 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.

3.5 Conclusions

This *in vitro* study showed promising and positive results in that supplementing the microbiota of ASD children with 65%B-GOS may manipulate gut bacterial populations 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.

Author's contribution

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®, used within this research.

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CHAPTER 4

Impact of exclusion diet on gut microbiota composition and metabolite production in autistic children

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Abstract

Evidence suggests that diet has a strong impact on the gut microbiota and its metabolic activity. Gluten- and casein-free diets are the most common dietary approaches in autism but, to date, it is not clear how and whether they can actually modulate autistic features, such as behavioural traits and/or GI problems. In this study, we recruited 30 autistic children, with formal diagnosis of ASD, who were following different diets: gluten-, casein- and dairy-free diet or un-restricted diet. We assessed the effect of these dietary habits on gut microbiota composition and metabolic profiles. In addition, we asked parents to fill in GI symptom diaries and 4-days food diaries in order to investigate nutrient intake and potential differences in GI issues. Results showed ASD children under gluten and casein-free diet to have significant reduction in abdominal pain and bowel movement and higher fibre intake, compared to the un-restricted dietary group. In addition, the exclusion diet group had reduced abundance of *Bifidobacterium* spp. and Veillonellaceae family, and increased *F. prausnitzii* and *Bacteroides* spp. In both groups, no significant differences were found in the urine metabolome, but significant correlations were found between bacterial populations and amino acids in faeces. Taken together, our findings indicate that exclusion diets impact gut microbiota composition and GI symptoms in ASD children but not enough to support their validity as treatment.

4.1 Introduction

Autism is a neurodevelopmental disorder characterized by impaired social interaction, verbal and non-verbal communication, and repetitive behaviour. In addition to cognitive aspects, ASD individuals might suffer from gastrointestinal (GI) problems (Kral *et al.* 2013).

Dietary interventions, including use of supplements, or exclusion diets have been suggested in ASD in order to reduce these issues. Gluten and casein-free (GFCF) diets are common within autistic individuals even though there is a lack of strong empirical evidence. Alterations in intestinal function have been often attributed to children who are on the autism spectrum (White 2003). It has been reported that autistic individuals might have increased gut permeability that could lead the passage of molecules, such as peptides from metabolism of gluten and/or casein, that could interact with central nervous system (CNS) and cause typical autistic traits (De Magistris *et al.* 2010). Observational studies reported how GFCF diets seem to alleviate GI problems and/or improve behavioural traits, according to parents/guardians reports, but association between the restricted diet and symptoms was not always apparent (Harris & Card 2012; Patel & Curtis 2007; Pennesi & Klein 2012). Some confounding outcomes have been seen in intervention studies, where only mood and behavioural aspects were evaluated. Some showed significant improvements in behavioural traits (Nazni 2008; Whiteley *et al.* 2010), while others reported no differences after treatment (Elder *et al.* 2006; Seung 2007; Johnson 2011). More recent randomised trials, showed that ASD individuals tolerated gluten and casein introduction in their diet but limitations in these studies, such as participant number and/or short treatment period, lead to careful consideration of these findings (Pusponegoro *et al.* 2015; Hyman 2016).

It is well known that the diet influences bacterial community and its role in health and well being of the host (Salonen & De Vos 2014). Differences in food intake have been reported to modify the gut microbiota since early life, depending on breast or formula feeding (Liu *et al.* 2016), and later on in life according to food intake, lifestyle (De Filippis *et al.* 2014), allergies and diseases (Girbovan 2017).

To date, little is understood about the relationship between the exclusion diets and the gut microbiome in the context of autism. Many of these diets focus on carbohydrate, because ASD individuals appear to be defective in intestinal digestive enzymes responsible for carbohydrate digestion (Williams *et al.* 2011), but evidence is still relatively weak and sometimes inconsistent, as in the case with GFCF diets. Further

studies on modified diets and food supplementation are needed not only to assess the efficacy of these diets, but also their impact on the gut microbiome and how its manipulation might influence autistic traits.

Therefore the aim of this study was to investigate the impact of gluten, casein and dairy-free diets on gut bacterial composition and metabolite production in autistic children who were following these restricted diets and in comparison to those on un-restricted diet.

4.2 Materials and Methods

4.2.1 Subjects

Thirty volunteers (8 female and 22 male; mean age 7.7 years-old; range 4-11 years) were included in the study. All participants had a formal diagnosis of ASD (Table 4.1) and previous history of antibiotic treatment in early life. None of the volunteers had been treated with antibiotics, prebiotics or probiotics within 4 weeks before sample collection. The study was approved by the University of Reading Research Ethics Committee (UREC15/41; Registration number: NCT02720900) prior to the start of the study and informed consent was obtained from the volunteers' parents.

Table 4.1: Diagnosis reported from parents by medical assessment (*). ASD: Autism Spectrum Disorders; ADHD: Attention Deficit Hyperactivity Disorder; PDD: Pervasive Development Disorder

Diagnosis*	**ASD**	**ADHD**	**Asperger**	**PDD**
Volunteers (n)	30/30	5/30	2/30	1/30

4.2.2 Dietary intervention and assessments

Food diary records were kept for 4 consecutive days (Appendix 4.1) and subjects were divided in 2 groups according to the diet they were following: 12 children were on an exclusion diet (gluten, casein and dairy free diet) and 18 children were on an un-restricted diet. At the front of the diary, detailed information on how to record food and beverages consumed using common household measures were provided. Food diaries were analysed using Diet-plan7 software (Forestfield Software Ltd.)

4.2.3 GI symptoms

Parents/guardians of child volunteers were asked to fill in daily questionnaires for GI function and symptoms (Appendix 4.2). The Bristol stool chart was used to assess faecal samples' type and consistency, together with the number of bowel movements,

abdominal pain, intestinal bloating and flatulence (none, present but well tolerated, present and interfering with but not preventing normal daily activities, preventing normal daily activities; Lewis & Heaton 1997). Concomitant medication, adverse events, changes in diet and behaviour were also recorded throughout the study, on a separate sample submission forms (Appendix 4.3).

4.2.4 Faecal sample collection and preparation

Faecal samples were collected once a week for 3 weeks using faecal collection kit (FC2040, Laboratories Ltd, UK), and volunteers were asked to keep them at -20°C until the visit day, when they were transferred to the laboratory and processed. Samples for DNA extraction and ^{1}H-NMR were weighed (~ 250 mg) and stored at -80°C until needed for analysis.

4.2.5 Urine sample collection and preparation

Urine samples were collected using sterile tubes (Mid-Stream Urine Specimen Collector; Pennine Healthcare, UK) or sterile pads (Sterisets Urine Collection Kit; MediBargains, UK) and stored at -20°C until a visit day when they were processed. Samples were transferred to 15 ml falcon tubes, centrifuged for 10 min at 1136 x g, supernatant transferred to 1.5ml Eppendorf tube (duplicate) and stored at -80°C.

4.2.6 DNA extraction and PCR

Total microbial DNA was extracted from faeces using the DNA stool mini kit (Qiagen, UK) by introducing three 1-min steps at 50 movements/s using TyssueLyser LT (Qiagen, UK) with 5-min incubation in ice between treatments as previously described by Candela *et al.* 2016. Briefly thermal disruption of the samples was performed by incubation at 95°C for 15 min, then centrifuged for 5 min at 4°C to pellet stool particles. 260 µl of 10 M ammonium acetate was added to the supernatant, incubated in ice for 5 min and centrifuged at full speed for 10 min at 4°C. Supernatants were collected and one volume of isopropanol added and samples incubated in ice for 30 min. The samples were centrifuged for 15 min at 4°C and the pellet washed with 70% (v/v) ethanol. They were re-suspended in 100 µl of TE buffer (10 mM Tris-Cl, pH 7.5; 1 mM EDTA, pH 8.0) and treated with 2 µl of DNase-free RNase (10 mg/ml, Sigma-Aldrich) at 37°C for 15 min. Proteins were removed by adding 15 µl of proteinase K (DNA stool mini kit; Qiagen, UK) and DNA was subsequently purified following the manufacturer's instructions.

DNA recovery was evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

4.2.7 16S rRNA gene amplification via next generation sequencing (NGS) and bioinformatics analysis

For each sample, the V3–V4 region of the 16S rRNA gene was PCR-amplified in 25 µl volumes containing 12.5 ng of microbial DNA, 2× KAPA HiFi HotStart ReadyMix (Kapa Biosystems, USA) and 200 nmol/l of S-D-Bact-0341-b-192 S-17/ S-D-Bact-0785-a-A-21 primers carrying Illumina overhang adapter sequences (Bio-Fab Research). Thermal cycling consisted of an initial denaturation at 95°C for 3 min, 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and a final extension step at 72°C for 5 min. Amplicons of 440 bp were purified with a magnetic bead-based clean-up system (Agencourt AMPure XP; Beckman Coulter) and sequenced on Illumina MiSeq platform using a 2×300 bp paired end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA). Libraries were pooled at equimolar concentrations, denatured and diluted to 4 nmol/l. Pair-ended amplicon reads (with corresponding quality scores) were trimmed, merged, clustered (operational taxonomic units [OTU] with 97% similarity), filtered from chimeric sequences using UPARSE (Edgar 2013), and taxonomically assigned using the GreenGenes database (version 12.10; McDonald *et al.* 2012). For downstream analysis, the OTU-table was normalised with cumulative sum scaling (CSS; Paulson *et al.* 2013) using the Qiime toolbox (*v*1.9; Caporaso *et al.* 2010). Beta-diversity was assessed through Bray-Curtis and Soresen distance and the factor-treatment analysed with redundancy analysis (RDA; Oksanen *et al.* 2015).

4.2.8 Metabolic analysis by ^{1}H-NMR

400 µL of urine samples were combined with 200 µL of phosphate buffer [0.2 M (pH 7.4) in D2O plus 0.001% TSP (3-(trimethylsilyl)-[2,2,3,3,−2H4]-propionic acid, δ 0.00)], mixed by vortexing, centrifuged at 1136 x *g* for 10 minutes, and then 550 µL was transferred into 5 mm NMR tubes for analysis. Faecal samples were pre-weighed (250 mg) and 700 µL of phosphate buffer and 2 glass beads were added in order to perform a bead-beating 5 min step at 25 movements/s using TyssueLyser LT (Qiagen, UK). Then, 500 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 one-dimensional (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 Tm 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 0.5 Hz line broadening.

4.2.9 Data Preprocessing and Analysis

All spectra were manually phased, baseline corrected and calibrated to the chemical shift of TSP using TopSpin (Bruker Biopsin, Rheinstetten, Germany). Spectra were digitised using an in-house MATLAB (version R2014a, The Mathworks, Inc.; Natwick, MA) and median fold normalisation was performed. The spectral region containing the water resonance was removed to minimise distortions in the baseline arising from imperfect water saturation. Principal components analysis (PCA) using mean-centred data was applied and orthogonal projection to latent structure discriminant analysis (OPLS-DA) models were constructed using for pairwise comparisons of the different experimental groups and time points. Colour represents the significance of correlation (r) for each metabolite to class membership. Predictive strength (Q^2Y) of the models was obtained using a 7-fold cross-validation method, and these were validated using permutation testing (number of permutations = 1000).

4.2.10 Statistical analysis

Statistical tests for food intake and GI symptoms were performed using Graphad Prism (version 5.0; Graph-Pad Software, 188 LaJolla, CA, USA). Normality test was used to assess whether the data were parametric or not parametric and unpaired Student's *t* test and Mann-Whitney tests were performed respectively on the data set.

4.3 Results

4.3.1 Dietary intake and bowel habit

Food diaries were analysed by comparing daily macronutrient and micronutrient intake according to different diets that the children were following (exclusion and un-restricted diet) and significant differences were seen only in Vitamin D intake (Table

4.2). ASD children who were not on the exclusion diet had significantly lower Vitamin D intake compared to ASD children on exclusion diet (P<0.01). In addition, Vitamin D intake seemed to be much lower, for both groups, than daily UK government recommendations (10 μg/day).

Vitamin C and B12, total sugar and protein intake was higher in both ASD groups compared to the nutrient intake requirements for typically developing children.

Table 4.2: Energy and nutrient intake in children on exclusion and un-restricted diets and comparison with the UK government recommendations for typically developing children. Mean: average of 4 consecutive days; SD: standard deviation. **: P<0.01

		Exclusion diet		**Un-restricted Diet**		**Typically developing children**
Daily dietary composition		*Mean*	*SD*	*Mean*	*SD*	*Adequate intake*
Energy Intake	Kcal	**1579.18**	394.19	**1478**	578.70	1430-1920
Protein Intake	g	**56.93**	17.30	**55.01**	15.68	19.7-42.1
Carbohydrate	g	**183.58**	35.20	**187.26**	74.99	191-333
Total sugars	g	**63.32**	27.58	**81.46**	36.44	19-33
Fibres	g	**17.19**	5.60	**15.36**	9.26	17.5-25
Saturated fatty acid	g	**22.22**	12.26	**22.28**	12.25	17.5-31
PUFA	g	**12.41**	6.39	**9.88**	7.97	10.5-18
MUFA	g	**22.88**	18.19	**19.39**	16.76	20.5-36
Vitamin C	mg	**73.64**	44.46	**70.94**	68.00	30-35
Vitamin D	μg	**2.72**	1.40	**1.21****	1.18	10
Vitamin B1	mg	**1.38**	0.49	**1.38**	0.66	0.6-1
Vitamin B2	mg	**1.44**	0.56	**1.41**	0.64	0.8-1.2
Vitamin B6	mg	**1.15**	0.33	**1.13**	0.53	0.9-1.2
Vitamin B12	μg	**3.04**	1.84	**3.82**	2.56	0.8-1.2
Iron	mg	**10.37**	4.30	**8.04**	2.99	6.1-11.3
Calcium	mg	**536.00**	247.89	**697.17**	357.52	450-1000

Scores extracted from GI symptoms diaries showed that exclusion diet had a significant impact on gastrointestinal problems (Figure 4.1). Significant reduction in abdominal pain (P<0.05) and bowel movement (P<0.001) has been reported in children following gluten, casein and dairy free diet.

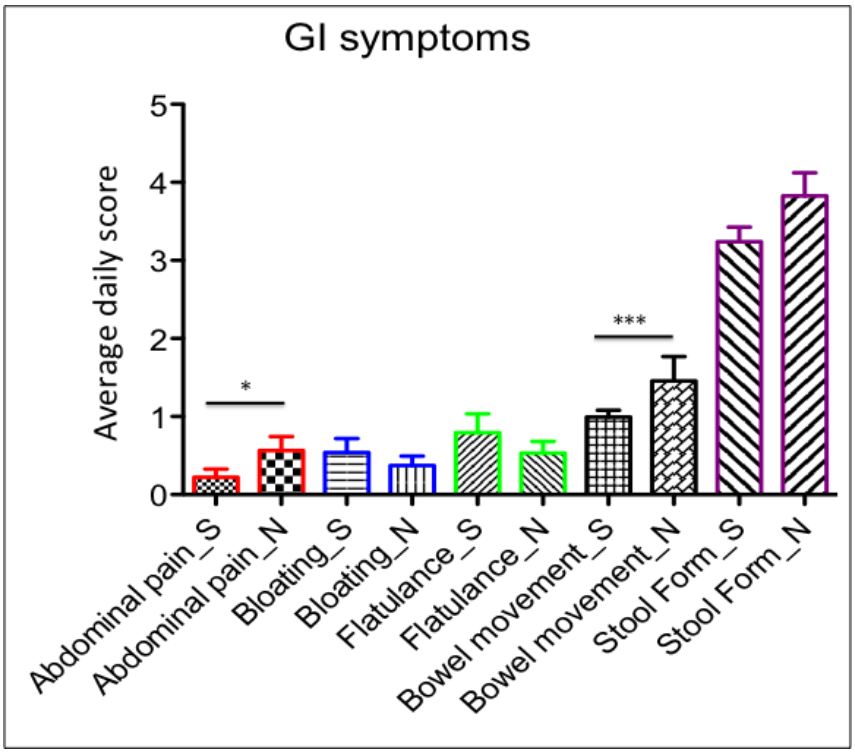
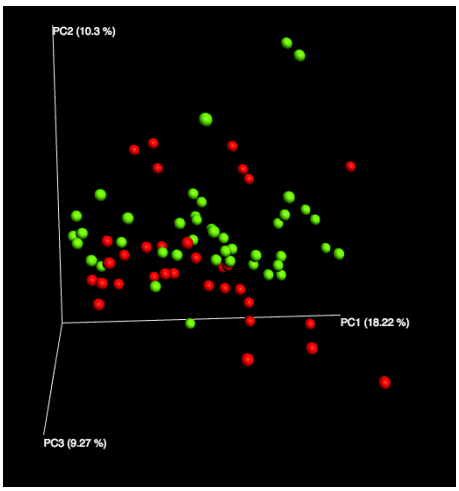


Figure 4.1: GI symptom assessment during 3 weeks data collection.
S: exclusion diets; N: un-restricted diet; *: P<0.05; ***: P<0.001.

4.3.2 Bacterial analysis

Beta-diversity was analysed in faecal samples of ASD children during the 3-weeks of sample collection. Soresen principal coordinate analysis (PCoA, Figure 4.2A) showed good separation between volunteers on exclusion diet (red dots) and un-restricted diet (green dots) considering presence or absence of particular bacterial species; separation that is lost in Bray-curtis PCoA (Figure 4.2B) when abundances were analysed.

A) 

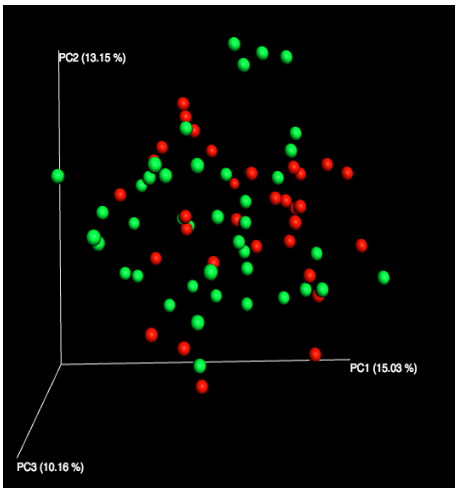
B) 

Figure 4.2: Comparison of the gut microbiota composition between ASD children following exclusion diet and ASD children following un-restricted diet. A) Sorensen PCoA; B) Bray-Curtis PCoA. Red dots: exclusion diet; green dots: un-restricted diet.

In order to assess the impact of the diets on the total gut microbiota composition, a RDA model was built. A 3% variance in the dataset was significant (P<0.004) and different bacterial groups were associated with the separation (Figure 4.3).

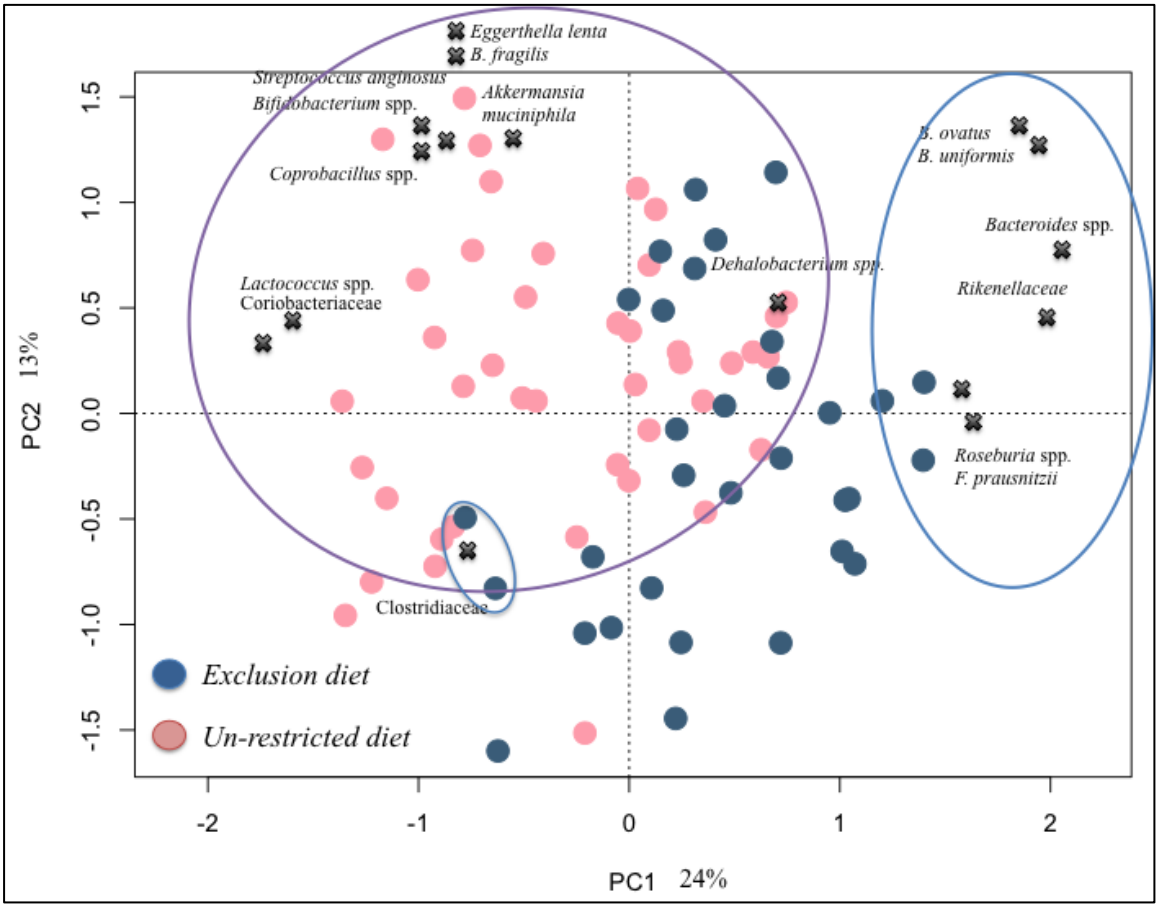


Figure 4.3: Microbial genera involved in the separation in the RDA plot. Blue dots: exclusion diet; pink dots: un-restricted diet.

Bacteroides spp. (Bacteroidaceae), Rikenellaceae, *Roseburia* spp. (Lachnospiraceae), *F. prausnitzii* (Ruminococcaceae) and Clostridiaceae were associated with the exclusion diet group, whereas *Eggerthella lenta, Bifidobacterium* spp. (Coriobaceriaceae), *B. fragilis* (Bacteroidaceae), *Akkermansia muciphila* (Verrucomicrobiacea), *Streptococcus anginosus, Lactococcus* spp. (Streptococcaceae), *Dehalobacterium* spp. (Dehalobacteriaceae) were associated with the un-restricted diet. In addition, our results showed bifidobacteria in lower abundances (3.5%) in the exclusion diet group compared to the un-restricted diet group (4.5%) and a reduction in the Veillonellaceae family.

4.3.3 ^{1}H-NMR analysis

Comparison of spectra profiles from urine samples did not show any significant differences in the general metabolism of ASD children when considering the different diets (Q^2Y= -0.0199). A significant OPLS-DA model was obtained by analysing faecal samples (Q^2Y=0.185, P <0.001), where changes in metabolic profile were driven by the diet. The PCA (Principal component analysis) score plot showed partial separation between the two groups; in particular for the exclusion diet, indicating its influences upon variation in data sets (Figure 4.4).

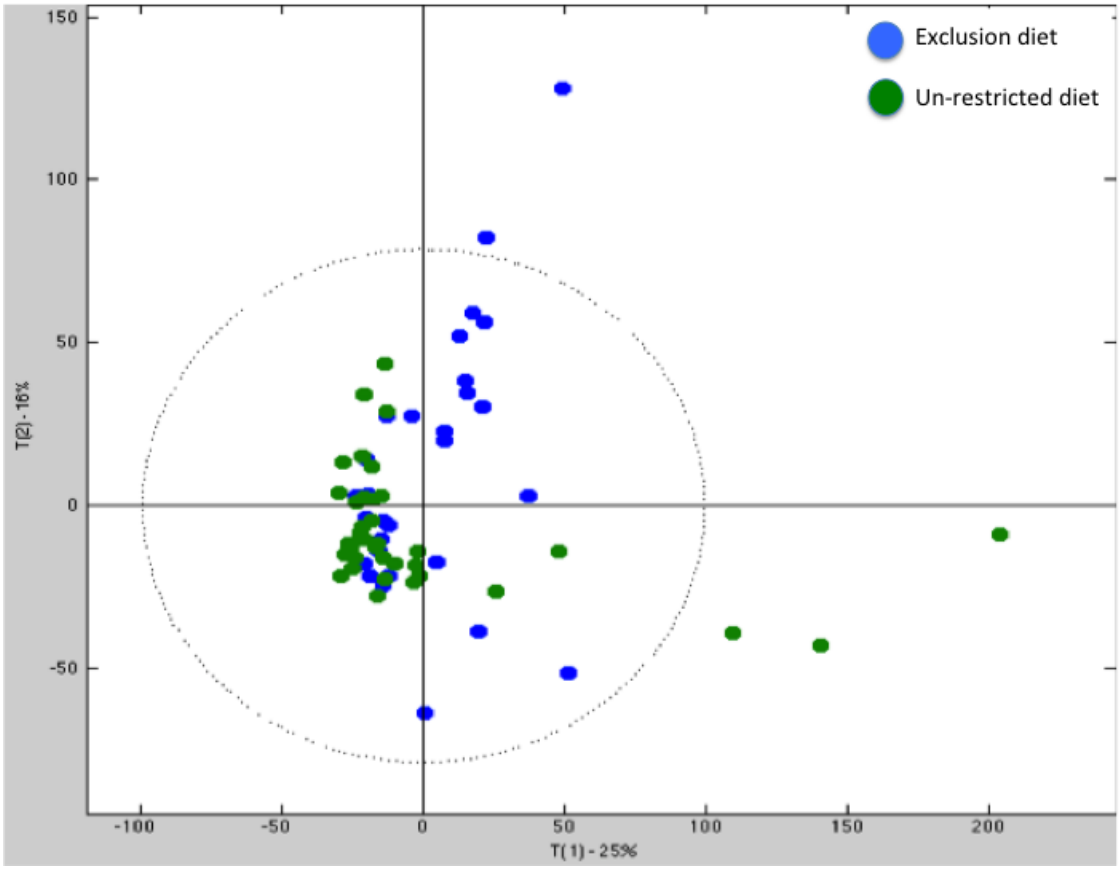
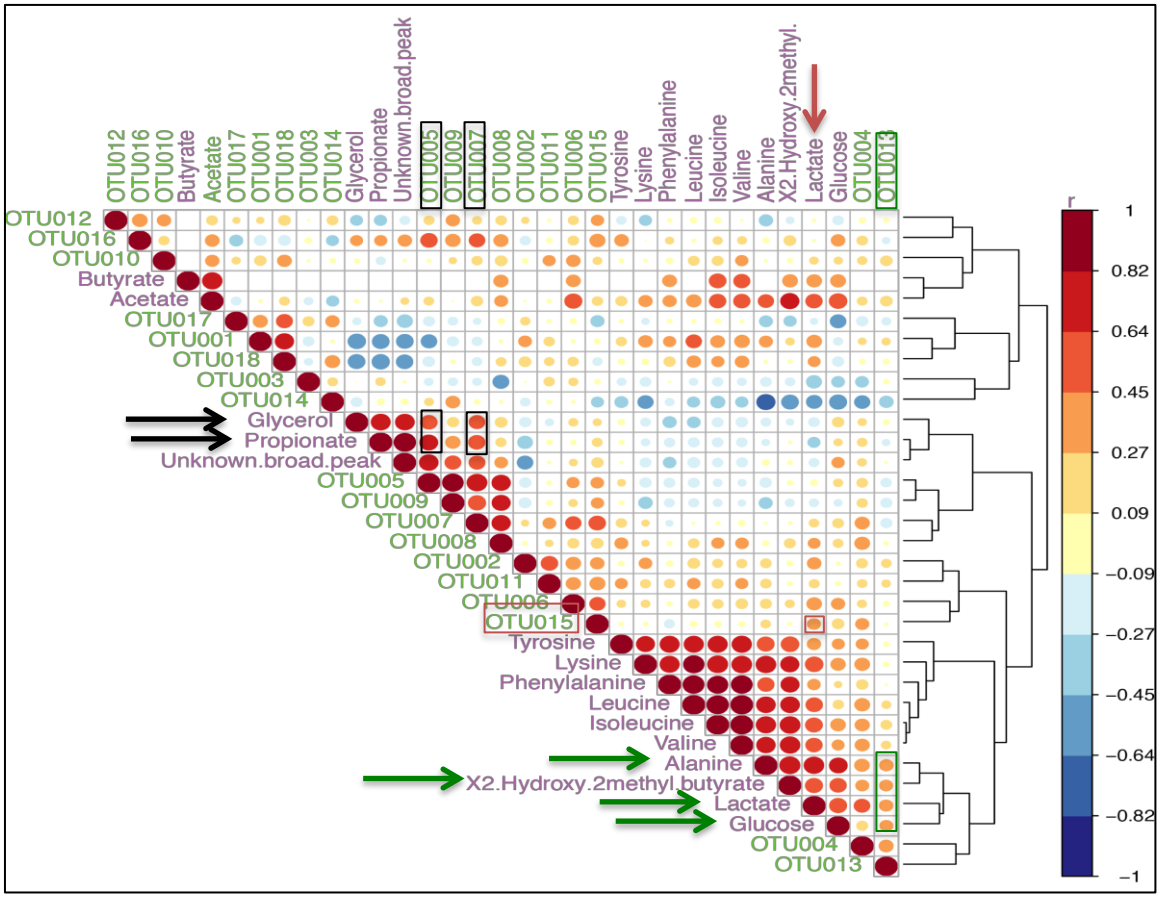


Figure 4.4: PCA score plot. PCA plot shows partial separation between children following exclusion diets and those whose diet was not restricted. Blue dots: exclusion diet; green dots: un-restricted diet.

Figure 4.5A and B summarises correlations between bacterial changes and metabolic variation in faecal samples of children following an exclusion diet and those on an un-restricted diet, respectively. During the exclusion diet (Figure 4.5A), *Bacteroides* spp. (OTU005 and OTU007) had a strong correlation with glycerol and propionate (black arrows), whereas Clostridiaceae (OTU013) were positively correlated with valine, 2-hydroxy-2-methilbutyrate, glucose and lactate (green arrows). The latter (red arrow) was also positively associated with *Roseburia* spp. (OTU015). In faecal

samples from children on the un-restricted diet (Figure 4.5B) positive correlations were identified for *Eggerthella lenta* (OTU004) and *Streptococcus anginosus* (OTU011) with lactate, tyrosine, 2-hydroxy-2-methilbutyrate, isoleucine, leucine, alanine and valine (black arrows); for Clostridiaceae (OTU012) and bifidobacteria (OTU001 and OTU002) with valine (orange arrow) and alanine (red arrow), respectively; and for *Lactococcus* spp. (OTU010) and *Coprobacillus* spp. (OTU017) with 2-hydroxy-2-methilbutyrate and propionate (green arrows).

A)



B)

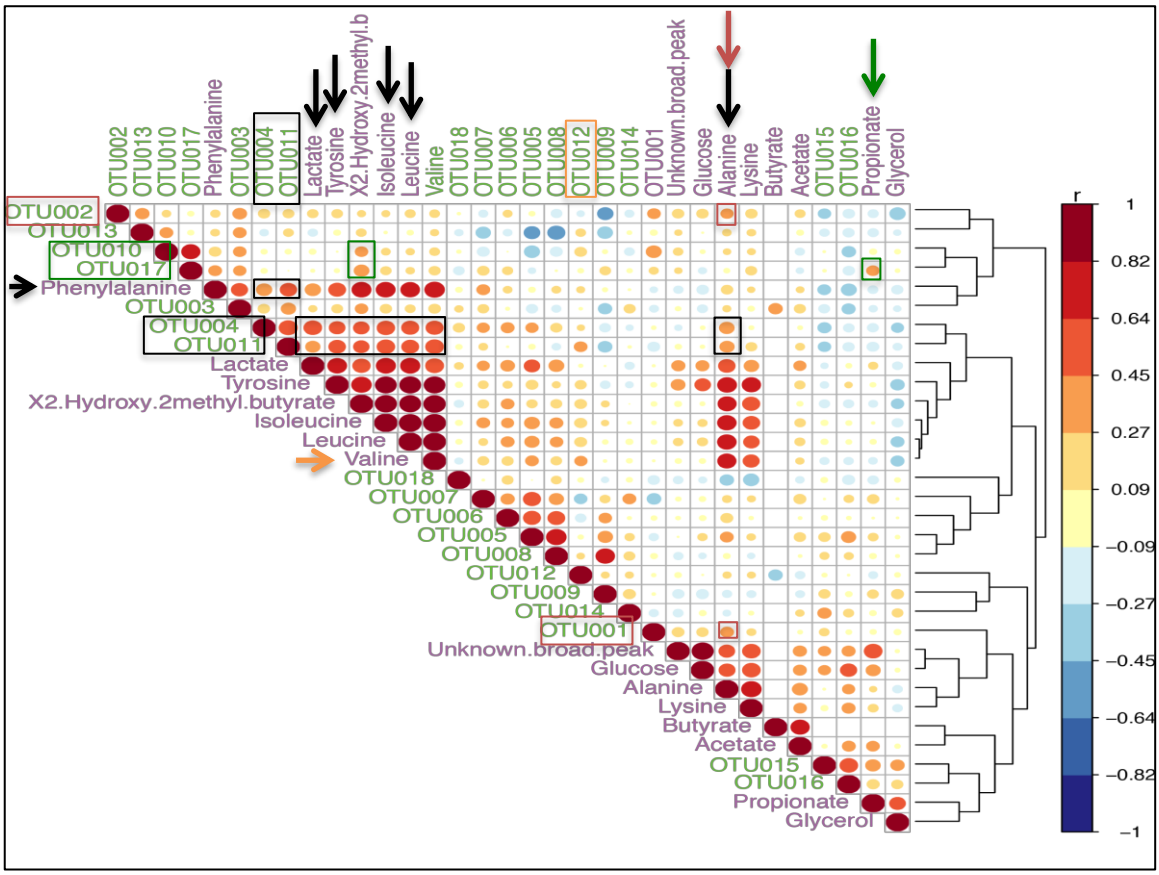


Figure 4.5: Summary of the correlation between bacterial changes and metabolic variation in faecal samples of children following exclusion diet (A) and those in un-restricted diet (B). OUTs: bacterial groups. **Arrows: metabolites identified; Squares: bacteria involved in the metabolic pathway.** OTU001: *Bifidobacterium* spp.; OUT002: *Bifidobacterium longum*; OTU003: Coriobacteriaceae; OTU004: *Eggerthella lenta*; OTU005: *Bacteroides* spp.; OTU006: *B. fragilis*; OTU007: *B. ovatus*; OTU008: *B. uniformis*; OTU009: *Rikenellaceae* spp.; OTU010: *Lactococcus* spp.; OTU011: *Streptococcus arginosus;* OTU012: Clostridiales; OUT013: Clostridiaceae; OTU014: *Dehalobacterium* spp.; OTU015: *Roseburia* spp., OTU016: *F. prausnitzii*; OTU017: *Coprobacillus* spp.; OTU018: *Akkermansia muciphila.*

4.4 Discussion

The effect of restricted diet in ASD children was investigated in this study. Diet has been previously seen to have a strong impact on the gut environment (De Angelis 2017), therefore we aimed to understand if and how it could impact on composition of gut microbiota and metabolic profile. The results showed significant differences in gut bacterial populations and faecal metabolome between the 2 diet groups (children following gluten and casein free diet and those whose diet was not restricted), but not in urine samples, indicating that diet has a strong effect on gut ecosystem but not on the general metabolism of ASD individuals.

Previous studies in ASD have mainly focused on the GFCF diet and the impact on GI symptoms or behaviour, showing inconsistent results (Mulloy *et al.* 2010). In our study, ASD children following gluten, casein and dairy-free diets had significant

reductions in abdominal pain and bowel movements, supporting the hypothesis that exclusion diets might alleviate these GI issues.

Lower abundances of bifidobacteria and Veillonellaceae were found in healthy volunteers following a gluten-free diet in a recent human intervention study by Bonder and colleagues (Bonder *et al.* 2016). These findings are reflected in our results, suggesting that dietary restriction might have bigger impact on the growth of these bacterial groups, than the type of disorder (eg autistic features). Veillonellaceae is considered to be a pro-inflammatory family of bacteria and reduction in its abundance could be considered a beneficial effect of this restricted diet. Reduction in *Bifidobacterium* spp. abundance was also confirmed by De Palma *et al.,* who associated the decrease of this bacterial group to the lower fibre intake in healthy participants during a one month gluten-free diet (De Palma *et al.* 2009). Our results are contrary to this suggestion since dietary records of ASD children in our study showed that those following GFCF diet had higher fibre intakes compared with autistic ones who were on un-restricted diet.

Similar outcomes were reported in a recent study conducted in Spain where researchers enrolled 105 ASD volunteers and 495 typically developing peers. Even though the autistic group consumed fewer carbohydrates (reported also in our study) they ate more legumes and vegetables. This could be an explanation for the higher fibre intake potentially derived from different sources (Marí-Bauset *et al.* 2016). The same research group obtained similar results, focusing only on the nutritional impact of GFCF in ASD individuals; their results showed higher fibre intake and, in addition, deficiency in Vitamin D intake, also supporting our findings (Marí-Bauset *et al.* 2015). Vitamin D is considered a neurosteroid that is active during brain development and its deficiency has been taken into consideration as potential environmental risk factor for ASD (Kocovska *et al.* 2012, Vinkhuyzen *et al.* 2017, Cannell *et al.* 2017). Our results reinforced this hypothesis, therefore the association between ASD and Vitamin D deficiency is worth to be further investigated.

Our metabolomic analysis showed amino acid (AA) and glucose as main metabolites present in the faecal samples in both dietary groups, as previously reported by De Angelis and colleagues (De Angelis 2015). It could be due to malassimilation of nutrients since results obtained from the food diaries showed increase in protein and total sugar intake. These results support the hypothesis that exclusion diet alone might not be enough to improve gut health.

Bacterial groups such *Clostridium* spp., *Bacillus* spp., *Lactobacillus* spp., *Streptococcus* spp., and proteobacteria have been associated with AA metabolism (Neis 2015) and this was confirmed by our results. Clostridiaceae and *Streptococcus anginosus* were positively correlated to alanine, tyrosine, isoleucine, leucine and phenylalanine. It is known that amino acids are precursors for neurotransmitters, such as tryptophan for serotonin or tyrosine for catecholamines, but little is known on the impact of the gut microbiota in these pathways and how the diet might modulate them (Tuohy *et al.* 2015). Alteration in AA metabolism has been seen in the urine of ASD children (Ming *et al.* 2012) and *in vitro* studies investigating the AA transporters in fibroblasts from skin biopsies of autistic individuals. Alterations were found for tryptophan, alanine and tyrosine that might lead to modifications in the neurotransmitter pathway (Fernell *et al.* 2007; Johansson *et al.* 2011).

Since AA are important for brain development and cognitive functions, Fernell and colleagues focused on the hypothesis that autistic individuals might have altered tyrosine and alanine transport mechanisms (L- and A-systems). They demonstrated that ASD children have aberrant AA transport systems and this might cause changes in levels of tyrosine in the brain, and therefore in dopamine synthesis (Fernell *et al.* 2007). Johansson *et al.,* focused not only on the tyrosine and tryptophan transporters, but also investigated the competition between alanine and tyrosine for transport across the brain-blood barrier (BBB). Their results showed a decrease in tryptophan transport into the brain, which might lead to a reduction of serotonin synthesis and a higher level of alanine. Synthesis of neurotransmitters do not require the presence of alanine, but the authors speculated that the high level might cause a reduction in essential amino acids important for brain activity (Johansson *et al.* 2011).

SCFAs have also been proven to have an effect on the CNS. In particular, injection of propionate in mice has been seen to cause autistic like behaviour (MacFabe *et al.* 2008). Our results showed strong correlations between *Bacteroides* spp. and propionate in faecal samples of ASD children on exclusion diets, confirming the role of the gut microbiota in metabolite production that might be associated with autistic traits. However, recently this hypothesis has been questioned since children with inborn propionic acidemia do not show typical autistic behaviour (Goof 2014). Also, the presence of lactate has been previously associated with psychological comorbidities (Dillon *et al.* 1987; Cowley *et al.* 1987) and its detection in both groups confirms *in vitro* results (Chapter 3, Grimaldi *et al.* 2017) and its potential role in ASD.

4.5 Conclusion

In summary, the results of this study give an insight on the impact of exclusion diets on gut microbiota composition and metabolic activity in ASD.

Apart from the improvement in GI symptoms in children following exclusion diets, our results suggested that dietary approach overall does not alter the general metabolism in ASD children, as reported by urine metabolome analysis.

We confirmed that gluten and casein-free diets modulated the gut microbiota revealing different bacterial populations associated with the two respective groups. In addition, faecal metabolic profiles showed amino acids as main metabolites identified, suggesting potential gut inflammation that might lead to malabsoption.

In conclusion, our results showed not only that exclusion diet might not be enough to improve gut health, but also that it is able to manipulate the gut environment and potentially be a factor that might interfere with other therapeutical approaches.

Author's contribution

The authors' contributions were as follows: RG conceived and carried out the research, managed all study visits and communicated with volunteers and drafted the manuscript; JRS and NG were involved in the interpretation of the metabolomics data; JB and IT helped in the experimental work; JLCM and DSN assisted with sequencing analysis; LG contributed in the mood and behaviour questionnaires evaluation; AC, GRG and JV were involved in the trail design and contributed to the critical revision of the manuscript; JV is employed by Clasado Biosciences Ltd, who provided the B-GOS product, marketed as Bimuno®, used within this research.

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CHAPTER 5

A double-blind, placebo-controlled, parallel-designed, prebiotic B-GOS intervention study in Children with Autism Spectrum Disorders (ASDs)

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Abstract

The main hypothesis of this study was to investigate the effect of B-GOS (83% GOS content) after 6 weeks treatment period on the gut microbiota composition and metabolic signature in autistic children. Furthermore, we also aimed to evaluate effect of B-GOS administration on GI dysfunction, mood, behaviour and sleep. We did not observe strong impact on GI issues after B-GOS intervention, but there were improvements in sleep habits. Our results also showed that anti-social behaviour varied over time depending on both exclusion diet (gluten and casein free diet) and treatment. 16S rRNA sequencing showed that B-GOS had a bifidogenic effect, confirming its prebiotic effect, as well as modulation of Lachnospiraceae family, in ASD children following un-restricted diets. The effect of B-GOS was significant also on the metabolome of this group. Butyrate and valerate were the main SFCAs produced and reduced amino acid excretion was detected in faecal samples of the intervention group. Urine samples were dominated by citrate, creatine, creatinine, DMA, DMG, malonate, carnitine, TMAO, and α-hydroxybutyrate in ASD children taking B-GOS, whereas PAG, phenylalanine and β-hydroxybutyrate were detected in the control group. To our knowledge, this is the first study where a prebiotic intervention has been evaluated in autism and the results are promising. Administration of B-GOS to autistic children may be useful in positively affecting the microbiota, metabolic profile and some traits associated with autism.

5.1 Introduction

Autism is a spectrum of disorders diagnosed in early life and interferes with the normal course of social, communicative, and cognitive development. Autistic Spectrum Disoder (ASD) individuals might also sufferer GI problems. The causes are still unknown but there are hypotheses behind these issues such as (1) dysbiosis in gut microbiota composition, in particular reduced number of bifidobacteria and increased *Clostridium* spp., *Desulfovibrio* spp., *Sutterella* spp. and/or Veillonellaceae; (2) altered dietary intake and (3) increased gut permeability. Dysfunction of the intestinal barrier might lead to the translocation of bacteria and/or metabolites that may have an impact on systemic metabolome, immune and nervous systems (Ding *et al.* 2016).

Previous studies have reported alterations in gut barrier function in ASD individuals (White 2003) and in order to reduce these problems, exclusion diets are often suggested. It was previously shown (as reported in chapter 4) that exclusion diet was able to modulate gut bacterial populations and reduce GI discomfort, but partial changes in the metabolomics analysis, such as the presence of amino acids in faecal samples, lead us to think that diet alone does not have an effect on problems with nutrient absorption, and potentially, on gut inflammation. Patel and Curtis (2007) reported improvement in 10 ASD children after 6 months of multiple behavioural, language and nutritional therapies in a controlled environment, speculating that multiple interventions might have greater potential in improving ASD symptoms.

Manipulation of the gut microbiota using pre- and probiotics has shown beneficial effects on human health (Holmes *et al.* 2012) but, to date, there are only a few studies that have focused on the impact of these food supplements in autism. Parracho *et al.* reported improvement behavioural changes after *Lactobacillus plantarum* WCSF1 administration in ASD children (Parracho *et al.* 2010). Adam and colleagues noticed a strong correlation between GI symptoms and severity of autism (Adams *et al.* 2011). Furthermore, they showed significant differences in SCFA levels in ASD individuals taking probiotics. Kaluzna-Czaplinska, after 2 months of oral supplementation with *Lactobacillus acidophilus,* found significant modifications in the level of D-arabinitol (DA) and the ratio of D-/L-arabinitol (DA/LA) in ASD children, and an improvement in concentration (Kaluzna-Czaplińska and Błaszczyk 2012). Recently, Tomova and colleagues confirmed the correlation of autism severity with GI dysfunction and showed that 4 months of mixed probiotic administration was able to modulate gut microbiota populations in ASD children (Tomova *et al.* 2015). Furthermore, probiotic therapy has

been demonstrated to reduce inflammation, as assessed by myeloperoxidase-MPO and copper in autistic children compared to non-autistic controls (Russo 2015).

To date, data on the effect of prebiotics in ASD are scarce. B-GOS is a galacto-oligosaccharide mixture that has been widely tested *in vitro* and *in vivo* studies, showing improvements in several aspects of human physiology (Vulevic *et al.* 2008) (Depeint *et al.* 2008) (Silk *et al.* 2009) (Vulevic *et al.* 2015) (Drakoularakou *et al.* 2009). It was previously shown that B-GOS has an impact on the faecal gut microbiota composition and metabolic profile of autistic children using an *in vitro* fermentation system mimicking conditions of the colon (Chapter 3).

Considering these promising results, the main hypothesis of this study was to investigate the effect of B-GOS after 6-weeks treatment period on gut microbiota composition and metabolic signature in autistic children. Furthermore, we also aimed to evaluate the effect of B-GOS administration on GI dysfunction, mood, behaviour and sleep.

5.2 Materials and Methods

5.2.1 Study design

A randomised, double-blind, placebo-controlled (Maltodextrin), parallel-designed prebiotic GOS mixture (B-GOS; Bimuno®) feeding study was conducted in children diagnosed with ASD (Figure 5.1). Both treatments were provided in powder form and supplied by Clasado Biosciences Ltd. (Reading, United Kingdom).

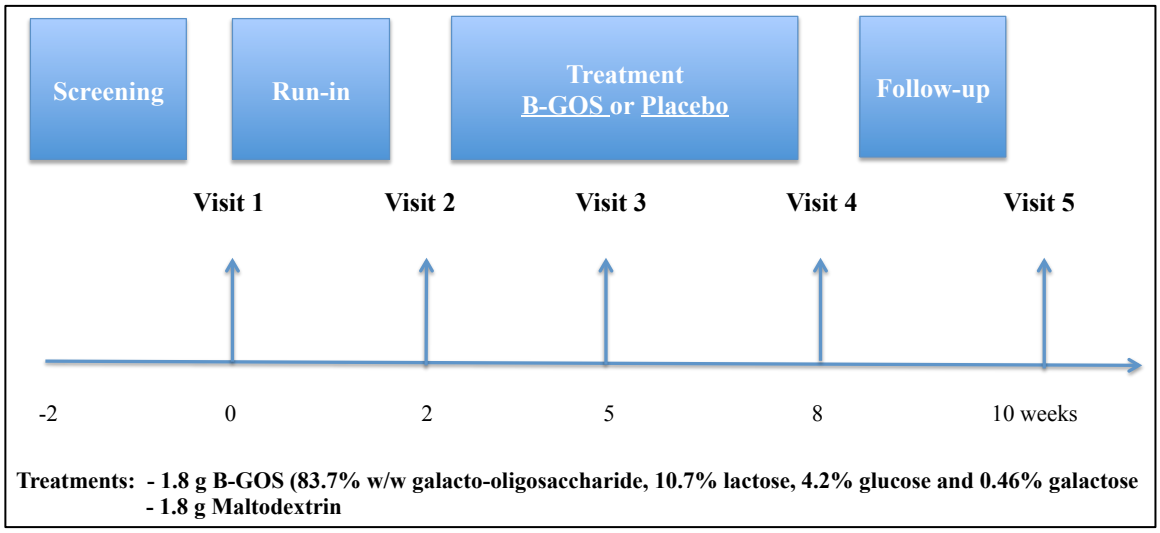


Figure 5.1: Study design and nutritional information on the treatments administrated throughout the study. Each treatment was pre-weighed and delivered in individual containers. Volunteers' parents were asked to mix the powder with drinks that children usually consume.

The study was conducted according to the guidelines of the Declaration of Helsinki, and the University of Reading Research Ethics Committee (UREC15/41; Registration number: NCT02720900) approved all procedures involving human subjects (questionnaires, volunteer forms, diaries and advertising documents Appendices 5.1 - 5.9).

Recruited subjects were divided into two groups A and B, according to dietary habits assessed by 4-day food diaries (as previously discussed in Chapter 4), children whose diet was not restricted and on restricted diet (mainly gluten, casein and dairy free) respectively. Within these two groups, children were randomly assigned to two feeding groups using a random number system. Group-I received placebo and Group-II received B-GOS during the 6 weeks feeding period. Volunteers were required to visit the University of Reading or the researcher visited the volunteers at home on five separate occasions during the study period in order to provide faecal (weekly collection) and urine samples.

5.2.2 Subjects

The study was powered using Hedwig Harvard Software and the calculation was based on changes in bifidobacterial number from a previous intervention study in human volunteers using B-GOS (Vulevic *et al.* 2008). A total of 41 autistic children (31 male and 10 female) with formal diagnosis of ASD were enrolled in the study in order to have a 95% probability that the study would detect, at a two-sided 5% significance level, an effect on the colonic bifidobacterial population.

Of these 41, 11 subjects withdrew from the study during the baseline period, largely due to difficulties and/or inconveniences associated with collecting samples. The remaining 30 volunteers were divided into group A (un-restricted diet; n=18) and B (exclusion diet; n=12) and each group randomly distributed between groups I and II (A-I: n=9, A-II: n= 9 and B-I: n=6, B-II: n=6). However, 4 subjects subsequently dropped out before the end of the first feeding arm. One left the study without giving a reason, two were withdrawn due to protocol violation, and one withdrew due to an adverse event (strong diarrhoea and abdominal pain observed after 2 days of treatment feeding).

In total, 26 volunteers completed the 10 weeks study (A-I: n=7, A-II: n= 7 and B-I: n=6, B-II: n=6; Table 5.1) providing 80% statistical power to the study.

Table 5.1: Diagnosis reported from parents by medical assessment (*). ASD: Autism Spectrum Disorders; ADHD: Attention Deficit Hyperactivity Disorder; PDD: Pervasive Development Disorder

Diagnosis*	ASD	ADHD	Asperger	PDD
Volunteers (n)	26/26	5/26	1/26	1/26
Exclusion diet B-GOS (B-II)	6/6			1/6
Exclusion diet Placebo (B-I)	6/6	3/6	1/6	
Un-restricted diet B-GOS (A-II)	7/7	1/7		
Un-restricted diet Placebo (A-I)	7/7	1/7		

Volunteers were assessed before the start of the trial and were selected according to certain exclusion and inclusion criteria. Inclusion criteria for participation in the study were signed consent form from volunteers' parents or guardians, age of 4-11 y inclusive, formal diagnosis of ASD. Volunteers taking probiotics, prebiotics, antibiotics or other

dietary supplement drugs that could affect the luminal microenvironment of the intestine within 4 weeks before the study were also excluded. Volunteers were instructed not to consume such products during the study and not to alter their usual diet or fluid intake.

5.2.3 Faecal sample collection and preparation

Faecal samples were collected weekly using faecal collection kit (FC2040, Laboratories Ltd, UK) and volunteers were asked to keep them at -20°C until the visit day when they were transferred to the laboratory for all the analysis. Samples for fluorescence *in situ* hybridisation (FISH) were stored in glycerol until the visit day and diluted 1 in 10 (wt:wt) in phosphate-buffered saline (1X PBS, 0.1 mol/L, pH 7.0; Oxoid, UK) and homogenised in a Stomacher 400 (Seward, Norfolk, United Kingdom) for 2 min at normal speed. Samples for DNA extraction and ^{1}H-NMR were weighed (250 mg) and stored at-80°C.

5.2.4 Urine sample collection and preparation

Urine samples were collected using sterile tubes (Mid-Stream Urine Specimen Collector; Pennine Healthcare, UK) or sterile pad (Sterisets Urine Collection Kit; MediBargains, UK) and stored at -20°C until visit day when they were processed. Samples were transferred to 15ml falcon tubes and centrifuged for 10 min at 1136 x g. The supernatants were transferred to 1.5ml Eppendorf tube (in duplicate) and stored at -80°C.

5.2.5 Fluorescence *in situ* hybridisation (FISH)

Synthetic oligonucleotide probes targeting specific regions of the 16S rRNA labelled with the fluorescent dye Cy3, as previously described in Chapter 2 were used for bacterial enumeration assessed by FISH analysis. Briefly, faecal homogenate samples were fixed for at least 4 hours (4 °C) in 4% (wt:vol) paraformaldehyde. Fixed cells were centrifuged at 1136 x g for 5 min at 25 °C and washed twice in 1 mL filtered PBS (0.1 mol/L, pH 7.0; Oxoid, UK). The washed cells were resuspended in 150 µL PBS/EtOH (1:1) and stored at -20 °C. The probes used (Eurofins Genomics, UK) were Bif164 for *Bifidobacterium* genus (Langendijk *et al.* 1995) and EUB 338 I-II-II for total bacteria (Daims *et al.* 1999).

5.2.6 DNA extraction and PCR

Total microbial DNA was extracted from faeces using the DNA stool mini kit (Qiagen, UK) by introducing three 1-min steps at 50 movements/s using TyssueLyser LT (Qiagen, UK) with 5-min incubation in ice between treatments as previously described by Candela *et al.* 2016. Thermal disruption of the samples was performed by incubation at 95°C for 15 min, then centrifuged for 5 min (1136 x *g)* at 4°C to pellet stool particles. Two hundred-sixty µl of 10 M ammonium acetate was added to the supernatant, incubated in ice for 5 min and centrifuged at full speed for 10 min at 4°C. The supernatants were collected, one volume of isopropanol added and the samples incubated on ice for 30 min. Samples were centrifuged (1136 x *g)* for 15 min at 4°C and the pellet washed with 70% (v/v) ethanol. They were resuspended in 100 µl of TE buffer (10 mM Tris-Cl, pH 7.5; 1 mM EDTA, pH 8.0) and treated with 2 µl of DNase-free RNase (10 mg/ml, Sigma-Aldrich) at 37°C for 15 min. Proteins were removed by adding 15 µl of proteinase K (DNA stool mini kit; Qiagen, UK) and DNA was subsequently purified following the manufacturer's instructions. DNA recovery was evaluated using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

5.2.7 16S rRNA gene amplification via next generation sequencing (NGS) and bioinformatics analysis

For each sample, the V3–V4 region of the 16S rRNA gene was PCR-amplified in 25-µl volumes containing 12·5 ng of microbial DNA, 2× KAPA HiFi HotStart ReadyMix (Kapa Biosystems, USA) and 200 nmol/l of S-D-Bact-0341-b-192 S-17/ S-D-Bact-0785-a-A-21 primers carrying Illumina overhang adapter sequences (Bio-Fab Research). Thermal cycle consisted of an initial denaturation at 95°C for 3 min, twenty-five cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and a final extension step at 72°C for 5 min. Amplicons of about 440 bp were purified with a magnetic bead-based clean-up system (Agencourt AMPure XP; Beckman Coulter) and sequenced on Illumina MiSeq platform using a 2×300 bp paired end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA). Libraries were pooled at equimolar concentrations, denatured and diluted to 4 nmol/l. Pair-ended amplicon reads (with corresponding quality scores) were trimmed, merged clustered (operational taxonomic units [OTU] with 97% similarity), filtered from chimeric sequences using UPARSE (Edgar 2013), and taxonomically assigned using the GreenGenes database (version 12.10; McDonald *et al.* 2012). For downstream analysis,

the OTU-table was normalised with cumulative sum scaling (CSS; Paulson *et al.* 2013) using the Qiime toolbox (*v*1.9; Caporaso *et al.* 2010). Beta-diversity was assessed through Bray-Curtis and Soresen distance and the factor-treatment analysed with redundancy analysis (RDA; Oksanen *et al.* 2015). Alpha-diversity was measured and expressed as observed species (97% similarity OTUs) and computed with 10 rarefied OTU tables. Comparison of Alpha-diversities was made through nonparametric t-test method (Monte Carlo, 999 permutations).

5.2.8 Metabolic analysis by ^{1}H-NMR

Four hundred μL of urine samples were combined with 200 μL of phosphate buffer [0.2 M (pH 7.4) in D2O plus 0.001% TSP), mixed by vortexing, centrifuged at 1136 x *g* for 10 minutes and then 550 μL was transferred into 5 mm NMR tubes for analysis. Faecal samples were pre-weight (250 mg) and 700 μL of phosphate buffer and 2 glass beads were added in order to perform a bead-beating 5-min step at 25 movements/s using TyssueLyser LT (Qiagen, UK). Then, 500 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 one-dimensional (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 Tm of 100 ms and a 90 pulse set at 7.70 μs. For each spectrum, a total of 128 scans was accumulated into 64 k data points with a spectral width of 12.001 ppm. The FIDs were multiplied by an exponential function corresponding to 0.5 Hz line broadening.

5.2.9 Data Preprocessing and Analysis

All spectra were manually phased, baseline corrected and calibrated to the chemical shift of TSP using TopSpin (Bruker Biopsin, Rheinstetten, Germany). Spectral features were picked using in-house MATLAB (version R2014a, The Mathworks, Inc.; Natwick, MA) script and median fold normalization was performed. The spectral region, containing the water resonance, was removed to minimise distortions in the baseline arising from imperfect water saturation. Principal components analysis (PCA) using mean-centred data was applied and orthogonal projection to latent structure discriminant analysis (OPLS-DA) models were constructed for pairwise comparisons of the different experimental groups and time points. Colour represents the significance of correlation

(R^2) for each metabolite to class membership. Predictive strength (Q^2Y) of the models was obtained using a 7-fold cross-validation method and these were validated using permutation testing (number of permutations =1000).

5.2.10 GI symptom diaries

Parents/guardians of children volunteers were asked to fill in daily questionnaires for GI function and symptoms. The Bristol stool chart was used to assess faecal sample type and consistency, together with number of bowel movements, abdominal pain, intestinal bloating and flatulence (i.e. low, present but well tolerated, present and interfering with but not preventing normal daily activities, preventing normal daily activities; Lewis & Heaton 1997). Concomitant medication, adverse events, changes in diet and behaviour were also recorded throughout the study, on a separate sample submission forms.

5.2.11 Behavioural and sleep assessment

Autism Treatment Evaluation Checklist (ATEC) was used to evaluate the effectiveness of treatment. In addition, parents/guardians were asked to complete 5-day sleep diaries before and after interventions in order to understand the qualitative impact of B-GOS supplementation on sleep disorder, if present.

5.2.12 Statistical analysis

Behavioural assessments and GI symptoms were analysed using SPSS software (SPSS Inc., Chicago, IL, USA). Mood and behavioural questionnaire analysis were performed considering the 4 ATEC scale scores (Communication, anti-sociability, Sensory and Behaviour) and the total ATEC score, for each time point (before T1, after treatment T2 and follow-up T3). One-way ANCOVA was used in order to test whether T2 scores differed between treatment groups, adjusting for baseline T1 scores as covariate. GLM 2x3 RMANOVAs were used to test whether there were any treatments by time interaction across time points. Each of those RMANOVAs was followed by a 2x2 RMANCOVA to test for any treatment by time interactions across T2 and T3 adjusting for baseline (T1) differences. This test was used in order to assess whether any treatment effect was consistent to follow-up.

GI symptoms were analysed using a linear mixed model. The fixed terms assessed in this model were diet, treatment, time (pre-treatment, during treatment, follow-up), weeks, diet x time, treatment x time and volunteer scores as a random effect.

Statistical test for bacterial composition results was performed using Graphad Prism (version 5.0; Graph-Pad Software, 188 La Jolla, CA, USA). One-way ANOVA tests and unpaired Student's *t* tests, including post hoc tests appropriate for individual data sets (Tukey post-test with significance set at $P< 0{\cdot}05$), were used.

5.3 Results

5.3.1 GI symptoms and sleep diaries

GI symptoms were quantitatively evaluated each day, by recording abdominal pain, intestinal bloating and flatulence, stool form and bowel movement. A general trend of reduction in GI symptoms was reported, but differences between treatments were not significant. The significant effect was observed for the interaction between the diet and time, for bowel movements ($P<0.01$) and flatulence ($P<0.05$).

Qualitative analysis was performed on sleep habits and 3 volunteers (23% of participants) benefited from B-GOS intervention. Parents reported that their children slept one hour longer than usual and one of them also noticed that the child had less problems falling asleep.

5.3.2 Mood and behaviour questionnaires

Mood and behaviour questionnaires were analysed taking into account age, diet and intervention. ASD children following exclusion diet, showed a significant reduction in the "anti-social behaviour" score after B-GOS intervention. The results from the ATEC questionnaire showed that anti-sociability varied over time depending on both exclusion diet and treatment ($P<0.05$; Figure 5.2).

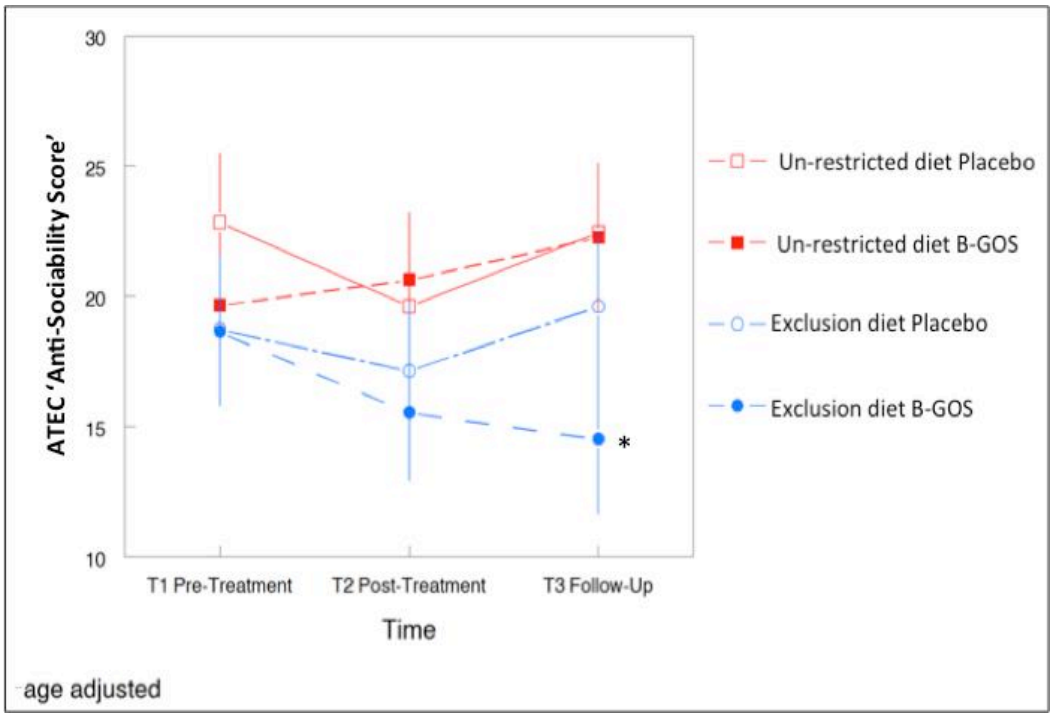
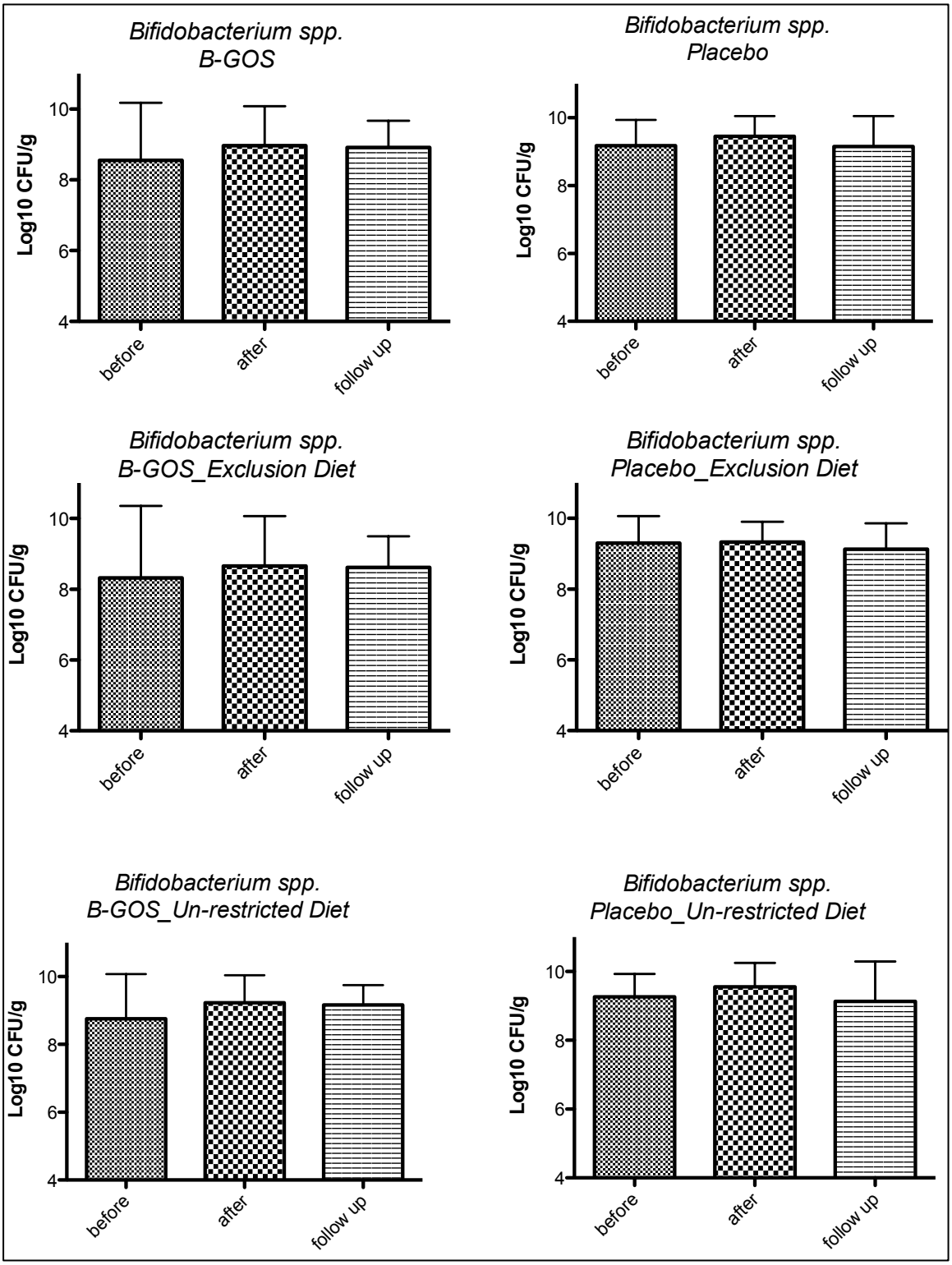


Figure 5.2: ATEC questionnaire. Results showed consistent reduction in anti-sociability score in children on exclusion diet and B-GOS intervention. Results were reported in mean ± standard error. Placebo: Maltodextrin. *: P <0.05.

5.3.3 Bacterial composition by FISH analysis

FISH analysis was performed on total bacteria and bifidobacteria of the faecal samples collected at baseline, after 6 weeks intervention and follow-up (Figure 5.3A and B). Despite an increase in number of *Bifidobacterium* spp. after B-GOS intervention (Figure 5.3A), FISH results did not show any significance considering treatment and the interaction between treatments versus diet.

A)


Bifidobacterium spp.
B-GOS
Bifidobacterium spp.
Placebo
Bifidobacterium spp.
B-GOS_Exclusion Diet
Bifidobacterium spp.
Placebo_Exclusion Diet
Bifidobacterium spp.
B-GOS_Un-restricted Diet
Bifidobacterium spp.
Placebo_Un-restricted Diet
Log10 CFU/g
10
8
6
4
before
after
follow up

B)

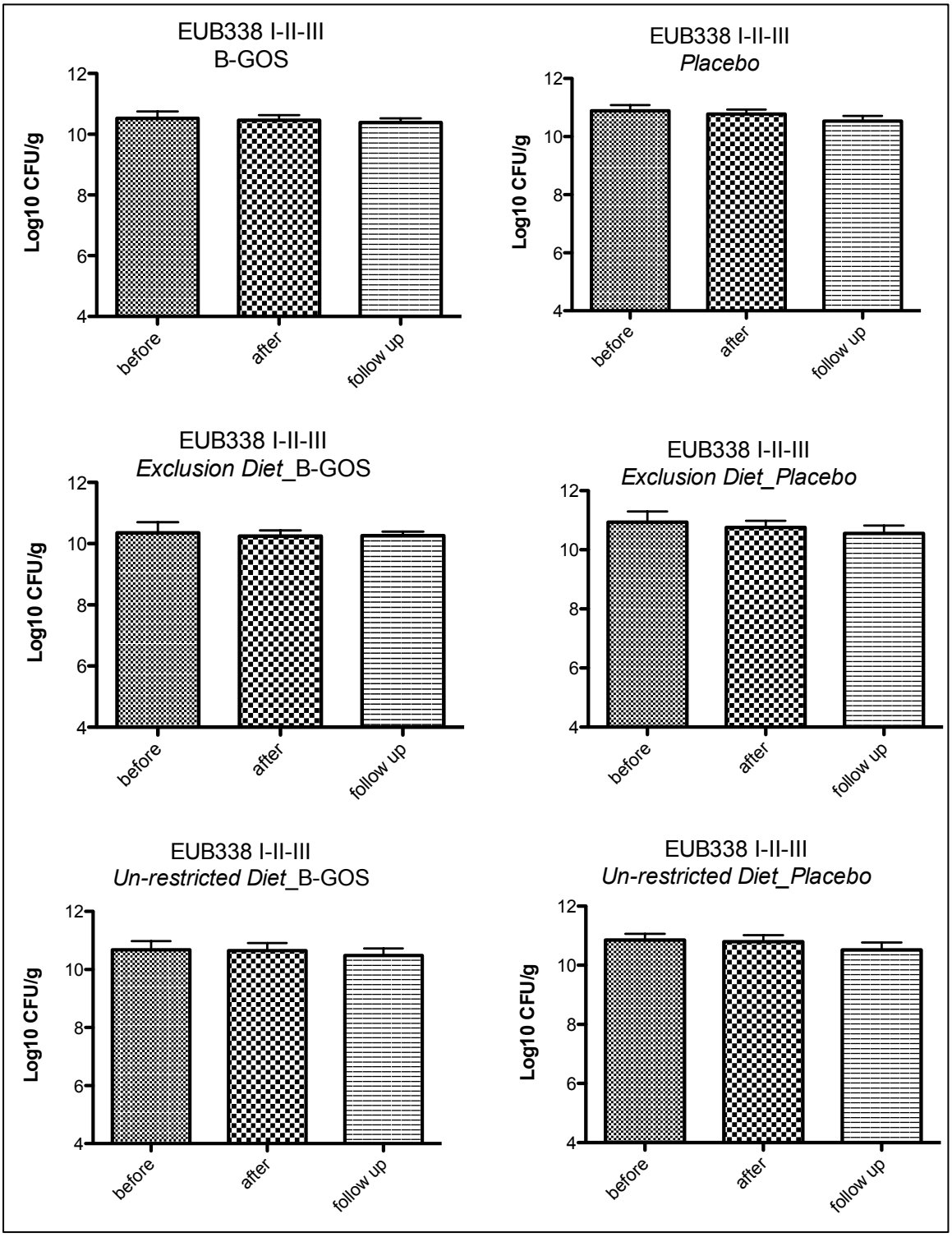
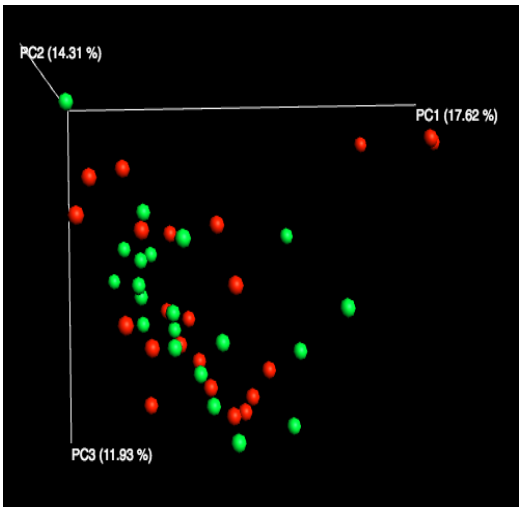


Figure 5.3: FISH analysis. Bifidobacteria (A) and total bacteria (B) numbers measured as Log_{10} CFU/g faeces before, after treatment and follow up in ASD children. Data were analysed considering treatment and interaction treatment/diet. B-GOS (n=13); Placebo (n=13); B-GOS_Exclusion Diet (n=6); Placebo_Exclusion Diet (n=6); B-GOS_Un-restricted Diet (n=7); Pacebo_Un-restricted Diet (n=7).

5.3.4 Metagenomics analysis

Beta-diversity of faecal samples collected at baseline (W0-W1-W2), last 3 weeks of treatment period (W6-W7-W8) and follow-up (W9-W10) were analysed. The only significant model was identified comparing bacterial populations in faecal samples from ASD children under un-restricted diet, before (baseline) and after B-GOS administration. Soresen and Bray-curtis principal coordinate analysis (PCoA) did not show any separation between the 2 groups (Figure 5.4A and B). Introducing the treatment as variable in RDA model, a 4% variance was significant (P<0.038). Figure 5.5 shows PCA (principal component analysis) score plot reporting bacterial populations positively associated with the separation after B-GOS supplementation in ASD children under un-restricted.

A) 

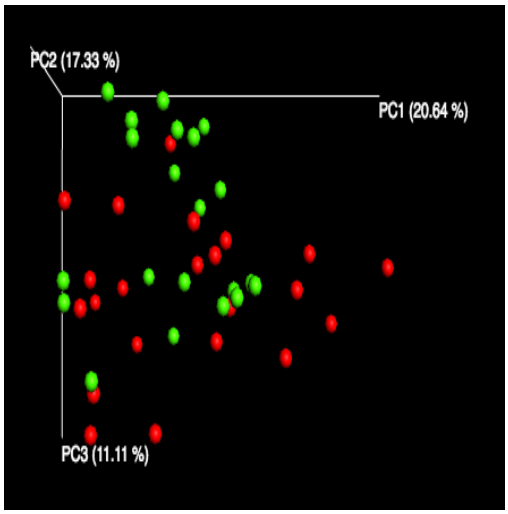
B) 

Figure 5.4: Comparison of the gut microbiota composition between baseline and after B-GOS administration in ASD children under un-restricted diet. A) Sorensen PCoA B) Bray-Curtis PCoA. Red dots: samples before B-GOS treatment; green dots: samples after B-GOS intervention

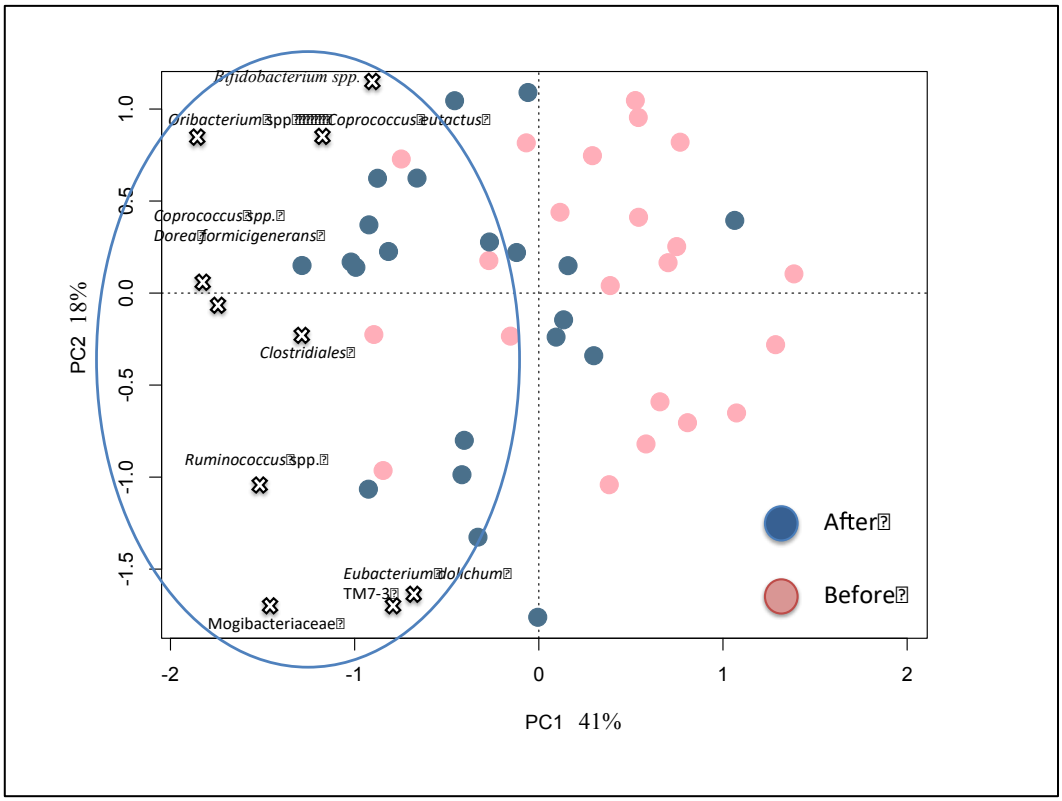


Figure 5.5: Microbial genera involved in the separation in the RDA plot after B-GOS treatment. Blue dots: after B-GOS intervention; pink dots: before B-GOS intervention.

B-GOS supplementation positively modulated some bacterial groups such as *Bifidobacterium* spp., *Ruminococcus* spp., Lachnospiraceae family (*Coprococcus* spp., *Dorea formicigenerans*, *Oribacterium* spp.), *Eubacterium dolchum*, TM7-3 family and Mogibacteriaceae.

In addition, rarefaction curves showed that B-GOS supplementation increased the diversity in gut microbial composition of ASD children in un-restricted diet, but the increase was not significant (Figure 5.6).

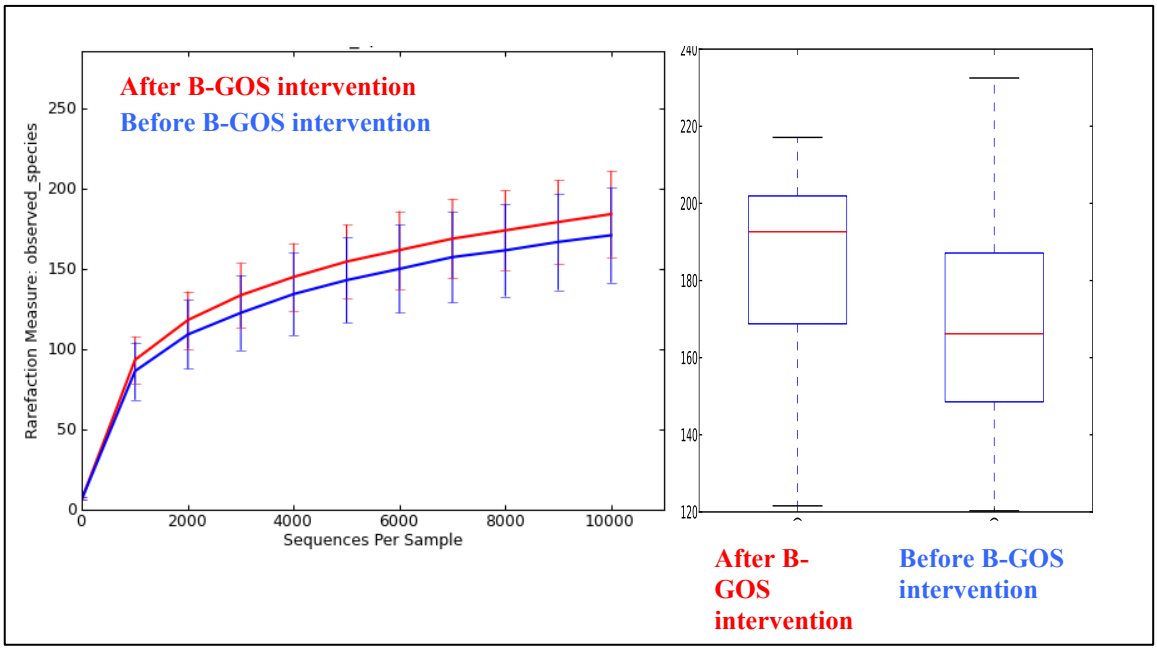


Figure 5.6: Comparison of bacterial richness and diversity before and after B-GOS treatment in ASD children following un-restricted diet. Rarefaction curves and box plots showed that B-GOS supplementation increased the diversity in gut microbial composition of ASD children in un-restricted diet.

5.3.5 ^{1}H-NMR analysis

Comparison of the spectra profiles from urine samples did not show any significant differences at baseline. Significance was identified in ASD children following un-restricted diet after B-GOS intervention, indicating that B-GOS supplementation contributed to the metabolic variation. A significant OPLS-DA model was obtained comparing metabolic profiles of ASD children taking placebo and those taking B-GOS, after 6 weeks intervention ($Q^2Y=0.065$; $P<0.01$; Figure 5.7). Urine spectra of autistic volunteers receiving B-GOS treatment contained greater amounts of creatinine, creatine, dimethylglycine (DMG), dimethylalanine (DMA), carnitine, malonate, citrate, adipate, trimethylamine-N-oxide (TMAO) and α-hydroxybutyrate compared to the autistic one taking maltodextrin. In addition, B-GOS supplementation seemed to reduce amount of phenylacetylglycine (PAG), phenylalanine and β-hydroxybutyrate in the intervention group.

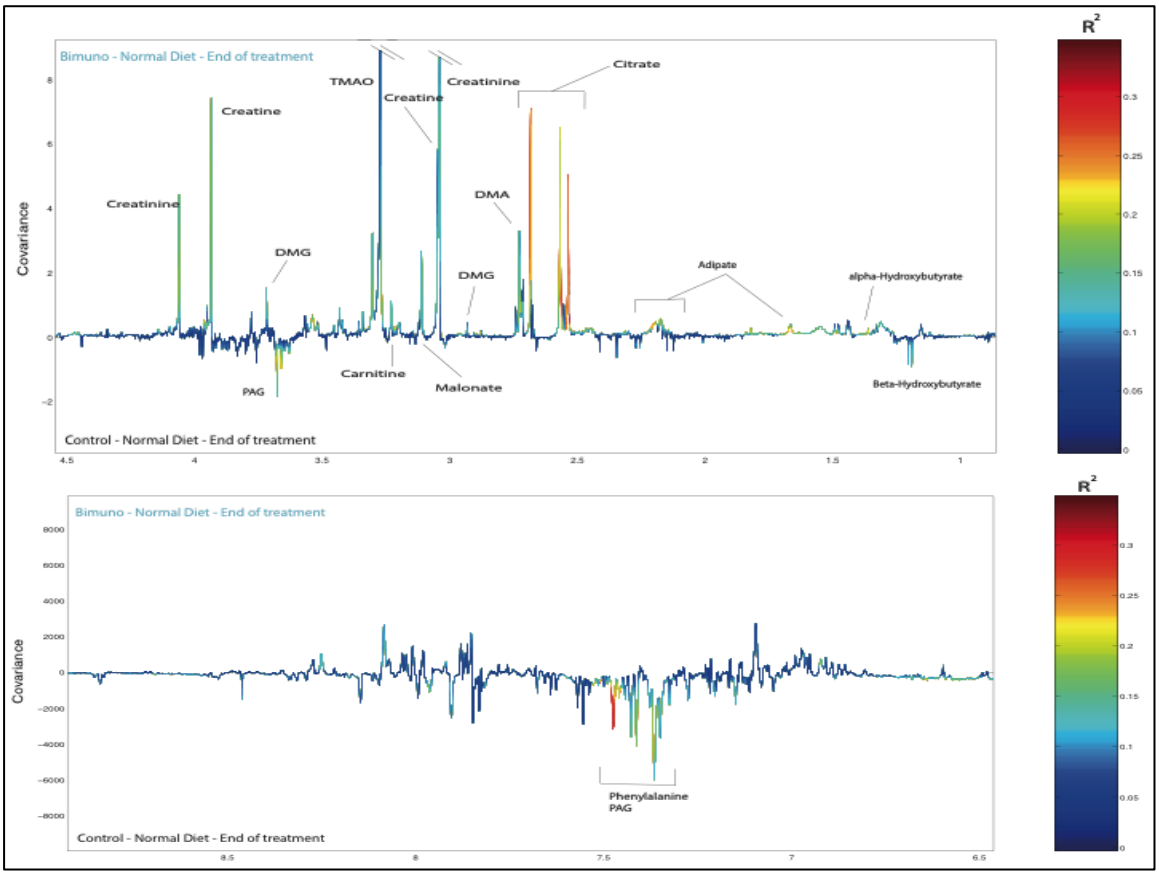


Figure 5.7: OPLS-DA obtained comparing the metabolic profile in urine samples of ASD children in un-restricted diet taking B-GOS to those taking placebo (control group). Compounds identified: Dimethylglycine (DMG); Dimenthylalanine (DMA); Creatinine; Creatine; PAG (Phenylacetilglycine); Cartine; Malonate; TMAO (Trimethylamine-N-oxide); Citrate; Adipate; Alpha-hydroxybutyrate; Beta-hydroxybutyrate; Phenylalanine.

Changes in metabolic profile were also seen after B-GOS intervention in faecal samples. At the baseline, a negative Q^2Y was associated with ASD children in un-restricted diet (Q^2Y= -0.3632), but after B-GOS supplementation, a significant OPLS-DA model was obtained (Q^2Y=0.2997, P <0.001). Ethanol, DMG and SCFAs were positively correlated with B-GOS intake, in particular butyrate and valerate. In addition, lower levels of amino acids and lactate were detected in the B-GOS group, compared to the control (Figure 5.8).

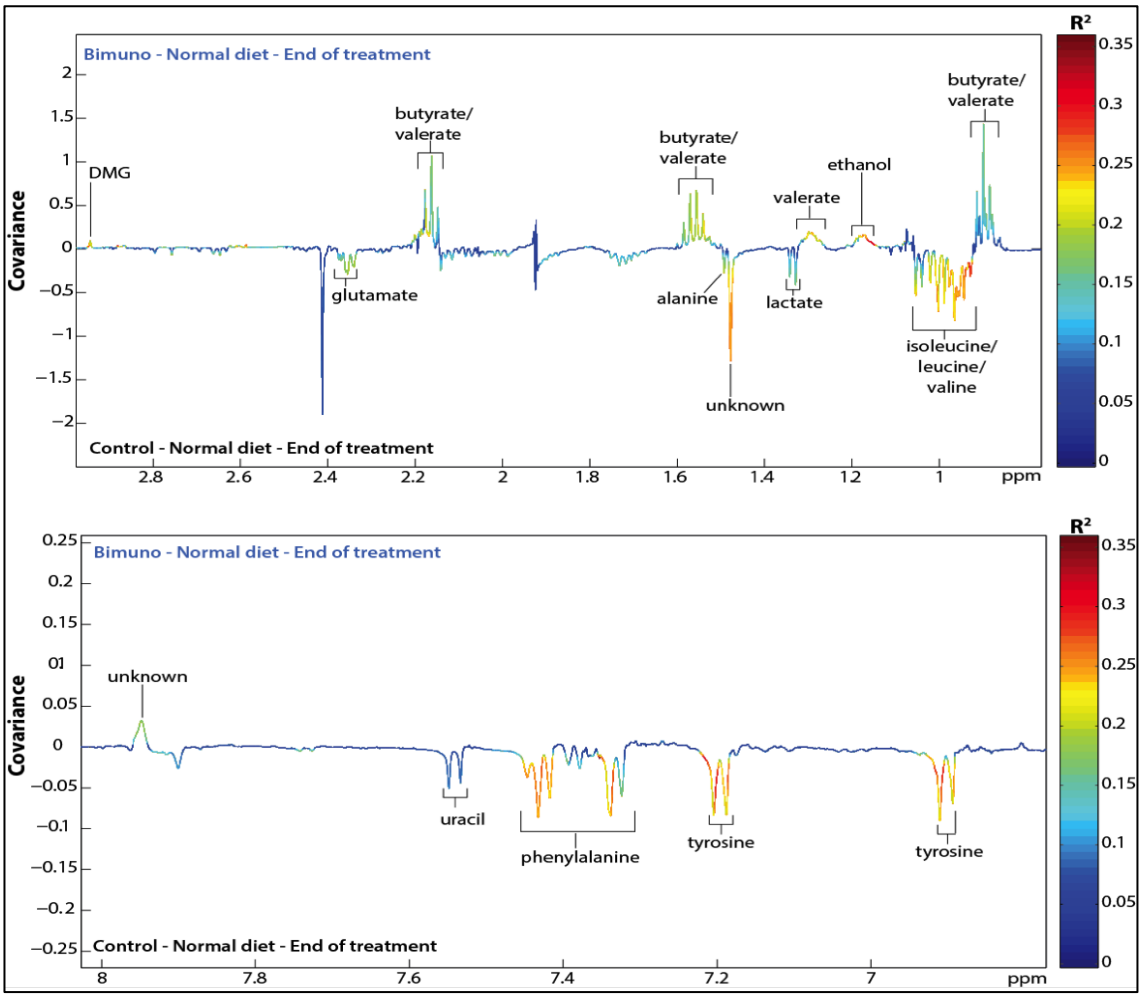


Figure 5.8: OPLS-DA obtained comparing the metabolic profile in faecal samples of ASD children in un-restricted diet taking B-GOS to those taking placebo (control group). Compounds identified: Dimethylglycine (DMG); glutamate; butyrate; valerate; ethanol; alanine; lactate; isoleucine; leucine; valine; uracil; phenylalanine; tyrosine.

5.4 Discussion

It has been suggested that pre- and probiotics have potential effects on the gut-brain axis (Li *et al.* 2017), therefore they have been considered a potential novel therapeutic approach for improvement of behavioural traits and GI discomforts associated with some ASD children. This study was designed to understand if a well-known prebiotic B-GOS was able to modulate the gut bacterial population and metabolic profile of ASD individuals and if it was able to impact other autistic traits, such as mood, behaviour and sleep.

Overall, our data did not show a strong impact on GI symptoms, sleep, mood and behaviour. The reason for this might in part be due to various difficulties reported by the parents in evaluating these aspects and related to impediments in communication skills typical of ASD children. Slight improvements were recorded on sleep diaries and,

combining B-GOS treatment with the restriction diet showed a significant reduction in anti-sociability scores, supporting the hypothesis that multiple intervention therapies might have a better impact on such psychological traits.

To date, metagenomic analyses in autism mainly focused on identifying potential biomarker in the gut bacterial composition, usually comparing faecal samples from autistic individuals with siblings and non-autistic controls. Finegold and colleagues found significant differences in *Firmicutes* and *Bacteroidetes* phyla, with the latter more abundant in the ASD individuals, and they identified some genera present in all the autistic participants, such as *Bacteroides, Clostridium, Faecalibacterium, Eubacterium, Ruminococcus, Roseburia, Dorea, Hespellia, Tucibacter* (Finegold *et al.* 2010). These genera have been also detected in our analysis but unfortunately we did not have a non-autistic control for comparison. Other studies showed reduced *Prevotella*, that may be explained by a low carbohydrate intake (Kang *et al.* 2013, Strati *et al.* 2017), whereas others focused on lower bifidobacterial numbers in ASD groups, compared to the typically developing children and significant effects of probiotics on lactobacilli (Adams *et al.* 2011b). All these results support the hypothesis that other bacterial populations might be involved in autism rather than only the clostridial group (Toh and Allen-Vercoe 2015), as it was initially suggested (Bolte 1998).

To our knowledge, this is the first study where 16S rRNA next generation sequencing has been used to better understand the impact of prebiotic intervention on gut microbiota in autism. Our results showed that B-GOS was able to modulate the gut microbiota composition in autistic children under un-restricted diets, exhibiting bifidogenic effects as well as changes in other bacterial groups, such as Lachnospiraceae family, known to be butyrate-producing bacteria. Mego and colleagues reported the same results in a recent human intervention study where regular consumption of GOS treatment induced changes in microbiota, and its modulation was correlated with reduction of gas production (Mego *et al.* 2017).

We supported our metagenomics results by ^{1}H-NMR, detecting butyrate and valerate as main SCFAs produced and matching previous *in vitro* data, where B-GOS supplementation modulated bacterial and metabolic changes in ASD (Grimaldi *et al.* 2017; chapter 3). It has been shown, in cell culture studies, that butyrate seems to regulate tyrosine hydroxylase (TH) mRNA levels and consequently it might regulate catecholamine pathway in the brain (DeCastro 2005; Shah 2006; Parab 2007; Nankova 2003; Patel 2005) thus, potentially positively impacting ASD (Nankova *et al.* 2014).

Therefore, showing that B-GOS was able to stimulate butyrate production in ASD *in vitro* and *in vivo*, suggests that it could have an effect indirectly on the CNS through the modulation of the gut bacterial populations.

B-GOS seemed able to modulate presence of glutamate in the intervention group. Glutamate is the precursor of GABA, neurotransmitters in the brain, and its alteration might influence the CNS of autistic individuals (Mazzoli and Pessione 2016). Intestinally, reduction of amino acids in faecal samples of ASD children taking B-GOS occurred. A presence of these compounds in stool samples has been associated with disorders related to gut inflammation, such as Inflammatory Bowel Disease (Marchesi *et al.* 2007), therefore our results suggest that B-GOS supplementation could help to improve gut health. In addition, our results could be associated with butyrate production, detected after B-GOS intervention, since improvements in barrier functions have been previously detected *in vitro* with sodium butyrate (Wang *et al.* 2012c).

Some confounding factors might impact on the analysis of urine samples, such as medication, diet and lifestyle. They are difficult to control especially when the sample size is small; this might explain our results, even though diet and medication intake were recorded during the trial. Strong correlations were identified in ASD children under un-restricted diets between citrate, creatine, creatinine and B-GOS. These metabolites were previously identified by Yap and colleagues who did not find any significant correlation with autism (Yap *et al.* 2010); in other studies, inconsistent levels of creatinine have been detected in ASD individuals and considered potential biomarkers for creatine deficiency syndrome (CDS), a metabolic disorder with similar features to autism (Wang *et al.* 2011a). In addition, they have been considered as biomarkers for other brain disorders such as Alzheimer's disease (Fukuhara *et al.* 2013) and schizophrenia (Cai *et al.* 2012), so it would be interesting to have a deeper understanding of their implications for ASD. Malonate, α- and β-hydroxybutyrate were also previously correlated to other mental diseases (An and Gao 2016) but there is not much evidence on how the same metabolic pathways may be altered in different psychological disorders.

Urinary NMR spectra were also dominated by dietary and microbial-derived metabolites such as dimethylamine (DMA), trimethylamine N-oxide (TMAO) in the B-GOS group and phenylacetylglutamine (PAG) in the control group. Elevated levels of DMA have been identified in urine of ASD children (Yap *et al.* 2010), however the data could not be correlated with our findings due to the absence of a non-autistic control. TMAO and PAG were reduced after antibiotic treatment (Wang *et al.* 2011b) (Yap *et al.*

2008) proving an impact of the gut microbiota on metabolic pathways. B-GOS was able to modulate the gut bacterial populations; therefore it might have an effect their production.

5.5 Conclusion

In the present study, we demonstrated that B-GOS intervention might have an effect on the microbiome and metabolome in ASD children. Interestingly, we showed that 6 weeks supplementation had a significant effect, in particular, in children on un-restricted diets. These results strengthen our previous assumption that exclusion diet, such as gluten- and casein-free diets, have modulatory effects on the gut environment, therefore further studies testing longer treatment administration should be carried out in order to show functionality effect also in children already following different therapeutic approaches. Other limitations such as lack of typically developing children as a control group, dose of the treatment and small sample size should also be considered. Regardless of all these issues, to date this is the first study that attempted to understand if and how prebiotic supplementation might impact various aspects of autism.

Author's contribution

The authors' contributions were as follows: RG conceived and carried out the research, managed all study visits and communicated with volunteers and drafted the manuscript; JRS and NG were involved in the interpretation of the metabolomics data; JB and IT helped in the experimental work; JLCM and DSN assisted with sequencing analysis; LG contributed in the mood and behaviour questionnaires evaluation; AC, GRG and JV were involved in the trail design and contributed to the critical revision of the manuscript; JV is employed by Clasado Biosciences Ltd, who provided the B-GOS product, marketed as Bimuno®, used within this research.

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CHAPTER 6

6.1 General discussion

In the last few years, prevalence rates of autism have increased, partly due to improvements in medical assessment and public awareness. Generally, it is considered as a childhood condition, but it does have influences throughout life (Lyall 2017). Studies that focus on more intervention to improve life course outcomes are required. The results presented in this thesis have highlighted the potential for B-GOS to be considered as a dietary approach in Autistic Spectrum Disorders (ASDs).

Recently, B-GOS has been seen to have an effect on cognitive function, via the gut-brain axis, in mice and healthy human studies (Williams *et al.* 2016) (Savignac *et al.* 2016) (Savignac *et al.* 2013) (Schmidt *et al.* 2015), supporting the hypothesis that modulation of the intestinal microbiota might help not only to improve GI symptoms associated with ASD, but potentially also impact on the central nervous system and typical autistic traits. Despite studies focusing on the impact of different food supplements in ASD individuals, there is not much information on the effect of prebiotics in autism.

Initially, prebiotic activity of purified B-GOS (65%GOS content) was investigated in faecal samples of healthy volunteers, compared to commercial B-GOS (52%GOS content), using a batch culture fermentation system. FISH was used to assess faecal microbiota composition and metabolic activities were analysed by GC and ^{1}H-NMR. 65%B-GOS was shown to selectivity enhance beneficial bacteria, confirming that reduction in impurities, such as sugars, is important to study activity and functional properties of prebiotics in *in vitro* systems (Chapter 2). In addition, reducing the presence of sugars, such as lactose, in prebiotic products is an important goal to achieve for *in vivo* application; in particular considering lactose intolerance, previously correlated with gastrointestinal and behavioural problems in ASD (Lucarelli *et al.* 1995).

Although batch cultures are considered acceptable models for preliminary studies of gut microbial fermentations, they have several limitations compared to *in vivo* conditions. These systems do not enable long fermentation periods since it is not possible to add extra nutrients or remove waste from the culture; therefore, bacteria will enter the stationary phase, terminating the fermentation. Furthermore, batch cultures only modelling one area of the colon.

It was therefore investigated whether purified B-GOS could modulate faecal microbiome and metabolome of autistic and non-autistic children using a more complex fermentation model. A three-stage continuous system was used in order to better simulate different conditions in the entire colon, through gradients of pH, substrate concentration and residence time (Chapter 3). B-GOS was able to significantly increase bifidobacteria in all vessels, representing different regions of the colon; and modulate other bacterial groups such as *Clostridium* spp., *Roseburia* spp., *Bacteroides* spp., *Atopobium* spp., *Sutterella* spp., *F. prausnitzii* and Veillonellaceae. Furthermore, in parallel with these population changes, functional alterations were also observed in both autistic and non-autistic models with the organic acids acetate and butyrate being increased, as well as ethanol.

Results from the *in vitro* work showed positive effects of B-GOS on faecal bacterial populations and metabolic activity; for this reason a parallel, double-blind, randomised human intervention study was designed in order to assess the effect of B-GOS *in vivo*. Exclusion diets, such as gluten, casein and dairy free diets, are often suggested for ASD individuals in order to reduce GI problems, but to date there are no studies investigating the impact of these restriction diets on gut microbiota and metabolism in autism. Diet has a huge impact on the gut microbiota and it provides the main substrates for bacterial composition and metabolism (Steegenga 2016, Proctor 2017).

Therefore, in Chapter 4 the effect of restricted diets was investigated in order to understand whether they could influence GI issues and if they impact on the general physiology of ASD children. Results highlighted that children following exclusion diets had significant reductions in abdominal pain and bowel movement, but presence of amino acids in faecal samples, previously associated with nutrient absorption and gut inflammation (Marchesi *et al.* 2007), proved that restricted diets might not be able to impact gut health.

In addition, significant differences in gut bacterial populations were reported at baseline. The gut microbiota of ASD children following special diets showed reduced bifidobacteria and Veillonellaceae, confirming previous studies in healthy volunteers (Bonder 2016) and reiterating the key research hypothesis that diet has a strong impact on the gut microbiota.

This was an important outcome that underlines diet as a potential confounding factor that might indirectly "compete" with other intervention approaches, such as

prebiotics; concept that was also confirmed in Chapter 5. Results showed that 6 weeks B-GOS administration had a significant impact on children whose diet was not restricted, increasing bifidobacteria, stimulating other bacterial groups such as Lachnospira family and modulating the metabolome. In this group, metabolic differences were detected in urine with increased DMA, DMG, creatine, creatinine, TMAO, citrate and alpha-hydroxybutyrate after prebiotic intervention, compared to the control group, showing that B-GOS was driving changes in the general metabolism. In addition, B-GOS was also able to modulate faecal metabolome, after 6 weeks intervention, stimulating butyrate production and reducing amino acids in faecal samples of ASD children taking the prebiotic, supporting the assumption that B-GOS intervention might help to improve gut health modulating the gut microbiome and metabolome. The *in vitro* (Chapter 3) in *in vivo* (Chapter 5) sections of this thesis back up cellular studies where a positive impact of butyrate was reported on gut barrier function (Wang *et al.* 2012, Miao et al. 2016).

Even though no big changes were detected in microbiome and metabolome in children following exclusion diets and taking B-GOS, significant improvements were seen in social behaviour, confirming the hypothesis that multiple therapies could have a stronger impact on psychological aspects.

Overall, the results from this project showed that B-GOS could be considered as potentially useful approach for ASD individuals but improvements in the study protocol could help to obtain stronger impacts, perhaps in combination with other dietary interventions.

6.2 Limitations

Despite relevant findings, the study protocol has limitations that should be taken into consideration for future studies.

The length of time was one example. Results supported the idea that a longer prebiotic intervention period is probably required in order to see a stronger effect, especially in individuals that are already following different therapeutic approaches, such as exclusion diets.

Lack of normally developing children as control group was another limitation. This was from the viewpoint of not only understanding differences between the ASD and non-ASD groups at baseline, but also to identify potential pathways through which prebiotics could impact in these groups.

Other limitations are the sample size and diagnosis confirmation through updated questionnaires.

6.3 Future work

Future work should focus on the importance of study protocol in relation to the specific considerations of this subject population, with a high dropout rate (predominantly during the baseline period) and relatively high inter-individual variability.

A parallel study is suitable for this subject population but additional logistics involved might have affected the completion rate, such as weekly sample collection and daily completion of detailed questionnaires and diaries across the whole study. Key questionnaires and diaries should be used in order to help a more direct interpretation of the impact of the prebiotic intervention on behaviour and sleep. In addition, identification of key time points for sample collection is also crucial in order to reduce stress in children that already suffer routine changes.

In addition to the human intervention study, a deeper insight into the mechanistic effect of butyrate in ASD is worth investigating. Recent mouse studies suggested potential correlation between altered gut barrier functions in ASD and IBD and how probiotics may impact on systemic immunity (Hsiao *et al.* 2013) (Lim *et al.* 2017), therefore it would be interesting to better understand how prebiotic modulates gut health in ASD. An animal study, using an autistic mouse model, could be carried out in order to understand how alteration of the gut microbiota by B-GOS influences butyrate production; and to understand the mechanistic effect of this SCFA on gut inflammation, permeability and catecholamine pathway.

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The University of Reading

School of Food Biosciences

Food Microbial Sciences Unit

APPENDIX 4.1

Bi²muno®	Screening No. ⌊ ⌋

VOLUNTEER 4 DAYS FOOD AND DRINK DIARY

PLEASE READ THROUGH THESE PAGES BEFORE STARTING YOUR DIARY

WE WOULD LIKE YOU TO KEEP THIS DIARY AND RECORD EVERYTHING THE CHILD EATS AND DRINKS OVER 4 DAYS (including one day during the weekend). Please include all food and drinks consumed at home and outside the home e.g. school, restaurants. It is very important that the child does not change what he/she normally eats and drinks just because you are keeping this record. Food diaries are very important part of the study because they help us to interpret the information we get from the child's study visits. The more details you add to the descriptions the more accurate our analysis will be and the better the feedback we can give you.

PLEASE KEEP THE CHILD'S USUAL FOOD HABITS

Please return the completed diary at your next visit at the University

We have given you 2 pages for each of the 4 days; please see below the empty sheets after some important guidelines and a useful example. Please write down the date, and indicate the next study visit. Use as many pages as you need for each day.

Issued on:

⌊ ⌋ ⌊ ⌋ ⌊ ⌋ ⌊ ⌋ ⌊ ⌋ ⌊ ⌋ ⌊ ⌋ ⌊ ⌋

day month year
(in letters)

Date of next visit:

⌊ ⌋ ⌊ ⌋ ⌊ ⌋ ⌊ ⌋ ⌊ ⌋ ⌊ ⌋ ⌊ ⌋ ⌊ ⌋

day month year
(in letters)

When should I complete the diary?

- You can choose which days to complete, but **ideally it must be 4 consecutive days** (one of the **days** must be during the **weekend**, the other **three** must be during **Monday-Friday**). You will need to record on the same days of the week for each diet diary.
- Do not record on the day before the intervention visits (i.e. the day when the child eats a standard evening meal and fast).
- Try to **write down what the child eats at the time the child eats it** rather than from memory at a later date. Each diary day covers a 24 hours period, so please include any food or drinks that the child may have had during the night. Remember to include foods and drinks between meals (snacks or nibbles, no matter how small the amount) including water.

How should I complete it?

- A **portion size guide has been included** to help you (please write down which **photo** you are referring to by each portion of food) but you may also weigh child's food portion if you prefer.
- Take a notebook with you if you eat out and try to include as much detail as possible (you could use the labels on food to help you or any details provided in the menu).
- **Keep the labels of ready meals or unusual foods** if they are clean and put in the back of the record book.
- Be as **specific as possible**, for example, Kellogg's cornflakes is better than cornflakes, Tesco's value baked beans etc. **Use brand names** and include details about food types such as semi-skimmed or full-fat milk.
- For **items that are rehydrated, e.g. pasta, rice, noodles and couscous**, it is important to state whether you are recording **dry or cooked weight**, if you do choose to weigh it out. The same applies to foods such as meat, and vegetables (i.e. raw or cooked weight).
- Record any **supplements** taken during diary recording days.
- For items that are eaten and drunk on a very regular basis such as **tea**, record **as much detail as possible** the first time i.e. amount of water, amount of milk, type of milk, the amount of sugar or sweetener, brand of tea bag and then record 'as usual' for these items in future.
- For any meals cooked from a recipe (**home made dishes**), complete the **recipe sheet** provided (please also see the example below). State how much the child ate as a proportion, e.g. one quarter, or half and remember to add this item to the main food diary too, e.g. 'Recipe 1:'.
- Record **how you cooked the child's food (boiled, steamed, fried, grilled, etc.).** If you fried the food, don't forget to record what you fried it in (olive oil; butter; lard, beef dripping, etc.).
- Remember to include any **fats/oils (butter, spread, oils etc.), condiments/sauces** (ketchup, mayonnaise, gravy salad dressing etc) added to food.
- If the child spills anything or doesn't finish a plate of food, add this to the remarks section and **include how much was spilt or left uneaten.**
- State whether the food was fresh, frozen, dried, canned, etc.

- For **takeaway dishes** from a restaurant or a friend's house, please record as much detail about the ingredients (and the portion size) as you can e.g. vegetable curry containing chickpeas, aubergine, onion and tomato. Also record **any other food or drink when the child eats away from home** (e.g. egg and cress sandwich from Marks and Spencer). Brand name or name of the restaurant will be very helpful.
- **Break down** foods like salads, sandwiches and pasta dishes into **individual ingredients** and add each on a separate line in the diary. Include the amount of each ingredient (weight or size).
- For butter, please specify whether it is **spreadable** or **block**, **salted** or **unsalted**.
- Please specify if the child is having a **heaped** or **level** tablespoon/teaspoon.
- If the weight is on the packaging of the food item, record that, noting down the individual ingredients and amounts. Write the weight of the portion that the child actually ate on your food recording sheets.
- An example of a **record sheet** is shown below.
- A table with some useful words to describe items is also included.

REMEMBER TO ALWAYS SPECIFY THE BRAND, INCLUDING SUPERMARKET BRANDS (E.G. SAINBURY'S BASIC OR SAINSBURY'S TASTE THE DIFFERENCE)

The table below lists common foods and drinks and gives examples of the words that you can use to describe these items. (Note: You may wish to use other relevant words that are not on this list)

Food / Drink	Examples of Descriptions
Soft drinks	No added sugar, diet, diluted / undiluted volume. If measured in a pub: pint / half-pint.
Biscuits	Varieties: shortbread / digestive / rich tea / custard cream / etc. With / without chocolate / cream-filled.
Bread	Wholemeal / brown / white / 50:50 / seeded / granary / fruit. Not pre-sliced, medium / thick slice, small loaf, toasted. Crusty / soft, baguette / French stick, bagel, pitta.
Breakfast Cereals	Include details about milk and sugar (type & quantity). Porridge: instant / homemade, quantity of raw oats, toppings.
Butter	Hard / spreadable, salted / unsalted Check butter & not buttery spreads like I Can't Believe It's Not Butter
Cakes & Buns	Type: Victoria / chocolate / fruit / carrot, muffin / sponge / etc. Filling: frosting / royal icing / marzipan / buttercream / jam.
Cheese	Variety: Cheddar (extra mature / mature / mild) / parmesan / brie / etc. Hard / soft / cream / cottage / processed / triangle. Half-fat / light.
Cream	Double / clotted / single / sour / long-life / spray / crème fraiche. Half-fat / light.
Tea	Include details about milk and sugar / sweetener (type & quantity). Cup / mug. Fruit / herbal / green. Filter / instant / decaffeinated / cappuccino / espresso.

Eggs	Medium / large. Scrambled (include details for milk & butter) / boiled / fried (include details for oil).
Fish & Shellfish	Type: salmon, cod, sardines, king prawns, etc. Fresh / canned (oil, brine or water) / frozen. Breadcrumbs / batter / cakes / fingers.
Fruit, Vegetables & Fruit Juice	Varieties: e.g. apples - brae burn, cox, gala, etc. State If peeled and add the weight of the peeled item, e.g. apples, potatoes, kiwi, banana. Canned (juice or syrup) / fresh / stewed / dried / frozen. Fruit juice: freshly squeezed / fresh (e.g. from chilled section) / from concentrate (e.g. long-life).
Gravies & Sauces	Instant / sachet / jar / fresh. With meat juices / milk (type & amount), thick / thin.
Meat & Poultry	Type: pork, chicken, beef, lamb, etc. With / without fat or skin, well-done / medium / rare. Cut: chop / steak / leg / drumstick / mince (lean) / sausages (pork or beef, thick or thin, low-fat) / bacon (streaky or back).
Milk	Skimmed / 1% fat / semi-skimmed / whole (or full-fat). UHT / soya / rice / flavoured / condensed.
Nuts & Seeds	Salted / unsalted / honey roasted / dry. Please state type of nuts & seeds, e.g. walnuts, peanuts, sesame seeds.
Oils	Varieties: olive oil / extra-virgin olive oil / vegetable oil / sunflower oil / rapeseed oil / sesame oil.
Pasta, Rice, Noodles & Couscous	Wholemeal / brown / white / pilaf / egg-fried. It is important to state whether weight is for the dried or cooked ingredient.
Pies & Quiches	Short crust / puff pastry / suet. Individual / family-sized / party-sized. Provide details on filling, e.g. steak and kidney.
Pizza	Size: 8” / 10” / 12”. Takeaway / chilled / frozen. Thick / thin / stuffed crust. List toppings.
Potatoes	Chips: frozen / fresh, oven-baked / fried, thick-cut / crinkle cut / French fries. Mash: remember to include butter, milk, cheese, etc. Roast: remember to include type & amount of fat used. New & Baked: with / without skin, butter.
Sandwiches	Spread / no spread / mayonnaise. List all fillings.
Spreads	Varieties: buttery (e.g. I can’t believe it’s not butter, Clover), olive oil-spread (e.g. Bertolli), vegetable oil spread (e.g. Flora), sunflower spread, soya spread. Low fat / full-fat.
Yoghurts	Creamy / full-fat / low fat / fat-free. Natural / Greek / fruit / toffee / fromage frais

Recipes

What to do if you make a dish containing more than one portion, e.g. lasagne, casserole, meat pie, stir fry, (please see the attached example).

In the **Recipe sheet**:

- **Date** of when the meal was cooked and then eaten
- **Name** of dish
- **Cooking method**
- **Ingredients**
  - List all ingredients – using brand names and amounts (if you are able to weigh things, that would be very useful)
  - It is important to include cooking oils/fats to your ingredients

In the **main diary (Food intake sheet)**:

- Record the **recipe name**, e.g. Lasagne – recipe and served portion.

Figure: Food portion sizes

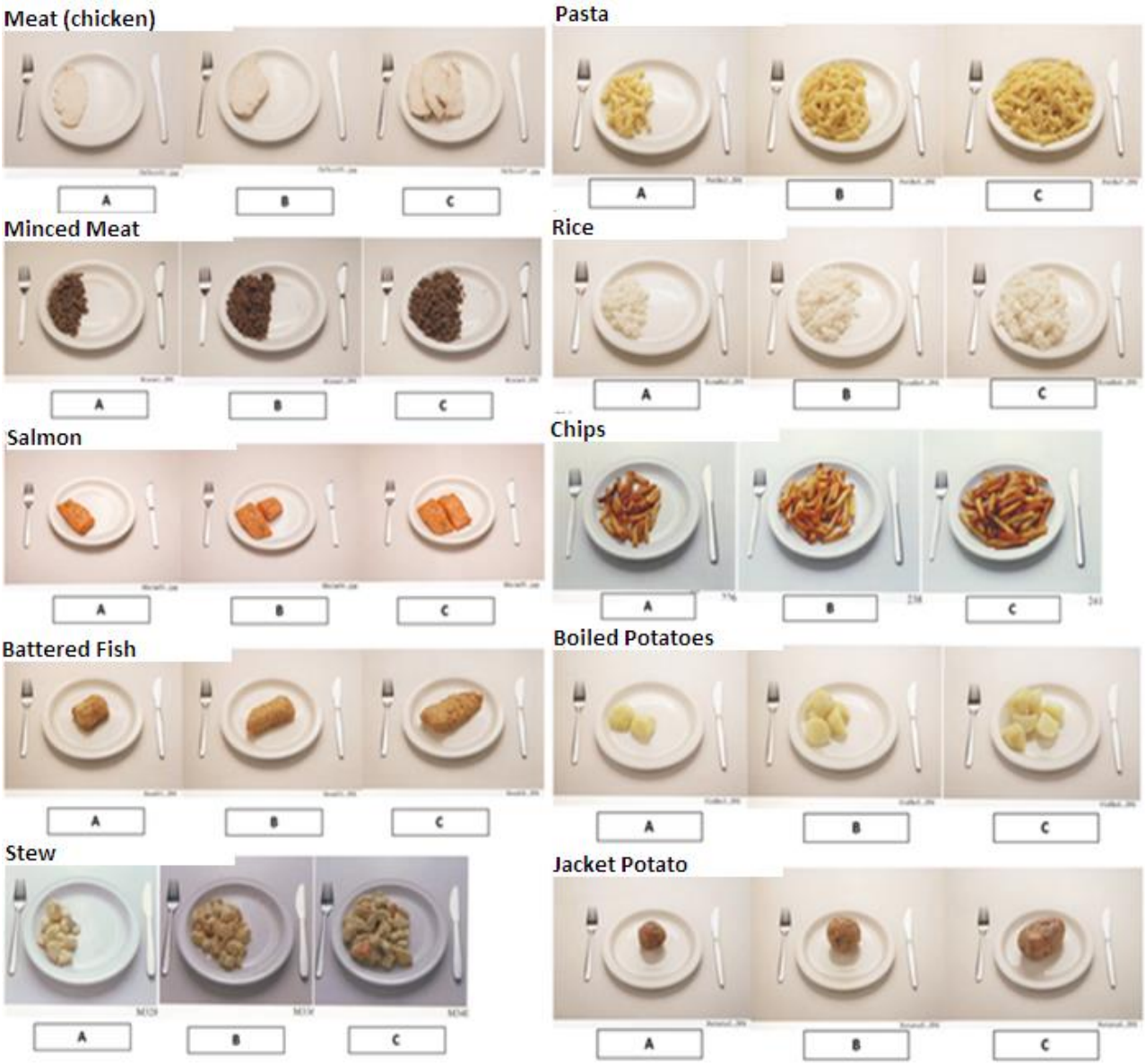
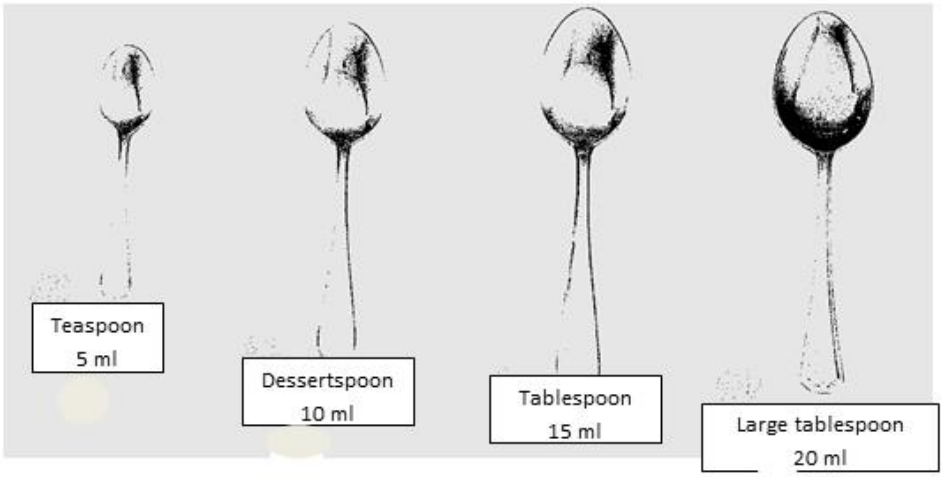
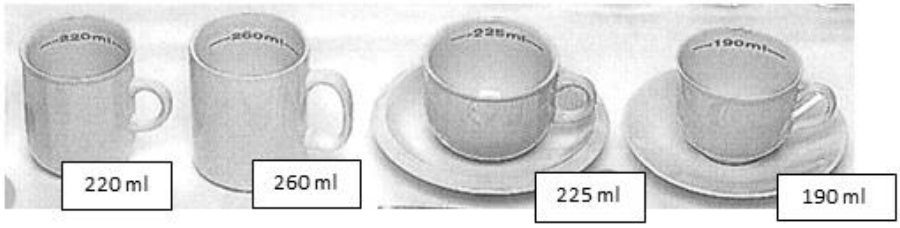
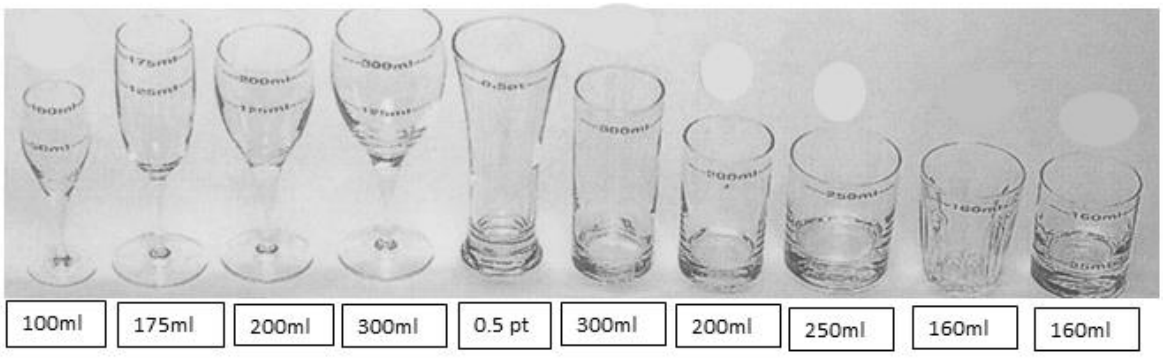


Figure: Food portion sizes







Common used spoons, cups and glasses sizes

Food intake sheet - EXAMPLE Page 1 out of 2

Volunteer Code:	**Day**: Mo Tu We Th Fr Sa Su (please circle)	**Date**: __/__/20_

TIME	BRAND NAME	FOOD/DRINK (description; how cooked; where bought; brand and any comments)	AMOUNT SERVED	LEFT OVER
08.00	Alpen Original	Muesli	Small bowl (A)	
	Tesco	Milk (**semi-skimmed**)	~100mL	
	Tesco	White toast (fresh baked large) bloomer	1 thick slice	
	Anchor	Butter, **spreadable**, **unsalted**	1 teaspoon	
	Robertson's	Marmalade orange (**thick cut**)	1 tablespoon	
10.30		Coffee (from machine), milk (semi-skimmed), no sugar	small cup (190ml)	teaspoon
13.00	Allison's	Bread: **Wholemeal** thick	2 slices	
	Cathedral city	Extra mature cheddar cheese	match box sized (B)	
	Utterly Butterly	Spread, **original**	1 tablespoon	
		Orange	large	Peel & pips
	Tesco	Diet Coca Cola	1 can (330ml)	
15.30	PG Tips	Tea - see recipe sheets	medium mug (220ml)	
		Twix bar	1 regular	
18.00		Chilli con carne – see recipe, page 2	medium portion 300g (B - stew)	
		Cauliflower, **boiled in salted water**	small portion (about 70g) (A – boiled potatoes)	
		Potatoes, **roasted in vegetable oil**	small portion (about 70g) (A)	
	Ski	**Low fat** Strawberry yoghurt	1 small yoghurt	
20.00	Blossom hill	**Red** Wine – Italian Shiraz	1 small glass (175ml)	
22.15		Tap water	Half pint	

Recipe sheet – EXAMPLE Page 2 out of 2

Please use this sheet for any recipes that you use whilst recording the child's intake for us. In the "portion served" box tell us how much of the recipe the child actually ate. Use this sheet also to tell us how the child would usually take tea on a regular basis.

INTERVENTION DIARY

Volunteer Code:	**Day**: Mo Tu We Th Fr Sa Su (please circle)	**Date**: __/__/20_

NAME of RECIPE AND INGREDIENTS (description, cooking method etc)	BRAND NAME	AMOUNT IN RECIPE (g)	PROPORTION SERVED
Tea (medium cup)			220mL
water		200mL	
semi skimmed milk	Tesco	30mL	
Tea bag	PG Tips	strong infusion	
Chilli con carne			¼ of the recipe
Lean minced beef	Tesco	440 g	
1 large onion, diced		Large white onion	
6 cloves of garlic, finely chopped		6 fat cloves	
tin of tomato puree	Tesco	small tin	
tin chopped tomatoes	Napoli	Large can (440g)	
tin red kidney beans, drained and washed	Tesco	Large can (440g)	
Plain flour	ASDA's smart price	**Heaped** tablespoon	
beef stock cube	OXO	1 cube	
1 large red pepper, deseeded and chopped		1 large pepper	
MILD CHILLI POWDER –	ASDA smart price	1 **heaped** teaspoon	
Salt and pepper to season		Pinch of	
Dried mixed herbs	Schwartz's	1 **level** teaspoon	
A little vegetable oil - (enough to cover the bottom of the casserole dish)	Waitrose	2 tablespoons	

General questions about the child's food/ drink in the last 4 days

Special diet

1. Does the child follow a special diet e.g. gluten free, casein free, vegetarian, cholesterol lowering, weight reducing?

No ☐

Yes ☐

If yes, please give details?

INTERVENTION DIARY **(Page 1 of the 1st day of completion)**

FOOD INTAKE SHEET

Volunteer Code: .....**Day**: Mo Tu We Th Fr Sa Su (please circle) **Date**: __/__/20_ Visit.....

TIME	BRAND NAME	FOOD/DRINK (**description; how cooked; where bought; brand and any comments**)	AMOUNT SERVED	LEFT OVER

Recipe Sheet **(Page 2 of the 1st day of completion)**

Use this page only for recipes and then state the amount the child consumed on the previous page

Volunteer Code:	**Day**: Mo Tu We Th Fr Sa Su (please circle)	**Date**: __/__/1_

NAME of RECIPE AND INGREDIENTS (description, brand etc)	BRAND NAME	AMOUNT IN RECIPE (g)	PORTION SERVED

The University of Reading

School of Food Biosciences
Food Microbial Sciences Unit

Bi²muno®	Randomisation No. ⌊ ⌋

APPENDIX 4.2

GI SYMPTOMS DIARY CARD

Issued on: ⌊ ⌋ ⌊ ⌋ ⌊ ⌋
day month year
(in letters)

The Bristol Stool Form Scale: to be used for the evaluation of patient stools!

Stool form	Appearance	Type
Separate hard lumps, like nuts (hard to pass). Result of slow transit		1
Sausage-shaped but lumpy		2
Like a sausage but with cracks on its surface		3
Like a sausage or snake-smooth and soft		4
Soft blobs with clear cut edges (easy to pass)		5
Fluffy pieces with ragged edges, a mushy stool		6
Watery, no solid pieces. Result of very fast transit		7

Bi²muno® **Week …**

Randomisation No. ⌊_____⌋

Date of first day of the week: ⌊__ __ | __ __ | __ __ __ __⌋ day month year

Please complete one row each day.

Day	No. of bowel movements	Stool form (average daily score)	Abdominal pain	Bloating	Flatulence	Product intake
	0=None **1**=One **2**=Two **3**=Three **>3**=More than three	**1**=Separate hard lumps, like nuts **2**=Sausage-shaped but lumpy **3**=Like a sausage or snake but with cracks on its surface **4**=Like a sausage or snake, smooth and soft **5**=Soft blobs with clear cut edges **6**=Fluffy pieces with ragged edges, a mushy stool **7**=Watery, no solid pieces	**0**=None **1**=Present but well tolerated **2**=Present and interfering with but not preventing normal daily activities (like work or sleep) **3**=Preventing normal daily activities			
Example	0 1 2 3 >3 ☐ ☒ ☐ ☐ ☐	1 2 3 4 5 6 7 ☐ ☐ ☒ ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☒ ☐ ☐	0 1 2 3 ☐ ☒ ☐ ☐	0 1 2 3 ☐ ☐ ☒ ☐	yes no ☒ ☐
1	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 5 6 7 ☐ ☐ ☐ ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes no ☐ ☐
2	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 5 6 7 ☐ ☐ ☐ ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes no ☐ ☐
3	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 5 6 7 ☐ ☐ ☐ ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes no ☐ ☐
4	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 5 6 7 ☐ ☐ ☐ ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes no ☐ ☐
5	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 5 6 7 ☐ ☐ ☐ ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes no ☐ ☐
6	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 5 6 7 ☐ ☐ ☐ ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes no ☐ ☐
7	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 5 6 7 ☐ ☐ ☐ ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes no ☐ ☐

At the end of Week … please answer:

	yes	no
Over the past week, do you consider that your child has felt better than in the previous week?	☐	☐
Over the past week, do you consider that your child has slept better than in the previous week?	☐	☐
Over the past week, do you consider that your child was less stressed than in the previous week?	☐	☐
Over the past week, do you consider that your child was more alert than in the previous week?	☐	☐

APPENDIX 4.3

Sample submission form

Screening No……..……

Date……..……

All participants

It is important that this section is filled out for all participants of the study when samples are submitted. The aims of this section are to understand the symptoms, health, treatments and diet of the participant at the time at which the samples were taken.

1. Symptoms, health and treatments

a) Please tick any of the following symptoms or state any other bowel symptoms that have occurred in the week prior to sample collection.

Bi²muno®	Randomisation No. ⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋⌊ ⌋	**Week …**

Please complete the row if there are any changes in GI symptoms

Day	No. of bowel movements	Stool consistency	Abdominal pain	Bloating	Flatulence	Product intake .
		1=Hard **2**=Formed **3**=Loose **4**=Watery	**0**=None **1**=Present but well tolerated **2**=Present and interfering with but not preventing normal daily activities (like work or sleep) **3**=Preventing normal daily activities			
Example	0 1 2 3 >3 ☐ ☒ ☐ ☐ ☐	1 2 3 4 ☐ ☒ ☐ ☐	0 1 2 3 ☒ ☐ ☐ ☐	0 1 2 3 ☒ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☒ ☐	yes ☐ no ☐
1	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes ☐ no ☐
2	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes ☐ no ☐
3	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes ☐ no ☐
4	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes ☐ no ☐
5	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes ☐ no ☐
6	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes ☐ no ☐
7	0 1 2 3 >3 ☐ ☐ ☐ ☐ ☐	1 2 3 4 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	0 1 2 3 ☐ ☐ ☐ ☐	yes ☐ no ☐

2. DIET

1. Has the child changed the diet during sample collection in the week …?

No ☐ Yes ☐

If yes, please give more details below

DAY	DIET	KIND OF SAMPLE COLLECTED			REASON FOR THE CHANGE
		Urine	Saliva	Stool	
1					
2					
3					
4					
5					
6					
7					

3. Behaviour

a) If bowel symptoms (section 1) and/or diet issues (section 2) were evident during the week … when the samples were taken, were these associated with worsened behaviour that is associated with ASD:

bowel symptoms? Yes ☐ No ☐

diet issues? Yes ☐ No ☐

b) If yes to either or both, please provide details:

When did it happen?
Please specify the which day of the week and which sample you were collecting

…….

How long ago did this occur?

……..

How long did this last?

……..

What aspects of behaviour changed?

……..

……..

4. Concomitant medications

Is the child taking any concomitant medication, including dietary supplement?

If YES, please fill in the concomitant medication section below

Concomitant medication log

Enter all medications taken during the week ...

MEDICATION NAME	**TYPE** (capsule, tablet, suppository, enema, syrup, *etc*.)	**DOSAGE**	**FREQUENCY** (once only, once a day, twice a day, once weekly, *etc*.)	**START DATE** (day, month, year)	**END DATE** (day, month, year)	**REASON FOR MEDICATION**

Thank you very much for completing the sample submission form as part of the study. If you have any questions or comments regarding the study, please do not hesitate to contact the study investigators.

Thank you very much for your time and effort.

Always

Unique

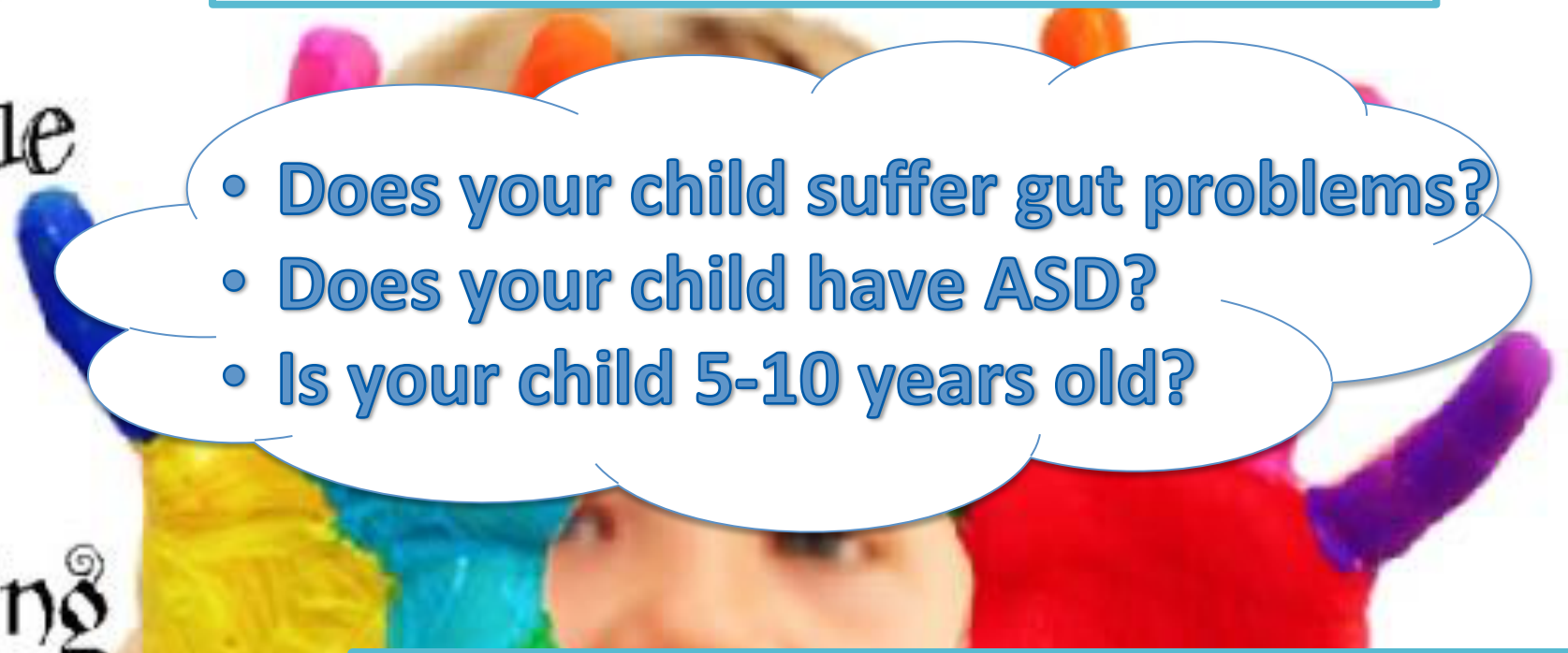
Totally

Interesting

SOMETIMES

Mysterious

VOLUNTEERS NEEDED



- Does your child suffer gut problems?
- Does your child have ASD?
- Is your child 5-10 years old?

If so, would you and your child be willing to participate in our prebiotic feeding study?

For further information please contact

Roberta Grimaldi

on r.grimaldi@pgr.reading.ac.uk or

07538209801

The University of Reading
School of Chemistry, Food
Biosciences and Pharmacy
Food Microbial Sciences Unit

APPENDIX 5.2

Volunteer Information Leaflet

Title: Effect of a prebiotic (B-GOS) supplementation on microbiota and gastrointestinal (GI) symptoms in children with autism spectrum disorders (ASD)

My name is Roberta Grimaldi, I am a research student working at the University of Reading studying the role of the gut bacteria in children with autism spectrum disorders (ASD), especially in relation to gut symptoms. My supervisors are Prof. Glenn R Gibson and Dr. Adele Costabile, who specialise in gut microbiology.

You and your child have been invited to take part in a volunteer research study at the University of Reading.

Before you decide, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish (friends, relatives or your GP, for example). Ask if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of the study?

The aim of this study is to investigate the effect of a dietary prebiotic food supplement (B-GOS, Bimuno - Clasado UK) on composition of the gut bacteria and their products in the gut of children with autism. We aim to recruit 42 children with autism into this study.

Please read the following inclusion/exclusion criteria to identify if your child is able to participate in the trial.

Exclusion Criteria - If the following applies to him/her, he/she will <u>not</u> be able to participate in the trial:

- Use of antibiotics, prebiotics or probiotics (in food products or as supplements) in the last 4 weeks prior to, or during the study period.
- Regular use of certain medications (see section 3.4).
- Children who have received bowel preparation for investigative procedures in the 4 weeks prior to the study.
- Children who have undergone surgical resection of any part of the bowel.

Inclusion Criteria – If the following applies to your child, he/she is suitable for the trial:

- Children aged 5-10 years with formal ASD diagnosis.
- Children's parent or guardian has given written informed consent to participate and is willing to participate in the entire study.

Why is this study being carried out?

It has been known for a long time that bacteria in the lower gut (bowel) can influence health. Some bad bacteria produce unhealthy substances that harm the gut. However, not all bacteria are harmful and most in the gut are actually positive. These produce beneficial substances that the human body can use.

In individuals with ASD, parents and health professionals have commonly recognised a higher incidence of dietary and/or bowel problems. It has been postulated that imbalances in the gut bacteria and/or metabolites present in the gut may be a contributing factor to these symptoms, with potentially bad (toxin-producing) bacteria colonising the gut. Studies to investigate such possibilities have led to inconsistent results and no studies have yet linked imbalances in the gut microbiota with imbalances of gut microbiota products.

Certain carbohydrates (so-called 'prebiotics') are not digested by the human gut and they provide food for beneficial bacteria and thus they improve the composition by preventing the growth of bad bacteria. Individuals could therefore benefit from these safe and effective dietary interventions to maintain the healthy gut bacteria and overall health.

This study is being carried out to establish the effect of one such prebiotic, called galactooligosaccharide (B-GOS) on the relative balance of gut bacteria and metabolites.

What will I be asked to do?

As the parent or guardian, we would like for you to discuss with your child (where appropriate), the potential participant, the purpose of the research study and what would be

involved for them. Where appropriate, it is important that you decide together whether you would like to participate.

The study will last 10 weeks and it will include 2 weeks baseline (before the treatment), 6 weeks treatment and 2 weeks fellow up.

Screening: If you decide to participate, we will ask you to give a brief medical history of your child, to fill in a 4-days food diary and to provide few personal details in order to determine his/her inclusion in this study. This is to provide some background information such as age and gender and will also provide some information on the recent diet and gut health of the participant to the study investigators.
Samples: If your child meets the inclusion criteria, you will be asked to provide your child's following samples: 20g of stool sample (weekly), 10ml of urine sample and 2ml of saliva sample (daily – or as often as possible). These will be used to screen some parameters that we are interested in (composition of gut bacteria and metabolites).

Treatment: We would like to form a group of volunteers that meet all the inclusion criteria that will then be randomly assigned a volunteer number and placed in one of two groups (one of which will start with B-GOS and the other Placebo). A placebo is a dummy treatment, which looks like the real thing but is not. It contains no active ingredient. To minimise any outside influence, neither you nor the investigator will know whether you are taking GOS or Placebo. Details about each stage of the research are provided in a table at the end of this document.
These products will be supplied to you in sachets of powder (1.8g - app. 1 teaspoon) and you will be asked to reconstitute them with water or other drinks or sprinkle them over your child's bowl of cereal. You will be provided with enough sachets to last until your next visit. You will be asked to give to your child one sachet each day. It may be easier to remember if you do that at the same time each day, *e.g.* in the morning, with their normal breakfast.

Gut symptoms and concomitant medication: During the 10 weeks you will be required to fill in a diary recording the number of faecal samples and gut symptoms your child experiences each day. The diary system works using a scale of severity requiring a tick in the appropriate box or a yes/no answer. In addition, any medication your child is taking or has during the study will also have to be recorded, including the type and dosage of the medication and the start and end date.

Behavioural assessment: Before and after the treatment period you will be asked to complete four mood and behaviour questionnaires (AQ, ATEC, EQ-SQ and SCAS-P) and 5-days sleep diary. They will give us additional information about the effect of the treatment related to social and communication difficulties.

Your child and/or you will either be asked to visit the Department of Food and Nutritional Sciences, University of Reading or the investigator will visit your home on 5 different occasions over the course of the 10 weeks study. At each visit, you will be asked to provide

your child's stool, saliva and urine sample. Specific containers and all relevant instructions will be provided for sample collection. Additionally, you will also be asked to fill in some questionnaires related to your child's sleep, mood and behaviour.

Are there any risks?

B-GOS has been widely used in a number of different feeding trials with various groups of volunteers and was well tolerated by all. Participation in this study does not pose any significant risk.

However, some people who have taken other similar prebiotics have reported an increase in gas production, with slight bloating feelings, some flatulence and mild diarrhoea. Cases of this are very rare. Occasionally, people have experienced gut cramping.

Confidentiality

Children's records will be kept strictly confidential. Children's study results will be recorded in a case record form for the study investigator but their name will not appear in this or on any report/publication of the results. Children's records will be kept by the investigators for five years. However, these records will only contain an identification code while information matching volunteer names with identification codes will be kept separately from these files by a departmental secretary. The only time data will be matched with volunteer names is for those volunteers that request to have their personal results discussed with them.

General

- This study has been subject to ethical review by the Southampton Research Ethics Committee and has been given a favourable ethical opinion for conduct.
- The travel expenses (with maximum £15 per visit) to the University will be covered upon receipt of train or bus tickets or at the rate of £0.45 per mile.
- The participation in this study is purely voluntary. Your children may leave the study at any time without giving a reason. You are free to ask the investigator for more information about this study before you give consent for your child to take part or once you have agreed to take part in the study.
- The general results from the study and those relating to children's individual samples may be obtained from Miss Roberta Grimaldi once the study is finished and all statistical analysis has been completed. No information relating to any other volunteer will be given. The information collected during this study and the overall results are still only experimental and although they will give us new information about how the bacteria in the human gut work and what they do, the results can not be used to tell the difference between health and ill-health.

What happens next?

If you would like to take part in this study, or have any questions then please contact the principal investigator Roberta Grimaldi, using the details below. Alternatively, if you are satisfied and happy to participate, then sign the consent forms and fill pre-trial questionnaires and send back to Roberta using the stamped/addressed envelope. Following Roberta will contact you and make arrangement for a visit.

Investigator
Miss Roberta Grimaldi

Department of Food and Nutritional Sciences
University of Reading, P.O. Box 226, Reading RG6 6AP

Telephone: +44 7538209801

E-mail: r.grimaldi@pgr.reading.ac.uk

Sponsor
Clasado Biosciences Ltd.
2-6 Church Street,
St Helier,
Jersey,
JE2 3NN
Telephone: 01534 715 100

Trial Day	Stage of the study	Procedures
-2 weeks	Screening period	Contact the Department of Food and Nutritional Sciences or arrange for an induction session during which you will be asked questions to determine inclusion criteria and you will get the chance to find out details on the study from the investigator. Once you consent to your child's participation in the study, you will be asked to fill in 4-day food diary during this period.
2weeks	Run-in Period	• You will be asked to fill in daily GI symptom diary, 5-day sleep diary and 4 questionnaires related to mood and behaviour of your child. • You will also be asked to collect daily (or as often as possible) urine (10ml) and saliva (2ml) samples and weekly stool samples (20g). You will be provided with instructions and home kits. You will have a choice of either storing samples and bringing them to the University at arranged visits or telephoning the study investigator and requesting collection of the samples. • Weekly telephone calls from the member of the research team will allow you to clarify any questions or concerns and for the research team to assess the compliance with sample collection, storage (where applicable), diary and questionnaires.
6 weeks	Intervention study	• The child will be requested to take 1 sachet of 1.8g prebiotic or placebo food supplement daily. Please reconstitute it with water and make your child take the sachet at the same time each day. • You will be asked to fill in daily GI symptom diary • You will be asked to collect daily (or as often as possible) urine (10ml) and saliva (2ml) samples and weekly stool samples (20g). You will be provided with instructions and home kits. You will have a choice of either storing samples and bringing them to the University at arranged visits or telephoning the study investigator and requesting collection of the samples. • Weekly telephone calls from the member of the research team will allow you to clarify any questions or concerns and for the research team to assess the compliance with sample collection, storage (where applicable), diary and questionnaires. • At the end of the treatment period, you will be asked to fill in 5-day sleep diary and 4

		questionnaires related to your child' behaviour and mood.
2weeks	Follow-up Period	• You will be asked to fill in daily GI symptom diary • You will be asked to collect daily (or as often as possible) urine (10ml) and saliva (2ml) samples and weekly stool samples (20g). You will be provided with instructions and home kits. You will have a choice of either storing samples and bringing them to the University at arranged visits or telephoning the study investigator and requesting collection of the samples. • Weekly telephone calls from the member of the research team will allow you to clarify any questions or concerns and for the research team to assess the compliance with sample collection, storage (where applicable), diary and questionnaires. • At the end of the follow-up period, you will be asked to fill in 4 questionnaires related to your child's behaviour and mood.
Trial Day	**Stage of the study**	**Procedures**
Visit 1	2 weeks before the start of the treatment period	At visit 1 (this could either be investigator visiting your home or you visiting the University of Reading), you will be provided with: • Home kits for collection of stool, urine and saliva samples and given instructions on how to use these kits and store samples. • You will also be provided with GI symptom and sleep diaries. • The regularity of sample collection, storage or any other concerns that you may have with trial procedures will be discussed. • Height and weight measurements will be taken either by the investigator or by you. • You will be asked to provide your child's stool sample on this visit from either that day or any 3 days after visit 1. • Visit will not take longer than an hour.
Visit 2	Start of the intervention period (2weeks)	At visit 2 (this could either be investigator visiting your home or you visiting the University of Reading) all remaining samples and diaries from the run-in period will be collected. You will be asked: • To fill in 4 questionnaires related to your child's behaviour and mood during this visit.

		• You will be provided with correct number of sachets for the first 3 weeks of treatment, home kits for collection of stool, urine and saliva samples, GI symptom diary and given all relevant instructions. • Weight of the child will be recorded either by the investigator or by you. • Concomitant medication will be checked and any changes to child's diet, GI symptoms or behaviour will be discussed with you. Any other concerns that you may have in relation to the study procedures will also be discussed (it will take about 30 minutes). • You will also be asked to provide your child's stool sample on this visit from either that day or any 3 days prior or after visit 2. • Weekly telephone calls from the member of the research team will be maintained during this period too. • Visit will take about 1 and half hours.
Visit 3	3 weeks after the start of the intervention period	At visit 3 (this could either be investigator visiting your home or you visiting the University of Reading) all remaining samples and diary from the first 3 weeks of the intervention period will be collected. You will be provided with: • Correct number of sachets for the remaining 3 weeks of treatment, home kits for collection of stool, urine and saliva samples, GI symptom and sleep diaries and given all relevant instructions. • Concomitant medication will be checked and any changes to the child's diet, GI symptoms or behaviour will be discussed with you. Any other concerns that you may have in relation to the study procedures will also be discussed (it will take about 30 minutes). • You will also be asked to provide your child's stool sample on this visit from either that day or any 3 days prior or after visit 3. • Weekly telephone calls from the member of the research team will be maintained during this period too. • Visit will take about 30-45 minutes.
		At visit 4 (this could either be investigator visiting your home or you visiting the University

Visit 4	End of the intervention period (3weeks)	of Reading) all remaining samples and diaries from the intervention period will be collected. • You will be asked to fill in 4 questionnaires related to your child's mood and behaviour during this visit. • You will be provided with home kits for collection of stool, urine and saliva samples, GI symptom diary and given all relevant instructions (it will take about 30 minutes). • Weight of your child will be recorded either by the investigator or by you. • Concomitant medication will be checked and any changes to your child's diet, GI symptoms or behaviour will be discussed with you. Any other concerns that you may have in relation to the study procedures will also be discussed (it will take about 30 minutes). • You will also be asked to provide your child's stool sample on this visit from either that day or any 3 days prior or after visit 4. • Weekly telephone calls from the member of the research team will be maintained during this period too. • Visit will take about 1 and half hours.
Visit 5	End of the trial (2weeks)	At visit 5 (this could either be investigator visiting your home or you visiting the University of Reading) all remaining samples and diary from the follow-up period will be collected. • You will be asked to fill in 4 questionnaires related to your child's behaviour and mod during this visit. • Weight of your child will be recorded either by the investigator or by you. • Concomitant medication will be checked and any changes to the child's diet, GI symptoms or behaviour will be discussed with you. • You will be asked to provide your child's stool sample on this visit from either that day or any 3 days prior or after visit 5. • Visit will take about 1 and half hours.

The University of Reading
School of Chemistry, Food
Biosciences and Pharmacy
Food Microbial Sciences Unit

APPENDIX 5.3

Volunteer Information Leaflet (Primary aged School children)

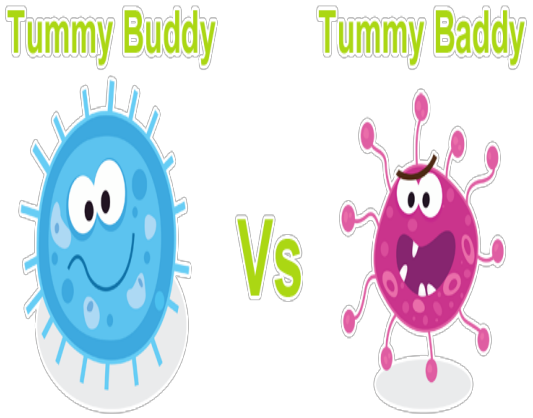
Would you like to help me answer a tricky question?

Hello my name is Roberta and I need your help!
I would like to understand is whether
eating a sugar can help
to stop your tummy problems.

Please read the sheet carefully and talk to your mum and dad to see if you can take part. If you have any questions please ask me.

What is the research about?

Some children have tummy problems, for example pain and toilet problems. ots and lots of bacteria live in the tumm Some of them are 'friendly' and some are 'unfriendly'.



These 'unfriendly' bacteria may cause the tummy problems. Eating this sugar could help to stop your tummy pain increasing the 'friendly' bacteria and reducing the 'unfriendly' bacteria.

What will I have to do?

You will need to do:

1) Take a prebiotic powder every day for 6 weeks with your Breakfast
(It does not have any smell or taste)

2) Give Roberta <u>10 poo samples</u>, so she can analyse your tummy bacteria and <u>urine and saliva</u> samples as often as possible. Your mum and dad will help you in using the kit to collect the sample.

3) Help your mum in completing diaries after the sample collection and how your tummy feels

Talk to your mum and dad, they will help you decide.
You can also talk to me and ask me as many questions as you want.

Miss Roberta Grimaldi

Department of Food and Nutritional Sciences
University of Reading, P.O. Box 226,
Reading RG6 6AP

Telephone: +44 7538209801

E-mail: r.grimaldi@pgr.reading.ac.uk

School of Chemistry, Food and Pharmacy
University of Reading
Whiteknights
PO Box 266, Reading RG6 6AP, UK

APPENDIX 5.4

Consent Form – Parent/Guardian consent

If the individual who will be providing the samples for the study are under 16 years of age and do not wish to consent for themselves or if they are unable to understand fully the study outlined in the information sheet, the parent/guardian is asked to provide consent for their children in the study.

1. I confirm that I am the parent/guardian of…….. (Please initial) ☐

2. I have read and had explained to me by …….. the accompanying information sheet relating to the project entitled "Effect of a prebiotic (B-GOS) supplementation on microbiota and gastrointestinal (GI) symptoms in children with autism spectrum disorders (ASD)". (Please initial) ☐

3. As the parent/guardian of the child involved in the study, I have had explained to me the purposes of the project and what will be required of my child and any questions I had, have been answered to my satisfaction. I agree to the arrangements described in the information sheet in so far as they relate to my participation. (Please initial) ☐

4. As the parent/guardian of the child involved in the study, I understand that participation is entirely voluntary and that my child has the right to withdraw from the study at any time without giving reason, and that this will be without detriment to any care or services they may be receiving or may receive in the future. (Please initial) ☐

5. As the parent/guardian of the child involved in the study, I authorise the Investigator to consult the General Practitioner of my child and I authorise their General Practitioner to disclose any information which may be relevant to my proposed participation in the project. (Please initial) ☐

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University of Reading
Whiteknights
PO Box 266, Reading RG6 6AP, UK

6. This project has been subjected to ethical review, according to the procedures specified by the University Research Ethics Committee, and has been given a favourable ethical opinion for conduct. (Please initial) ☐

7. As the parent/guardian of the child involved in the study, I have received a copy of this consent form and of the accompanying information sheet. (Please initial) ☐

8. As the parent/guardian of the child involved in the study, I consent to my child providing urine and saliva sample daily and one faecal sample weekly. (Please initial) ☐

9. As the parent/guardian of the child involved in the study, I wish to receive a summary of the results once the study is complete and analysed statistically. (Please initial as appropriate)

   Yes ☐ No ☐

10. As the parent/guardian of the child involved in the study, I have had explained to me that my child and my contact details and personal information are kept strictly confidential and only the investigator can have access to them. My child and me will be matched to an identification code and my child and my personal details will be kept separately from this file. (Please initial) ☐

11. As the parent/guardian of the child involved in the study, I have had explained to me that consent for my contact details and personal information to be added to the Nutrition Unit Volunteer Database is entirely voluntary. Accordingly I consent as indicated below:

    I consent to my contact details being stored on the Nutrition Unit Volunteer Database. (Please initial as appropriate)

    Yes ☐ No ☐

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University of Reading
Whiteknights
PO Box 266, Reading RG6 6AP, UK

I consent to the information that I provided in the pre-study questionnaire being stored on the Nutrition Unit Volunteer Database. (Please initial as appropriate)

Yes ☐ No ☐

Name ……

Signed ……

Date ……

I consent to the GP of my child being informed of their participation in the study and of my screening results and give his/her contact details below.

Name ……

Address ……...……

……

Telephone …….

Witnessed by

Name …….

Signed …….

Date ……..…

APPENDIX 5.5

Assent Form – Children

WOULD YOU LIKE TO HELP ROBERTA?

1. My name is……
   (Please colour the box) ☐

2. I talked to my mum and dad about Roberta's study
   (Please colour the box) ☐

3. I am happy to help Roberta in her study
   (Please colour the box) ☐

SIGNATURE ……..

DATE……..

The University of Reading

Head of Department
Professor Bob Rastall

Department of Food and Nutritional Sciences
Food Microbial Sciences Unit

Whiteknights
Reading RG6 6AP

Phone +44 7538209801
Email r.grimaldi@pgr.reading.ac.uk
Web www.food.rdg.ac.uk/

date

APPENDIX 5.6

Dear Dr.

Your patient [*name*] has been enrolled into a trial conducted by the University of Reading, on behalf of Clasado Biosciences Ltd., St Helier, Jersey.
Details of the trial procedure can be obtained from the Department of Food and Nutritional Sciences at the University of Reading, at the address given above. Briefly, your patient was asked to take a new food supplement, Bi^{2}muno®, which is a prebiotic.

GI bacteria secrete detrimental as well as beneficial compounds and overgrowth of certain species or imbalances (i.e. increases in less beneficial and decreases in beneficial bacteria) in the gut microbiota have been associated with ASD. Certain carbohydrates (so-called 'prebiotics') are not digested by the human gut and they provide food for beneficial bacteria and thus they improve the composition by preventing the growth of bad bacteria. Individuals could therefore benefit from these safe and effective dietary interventions.

We have provided the parents of your patient with an information leaflet, giving details about Bi^{2}muno® and the trial procedures. The parents of your patient were asked to sign an informed consent and the patient has undergone screening before his/her inclusion into the study. Your patient is now receiving daily dose of either Bi^{2}muno® or placebo and will continue to receive the treatment, in this parallel study, for the duration of 10 weeks. No other change to diet or lifestyle is required. At the end of the study, it will be possible for the parents of your patient to find out when the patient was receiving active or placebo product.

Should you require any further information, please do not hesitate to contact me.

Yours sincerely,

Roberta Grimaldi

Randomisation number:
Date:

APPENDIX 5.7

ATEC Questionnaire

Please circle the letters to indicate how true each phrase is:

I. Speech/Language/Communication: *[N] Not true [S] Somewhat true [V] Very true*

N S V 1. Knows own name
N S V 2. Responds to 'No' or 'Stop'
N S V 3. Can follow some commands
N S V 4. Can use one word at a time (No!, Eat, Water, etc.)
N S V 5. Can use 2 words at a time (Don't want, Go home)
N S V 6. Can use 3 words at a time (Want more milk)
N S V 7. Knows 10 or more words
N S V 8. Can use sentences with 4 or more words
N S V 9. Explains what he/she wants
N S V 10. Asks meaningful questions
N S V 11. Speech tends to be meaningful/ relevant
N S V 12. Often uses several successive sentences
N S V 13. Carries on fairly good conversation
N S V 14. Has normal ability to communicate for his/her age

II. Sociability: *[N] Not descriptive [S] Somewhat descriptive [V] Very descriptive*

N S V 1. Seems to be in a shell – you cannot reach him/her
N S V 2. Ignores other people
N S V 3. Pays little or no attention when addressed
N S V 4. Uncooperative and resistant
N S V 5. No eye contact
N S V 6. Prefers to be left alone
N S V 7. Shows no affection
N S V 8. Fails to greet parents
N S V 9. Avoids contact with others
N S V 10. Does not imitate
N S V 11. Dislikes being held/cuddled
N S V 12. Does not share or show
N S V 13. Does not wave 'bye bye'
N S V 14. Disagreeable/not compliant
N S V 15. Temper tantrums
N S V 16. Lacks friends/companions
N S V 17. Rarely smiles
N S V 18. Insensitive to other's feelings
N S V 19. Indifferent to being liked
N S V 20. Indifferent if parent(s) leave

III. Sensory/Cognitive Awareness: *[N] Not descriptive [S] Somewhat descriptive [V] Very descriptive*

N S V 1. Responds to own name
N S V 2. Responds to praise
N S V 3. Looks at people and animals
N S V 4. Looks at pictures (and T.V.)
N S V 5. Does drawing, coloring, art
N S V 6. Plays with toys appropriately
N S V 7. Appropriate facial expression
N S V 8. Understands stories on T.V.
N S V 9. Understands explanations
N S V 10. Aware of environment
N S V 11. Aware of danger
N S V 12. Shows imagination
N S V 13. Initiates activities
N S V 14. Dresses self
N S V 15. Curious, interested
N S V 16. Venturesome - explores
N S V 17. "Tuned in" — Not spacey
N S V 18. Looks where others are looking

IV. Health/Physical/Behavior:

Use this code: [N] Not a Problem [MI] Minor Problem [MO] Moderate Problem [S] Serious Problem

N MI MO S 1. Bed-wetting
N MI MO S 2. Wets pants/diapers
N MI MO S 3. Soils pants/diapers
N MI MO S 4. Diarrhea
N MI MO S 5. Constipation
N MI MO S 6. Sleep problems
N MI MO S 7. Eats too much/too little
N MI MO S 8. Extremely limited diet
N MI MO S 9. Hyperactive
N MI MO S 10. Lethargic
N MI MO S 11. Hits or injures self
N MI MO S 12. Hits or injures others
N MI MO S 13. Destructive
N MI MO S 14. Sound-sensitive
N MI MO S 15. Anxious/fearful
N MI MO S 16. Unhappy/crying
N MI MO S 17. Seizures
N MI MO S 18. Obsessive speech
N MI MO S 19. Rigid routines
N MI MO S 20. Shouts or screams
N MI MO S 21. Demands sameness
N MI MO S 22. Often agitated
N MI MO S 23. Not sensitive to pain
N MI MO S 24. "Hooked" or fixated on certain objects/topics
N MI MO S 25. Repetitive movements (stimming, rocking, etc.)

The University of Reading

School of Food Biosciences

Food Microbial Sciences Unit

Bi²muno®	Screening No. ⌊ ⌋

APPENDIX 5.8

VOLUNTEER 5 DAYS SLEEP DIARY

*You can choose which days to complete, but **it must be 5 consecutive days.***

Please return the completed diary at your next visit at the University

We have given you 1 page for each of the 5 days; please see below the empty sheets.
Please write down the date, and indicate the next study visit.

Issued on:

⌊ ⌋ ⌊ ⌋ ⌊ ⌋
day month year
(in letters)

Date of next visit:

⌊ ⌋ ⌊ ⌋ ⌊ ⌋
day month year
(in letters)

Starting Day: Mo Tu We Th Fr Sa Su (please circle) **Date:** __/__/20__

DAY 1

Morning questions

Answer the following questions about your child's sleep last night

1. What time did your child go to bed last night? _____
2. What time did your child fall asleep last night? _____
3. Did your child wake up during the night last night?

YES NO (circle one)

a) If YES, how many times _____
b) If YES, how much time were they awake_____

4. Last night, did your child take anything to help them sleep?

YES NO (circle one)

a) If YES, what did they take _____
b) If YES, what time did they take it_____

5. What time did your child get up for the day today? _____
6. About how many hours did your child sleep last night? _____

Bedtime question:

Answer this question about your child's day before they go to bed

7. Did your child take any naps during the day or evening today?

YES NO (circle one)

a) If YES, how much time total did your child sleep during the day and evening today _____

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APPENDIX 5.9

Instructions to collect stool and urine samples

Stool and urine samples are biologically active and hence, safe handling is essential. Please read these instructions before you collect a stool or urine sample from your child. These guidelines are to aid you in sample collection as easily and safely as possible using the sample collection kit provided for collecting stool and urine samples.

The sample collection kit comprises of the following:

- two stool collection pots
- a paper loop to fit over the toilet seat
- a urine collection container (specimen jar)
- two polythene bags to insert the sampling tubes for freezing (one for stools and one for urine)
- 2 larger protective plastic tubes

Step-by-step instructions for stool sample collection (Pictures below)

1. Open the envelope and insert the paper loop over the toilet seat (step 1-2).
2. Ask your child to provide a stool sample onto the paper loop.
3. Use the spatula inside the inner (smaller) sampling pot to scoop a portion (approximately four peas worth) of stool sample from the middle of the stool sample. Please try to minimise the urine getting into the stool sampling tube (step 5).

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Stool sample pot

4. Insert the spatula with the stool sample into the inner (smaller) sampling pot and tightly seal.
5. Repeat this with the second tube, so that we have a replicate sample.
6. Place both tubes with the samples into the larger protective plastic tubes provided and ensure that the protective tube is tightly sealed.
7. Place the protective tubes into the polythene bag provided and seal the bag, folding twice around the strap fixed and then folding the side straps into the bag.
8. Freeze the samples immediately. It is essential that this is performed within ten minutes of the sample being collected.
9. Dispose of the rest of the sample and the paper loop down the toilet.
10. Please contact the study investigators as soon as possible once the sample has been collected from the child and stored in the freezer and they will arrange for the sample to be collected within two weeks.

Step-by-step instructions for urine sample collection

1. Ask your child the parents to provide a urine sample from their child into the urine collection container (specimen jar).

2. Wash your hands

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3. Peel open the pack
4. A) help the child to pass a SMALL QUANTITY of urine into the toilet and stop
   B) help the child to pass a FURTHER QUANTITY of urine directly into the container and stop
   C) Finish helping the child to pass urine directly into the toilet
   D) detach the funnel from the container
   E) discard the funnel
   F) FASTEN THE LID SECURELY
5. Wash your hands and write your child's ID number on the label provided.
6. Place the protective tubes into the polythene bag provided and seal the bag, folding twice around the strap fixed and then folding the side straps into the bag.
7. Freeze the samples immediately. It is essential that this is performed within ten minutes of the sample being collected.
8. Please contact the study investigators as soon as possible once the sample has been collected and stored in the freezer and they will arrange for the sample to be collected within two weeks.

If you have any questions regarding these guide lines please do not hesitate to contact the study investigators.