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Production of Bioactive Metabolites by Intestinal Bacteria

A Thesis Presented to the National University of Ireland for the Degree of Doctor of Philosophy

By

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“Somewhere, something incredible is waiting to be known.”
Carl Sagan

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Declaration

This Thesis has not been previously submitted, in part or in whole, to this or any other university for any degree and is, unless otherwise stated, the original work of the author.

Author Contribution

All of the work described herein was performed independently by the author, with the following exceptions:

Chapter 3

Dr. Rebecca Wall assisted with the design of the experiments and tissue analyses. Dr. Orla O'Sullivan conducted bioinformatic analysis of pyrosequencing data.

Chapter 4

Tatiana M. Marques is second author of this chapter (published paper). She contributed for animal feeding, culling, and dissection, along with fatty acid analyses by gas chromatography.

Chapter 5

Mr. Patrick Fitzgerald performed the animal behavioural tests. Dr. Gerard Clarke performed HPLC analysis of brain monoamines and Ms. Paula O'Connor conducted GABA analysis of bacterial culture samples.

Chapter 6

Tatiana M. Marques and Devon Kavanaugh are co-authors of this chapter. TMM performed animal feeding, culling, and dissection, along with short chain fatty acid analysis by gas chromatography. DK performed serum cytokine and caecal secretory IgA analysis, faecal pellet preparation for 454 pyrosequencing, and contributed to the analysis of sequencing results. Dr. Orla O'Sullivan conducted bioinformatic analysis of pyrosequencing data.

_____

Tatiana Milena Marques

Abstract

The adult intestinal microbiota comprises a microbial ecosystem of approximately 100 trillion microorganisms, with specific bacterial communities exhibiting distinct metabolic capabilities. Bacteria produce a range of bioactive compounds to survive unfavourable stimuli and to interact with other organisms, and generate several bioactive products during degradation of dietary constituents, the host is not otherwise capable of digesting. This thesis addressed the impact of feeding potential probiotic bacteria, capable of producing bioactive metabolites, such as conjugated linoleic acid (CLA) and gamma-aminobutyric acid (GABA) and other dietary strategies such as pure fatty acids and oligosaccharides on gut microbiota composition, short chain fatty acid (SCFA) production and modulation of metabolism in animal models.

The aim of the first experimental chapter (Chapter 2) was the optimization of a gas chromatography (GC) method for the analysis of SCFA in intestinal samples. The method involved a direct aqueous injection into a GC system fitted with a high polarity capillary column that proved to be rapid, efficient and accurate for the detection of SCFA in faecal/caecal content, provided that extracted samples were not stored for long periods and the GC system was maintained in good operating conditions. The method was applied in the analysis of SCFA levels in caecal samples obtained in animal studies described in Chapters 3, 4 and 6 of this thesis.

In Chapter 3, *t*10, *c*12 CLA, a CLA isomer known to decrease fat mass and cause steatosis in rodents, was fed to mice and its effects on intestinal microbiota composition and production of SCFA in the large intestine were investigated. Animals receiving dietary *t*10, *c*12 CLA for 8 wk presented a significantly altered gut microbiota composition, harbouring higher proportions of *Bacteroidetes* ($p=0.027$),

including higher proportions of *Porphyromonadaceae* ($p=0.002$) previously linked with negative effects on lipid metabolism and induction of hepatic steatosis. Moreover, significantly higher levels of SCFA ($p<0.05$) were detected in the *t*10, *c*12 CLA–supplemented group compared with the control group probably due to the marked changes in microbiota composition. The data indicate that the effects of dietary *t*10, *c*12 CLA on lipid metabolism in mice may be partially mediated by alterations in gut microbiota composition and functionality. In the following chapter (Chapter 4), human-derived CLA-producing *Bifidobacterium breve* strains were fed to mice for 8 wk to compare their effect on fat composition and distribution and the composition of the gut microbiota. Fatty acid metabolism and microbiota composition were affected by *B. breve* DPC 6330 and *B. breve* NCIMB 702258 administration compared to unsupplemented controls. However, the responses were different indicating that the modulation of the gut microbial community by ingested microorganisms is most likely strain-dependent.

The metabolic and behavioural effects of dietary administration of GABA-producing *Lactobacillus brevis* DPC 6108 and pure GABA were investigated in Chapter 5. In a first study, dietary *Lb. brevis* DPC 6108 significantly increased ($p<0.05$) serum insulin and decreased ($p=0.0511$) anxiety-like behaviour in healthy rats. These data led to a second study using diabetic rats (type 1 diabetes induced by streptozotocin injection) to evaluate if pure GABA and *Lb. brevis* DPC 6108 exerted protective and/or regenerative effects on islet pancreatic β-cells and reversed diabetes. *Lb. brevis* DPC 6108 administration attenuated high levels of glucose, but did not change insulin levels in diabetic animals. Behavioural changes seen as a result of GABA and *Lb. brevis* DPC 6108 administration in healthy animals

were not observed in diabetic animals. Moreover, one single dose of STZ significantly increased glucose ($p<0.001$) and decreased insulin and c-peptide ($p<0.05$) in diabetic animals indicating an extensive loss of β-cell mass that could not be reversed by dietary GABA or *Lb. brevis* DPC 6108. As GABA beneficial effect may be primarily due to modulation of inflammatory response, these data indicate that animal models of auto-immune-induced diabetes may constitute better models to study protective and/or regenerative effects of pancreatic cells by GABA administration.

In Chapter 6, the effect of dietary bovine milk oligosaccharides (BMO), 6'sialyllactose (6'SL) and the commercial prebiotic, Beneo Orafti P95 oligofructose (P95), on murine gut microbiota composition and functionality was assessed. Oligosaccharides supplementation had an impact on gut microbiota composition and, overall, P95, BMO and 6'SL supplementations were associated with depletion or reduction of less favourable bacteria such as *Moraxellaceae*, *Vibrionaceae* and *Porphyromonaceae*. Moreover, levels of SCFA were generally unaffected by dietary BMO, 6'SL and P95, and indigenous *Bifidobacterium* were undetectable in all groups tested. The study demonstrated that ingestion of BMO and 6'SL is a safe and effective approach to modulate populations of the intestinal microbiota, but future studies (e.g using a synbiotic approach) are necessary to assess prebiotic properties of these oligosaccharides. In Chapter 7 (General discussion) the major findings of all studies were reviewed and discussed.

Publications

T. M. Marques, J. F. Cryan, F. Shanahan, G. F. Fitzgerald, R. P. Ross, T. G. Dinan & C. Stanton. (2013) Gut microbiota modulation and implications for host health: dietary strategies to influence the gut-brain axis. *Innovative Food Science and Emerging Technologies*. In Press http://dx.doi.org/10.1016/j.ifset.2013.10.016

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R. Wall, **T. M. Marques**, O. O'Sullivan, R. P. Ross, F. Shanahan, E. M. Quigley, T. G. Dinan, B. Kiely, G. F. Fitzgerald, P. D. Cotter, F. Fouhy, C. Stanton. (2012) Contrasting effects of *Bifidobacterium breve* NCIMB 702258 and *Bifidobacterium breve* DPC 6330 on the composition of murine brain fatty acids and gut microbiota. *American Journal of Clinical Nutrition, 95*(5), 1278-1287. PMID: 22492373

E. F. Murphy, P. D. Cotter, S. Healy, **T. M. Marques**, O. O'Sullivan, F. Fouhy, S. F. Clarke, P. W. O'Toole, E. M. Quigley, C. Stanton, P. R. Ross, R. M. O'Doherty, F. Shanahan. (2010) Composition and energy harvesting capacity of the gut

microbiota: relationship to diet, obesity and time in mouse models. *Gut, 59*(12), 1635-1642. PMID: 20926643

T. M. Marques, R. Wall, R. P. Ross, G. F. Fitzgerald, C. A. Ryan, C. Stanton. (2010) Programming infant gut microbiota: influence of dietary and environmental factors. *Current Opinion in Biotechnology, 21*(2), 149–156. PMID: 20434324

Abbreviations

5-HIAA 5-hydroxyindoleacetic acid

5-HT 5-hydroxytryptamine/serotonin

AAD Antibiotic-associated diarrhoea

AADC Aromatic-L-amino acid decarboxylase

AAP American Academy of Pediatrics

ACC Acetyl-CoA carboxylase

aP2 Adipocyte-specific fatty acid binding protein

ARA Arachidonic acid

ATB Antibiotic

BDNF Brain-derived neurotrophic factor

BMI Body mass index

BMO Bovine milk oligosaccharides

CALA Conjugated a-linolenic acid

C/EBP CAAT/enhancer binding protein

CFS Chronic fatigue syndrome

CFU Colony-forming unit

ChREBP Carbohydrate response element-binding protein

CLA Conjugated linoleic acid

CNS Central nervous system

DA Dopamine

DNL *de novo* lipogenesis

DSS Dextran sodium sulfate

DHA Docosahexaenoic acid

DOPAC 4-dihydroxyphenylacetic acid

DPA Docosapentaenoic acid

EAE Experimental autoimmune encephalomyelitis

EPA Eicosapentaenoic acid

EPM Elevated Plus Maze

FA Fatty acids

FAS Fatty acid synthase

FAMEs Fatty acid methyl esters

FHI Food for Health Ireland

FOS Fructooligosaccharides

FST Forced Swim Test

GABA Gamma-aminobutyric acid

GAD Glutamate decarboxylase

GC Gas chromatography

GF Germ-free

GIP Gastric inhibitory polypeptide

GIT Gastrointestinal tract

Gla Gamma-carboxyglutamyl

GLP-1 Glucagon-like peptide-1

GLUT4 Insulin-dependent glucose transporter 4

GOS Galactooligosaccharides

GPR41 G-protein coupled receptor 41

GPR43 G-protein coupled receptor 43

GRAS Generally Recognized as Safe

HF High-fat

HPLC High-performance liquid chromatography

HMO Human milk oligosaccharides

HPA Hypothalamus-pituitary-adrenal

HSL Hormone-sensitive lipase

HVA Homovanillic acid

IBD Inflammatory bowel disease

IBS Irritable bowel syndrome

IgA Immunoglobulin A

IL Interleukin

LCFA Long chain fatty acids

LPL Lipoprotein lipase

LPS Lipopolysaccharides

LXR- α Liver X receptor α

MCT Monocarboxylate transporter

MDSD Multiple low-dose streptozotocin-induced diabetes

MRS Man, Rogosa, and Sharpe (media)

MS Maternal separation

MSG Monosodium glutamate

MUFA Monounsaturated fatty acids

n-3 PUFA Omega-3 polyunsaturated fatty acids

n-6 PUFA Omega-6 polyunsaturated fatty acids

NA Noradrenaline

NAFLD Non-alcoholic fatty liver disease

NEC Necrotizing enterocolitis

NOD Non-obese diabetic

OTUs Operational taxonomical units

PCoA Principal coordinate analysis

PPAR- γ Peroxisome proliferator-activated receptor γ

PSA Polysaccharide A

PUFA Polyunsaturated fatty acids

PYY Peptide YY

qPCR Quantitative real-time PCR

RSD Relative standard deviation

SCD1 Stearoyl-CoA desaturase

SCFA Short chain fatty acids

SEM Standard error of the mean

SFA Saturated fatty acids

SIDS Sudden infant death syndrome

SREBP-1c Sterol regulatory element binding protein 1c

STZ Streptozotocin

TCA Trichloroacetic acid

TFA Trifluoroacetic acid

TLR4 Toll-like receptor 4

TNF-α Tumour necrosis factor-α

T1D Type 1 diabetes

T2D Type 2 diabetes

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Chapter 1

Literature reviews

Chapter 1.1

Programming infant gut microbiota: influence of dietary and environmental factors

In Current Opinion in Biotechnology, 2010, 21(2):149-156

1.1.1 ABSTRACT

The neonatal period is crucial for intestinal colonisation, and the composition of this ecosystem in early life is influenced by such factors as mode of birth, environment, diet and antibiotics. The intestinal microbiota contributes to protection against pathogens, maturation of the immune system and metabolic welfare of the host, but under some circumstances can contribute to the pathogenesis of certain diseases. Because colonisation with non-pathogenic microbiota is important for infant health and may affect health in later life, it is important to understand how the composition of this microbial organ is established and by which dietary means (e.g. supplementation with prebiotics/probiotics/food ingredients) it can be programmed in order to achieve an ecosystem that is valuable for the host.

1.1.2 INTRODUCTION

Microbial colonisation of the sterile infant intestine is an intricate process which is influenced by many factors (O'Toole et al., 2010) including mode of delivery (Grolund et al., 1999), type of feeding (Orrhage & Nord, 1999) and antibiotic therapy (Burman et al., 1999; Kalenic et al., 1993). Within the first year of life, the enteric microflora is highly dynamic but microbial diversity is low, and after the initial year, the microbial population stabilises and resembles that of the adult (Palmer et al., 2007). Whilst traditional culture-based techniques have been used in the past to determine the microbial load of the infant intestine, less biased DNA-based techniques including the use of the 16s ribosomal RNA gene have recently confirmed the dominance of species of *Bifidobacterium, Clostridium* and

Bacteroides in the early microbiota (Hopkins et al., 2005; Penders et al., 2006; Sakata et al., 2005; Wang et al., 2004). However, using sequencing techniques Wang et al. (2004) found that 10% of species from faecal samples of infants after the first two months of life were unidentifiable, whereas 30% unidentified species were observed after the first year of life, highlighting the complexity of the microbiota and the importance of the development of new and more powerful fingerprinting techniques. In a more recent study by Rajilic-Stojanovic et al. (2009), a phylogenetic microarray (referred to as the human intestinal tract chip or 'HITChip') was developed and applied for comparing the effect of ageing on the intestinal microbiota of young and elderly adults. Because of the good reproducibility and the possibility for relative quantification of microbial groups, this technique might be a suitable tool for determining the microbial diversity of the infant gastrointestinal tract in future studies. Another high-throughput alternative test is the recently launched GA-mapTM microarray that will enable screening of the infant gut microbiota based on sets of unique probes that are highly specific to their target group of bacteria. It is envisaged that by providing an 'overall map' of the enteric microbiota, this test will give valuable information to assist in disease intervention (Genetic Analysis AS, 2009).

The mutualistic interactions between the enteric microbiota and the human host are essential for health (Dethlefsen et al., 2007). Indeed, the enteric microbiota can secrete molecules (so called 'pharmabiotics') (Shanahan et al., 2009) that inhibit host pathogens, metabolise compounds that harm the host to less toxic substances (Dethlefsen et al., 2007; Wall, Ross, Ryan et al., 2009) and produce a range of bioactive compounds such as conjugated linoleic acid (CLA), short chain

fatty acids (SCFA) and gamma-aminobutyric acid (GABA) that may play a role in the protection from lifestyle illnesses such as cancer, obesity and cardiovascular diseases (Wall, Ross, Ryan et al., 2009). Moreover, the microbiota contribute to biochemical pathways that humans cannot process because of the lack of proper genes (Kovatcheva-Datchary et al., 2009), such as fermentation of indigestible dietary polysaccharides, metabolism of complex proteins and synthesis of vitamins (Resta, 2009; Wall, Ross, Ryan et al., 2009). The infant gut microbiota can also significantly influence the maturation of the immune system in early days of life (Gottrand, 2008; Kelly et al., 2007; Shanahan et al., 2009). Remarkably, colonisation of the newborn intestine plays a key role in the development and fine-tuning of the intestinal immune responses. Disruption to this process, due for example to antibiotic therapy, may have long-term health consequences, giving rise to immune-related disorders such as eczema, allergic rhinitis and inflammatory bowel disease (IBD) (Conroy et al., 2009; Kelly et al., 2007). For example, in a study conducted by Wang et al. (2008), the intestinal microbial diversity of 18-month-old infants suffering from atopic eczema was reduced in comparison to healthy infants of the same age.

As the infant enteric microbiota is more variable in its composition and less stable over time compared to the adult (Palmer et al., 2007), the use of nutritional strategies in order to shape/programme its composition to favour a more beneficial bacterial population may be a good opportunity to avoid future health problems. Probiotics and prebiotics are widely used as supplements in infant formulae and many studies have confirmed their efficacy in changing the microbiota composition by stimulating the growth of bifidobacteria (Saulnier et al., 2009) and therefore

helping in the treatment and prevention of certain illnesses (O'Hara & Shanahan, 2007; Parracho et al., 2007).

This review will discuss the current knowledge of the microbial diversity in infants and the metabolic capabilities that the enteric microbiota possesses. Furthermore, the impact of diet and dietary supplementation (with probiotics and prebiotics) on the evolution of the microbial diversity in the developing infant will be reviewed.

1.1.3 DEVELOPMENT OF THE INFANT GUT MICROBIOTA

At birth, the newborn infant gastrointestinal tract is almost sterile (DiGiulio et al., 2008; Jimenez et al., 2008; Satokari et al., 2009), but is rapidly colonised in the first days of life, reaching a stable population similar to that of an adult when the infant is around two years old and there is the introduction of solid foods (Dethlefsen et al., 2007; Palmer et al., 2007; Reinhardt et al., 2009; Wall, Ross, Ryan et al., 2009). Immediately after birth, the newborn gut environment is colonised by facultative anaerobic bacteria such as *Enterobacteriaceae*, streptococci and staphylococci (Adlerberth & Wold, 2009; Morelli, 2008; Penders et al., 2006). These first colonisers belong to species with pathogenic potential and might be harmful (Morelli, 2008), however most interactions between humans and microorganisms do not result in disease (Dethlefsen et al., 2007). Instead, these bacteria gradually consume oxygen and produce new metabolites, preparing the intestinal environment for the establishment of a strict anaerobic bacterial population dominated by *Bifidobacterium, Clostridium* and *Bacteroides*, bacterial groups that may play a role in the neonatal gut maturation (Adlerberth & Wold, 2009; Morelli,

2008; Penders et al., 2006). Recently, the more strict hygienic conditions during delivery, short hospital stays and practise of rooming-in (where the mother, not health-care workers mainly handles the baby) have reduced bacterial exposure and altered the colonisation pattern with skin-derived staphylococci been the first colonisers of the infant gut instead of faecal *Enterobacteriaceae* (Adlerberth & Wold, 2009; Morelli, 2008).

The bacterial community colonising the newborn infant gut is dynamic and originates from the environment, mainly from the mother (Dethlefsen et al., 2007; Penders et al., 2006). A broad range of factors can affect the bacterial composition of the gut including mode of delivery (caesarean section or vaginally), type of feeding (exclusive breast-feeding versus formula), gestational age, antibiotic use, hospitalisation, surrounding environment and maternal infection or illness (Adlerberth & Wold, 2009; Biasucci et al., 2008; Morelli, 2008; Penders et al., 2006; Reinhardt et al., 2009) (Fig. 1.1).

Mode of delivery and type of feeding exert the most significant influences on the development of the microbiota in the infant (Butel et al., 2007; Morelli, 2008; Wall, Ross, Ryan et al., 2009). Vaginally born infants are initially colonised by faecal and vaginal bacteria from the mother, whereas infants born via caesarean section are colonised by bacteria from the hospital environment (health-care workers, air, equipment, other newborns) (Biasucci et al., 2008; Morelli, 2008; Penders et al., 2006; Reinhardt et al., 2009). Newborns delivered by caesarean section have in general lower numbers of *Bifidobacterium*, reduced levels of members of the *Bacteroides fragilis* group and higher amounts of *Clostridium difficile* compared to vaginally born infants (Martin & Walker, 2008; Morelli, 2008;

Penders et al., 2006). Moreover, the growth of *Bacteroides*, *Bifidobacterium* and *Escherichia coli* is delayed in infants born by caesarean section (Adlerberth & Wold, 2009; Biasucci et al., 2008; Morelli, 2008).

Traditionally, it has been considered that the microbiota of breast-fed infants are dominated by bifidobacteria and also colonised in lesser quantities by some facultative anaerobic bacteria such as streptococci, staphylococci, enterococci, lactobacilli and enterobacteria, whilst the microbiota of formula-fed infants are more diverse and include bacterial groups such as *Bacteroides, Clostridium* and *Enterobacteriaceae* (Martin et al., 2008; Martin & Walker, 2008; Penders et al., 2006; Wall, Ross, Ryan et al., 2009). However, the effect of diet on the composition of the infant gut microbiota, more specifically in the predominance of bifidobacteria in breast-fed infants is still controversial. Some reports have not found differences amongst the types of feeding (Adlerberth & Wold, 2009; Palmer et al., 2007) and even suggested that modern formulae are more faithful replicas of breast milk, with the addition of prebiotics such as galactooligosaccharides (GOS) and fructooligosaccharides (FOS), increasing the number of bifidobacteria and lactobacilli in the gut of formula-fed infants to a similar number found in breast-fed infants (Adlerberth & Wold, 2009; Boehm & Moro, 2008; Penders et al., 2006).

The gut colonisation pattern of preterm infants differs from that of full-term infants both temporally and qualitatively (Mshvildadze et al., 2008). The several immaturities of the preterm infant gut, the long time spent in the neonatal intensive care unit and the use of broad spectrum antibiotics delay the establishment of a beneficial bacterial community and enable the growth of potentially pathogenic bacteria (Martin & Walker, 2008; Mshvildadze et al., 2008).

Antibiotics negatively affect the composition of the infant gut microbiota by decreasing the numbers of obligate anaerobes (e.g. *Bifidobacterium* and *Bacteroides*) (Martin & Walker, 2008; Reinhardt et al., 2009). However, the effects differ between antibiotics (Penders et al., 2006) and usually most families and genera of gut microorganisms return to typical levels within weeks of exposure (Dethlefsen et al., 2007).

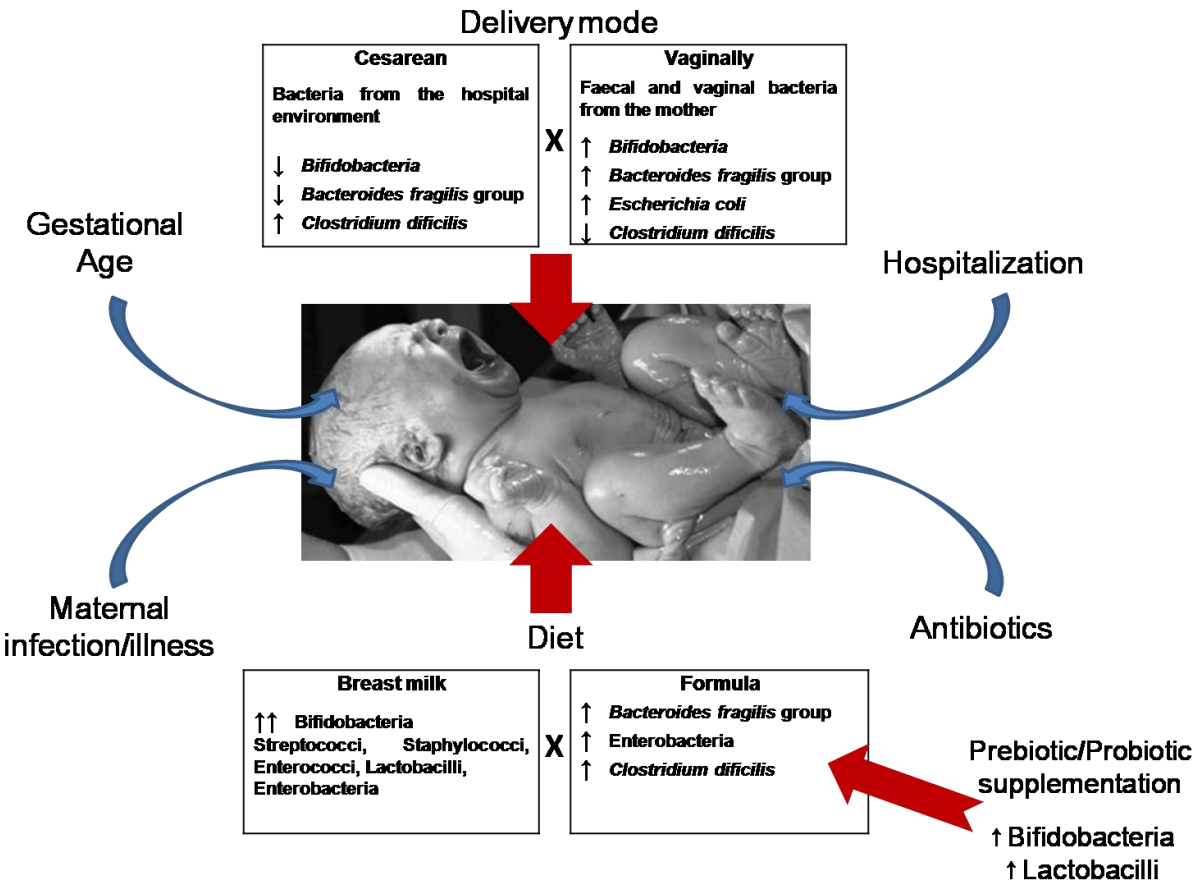


Figure 1.1 Factors affecting the infant gut composition and the differences in the bacterial colonisation influenced by the delivery mode and the diet.

1.1.4 IMPLICATIONS OF MICROBIOTA FOR HOST HEALTH

The enteric microbiota plays an important role in host health, being involved in nutritional, immunological and physiological functions. Along the epithelium, enteric bacteria complement the natural defence barrier against exogenous microbes, thereby preventing invasion by pathogens. In addition, the enteric microbiota has an important role in influencing the normal structural and functional development of the mucosal immune system (Round & Mazmanian, 2009). The molecular interactions between enteric bacteria and the host seem to direct the development of immune responses, and in turn the immune system shapes the composition of the microbiota. Most of the information regarding the effects of the microbiota on the host immune system comes from studies using germ-free animals. Germ-free animals show extensive defects in the development of the gut-associated lymphoid tissue with fewer and smaller Peyer's patches without germinal centres and smaller T cell zones. Furthermore, the lamina propria contains essentially no immunoglobulin A (IgA), plasma cells or CD4 cells, and intraepithelial lymphocytes are also rare compared with conventional animals (Macpherson & Harris, 2004). Germ-free animals are therefore more susceptible to infection compared with conventional animals. However, colonisation of animals with a single bacterium (*Bacteroides fragilis*) capable of producing polysaccharide A (PSA) has been shown to correct mucosal and systemic immune defects, including correcting systemic T cell deficiencies and restoring the balance between T_H cell subsets (Mazmanian et al., 2005).

Because the microbiota influences the developing immune system, variations from the normal bacterial colonisation pattern through modern

strategies such as caesarean section, formula-based diet, hygiene and the excessive use of antibiotics in infants, may change the outcome of immune development and thus potentially predispose to certain inflammatory diseases in later life. Indeed, increased rates of several immune-mediated disorders, such as IBD, atopy, asthma and rheumatoid arthritis have occurred in recent years in the 'Western' populations. This increase may in part, be because of changes in host–microbe interactions caused by implementation of different antimicrobial strategies (i.e. excessive use of antibiotics, hygiene and Western diets).

Recent evidence from animal and human studies indicate that the composition of the gut microbiota may also be involved in several extra-intestinal disorders such as obesity (Backhed et al., 2004; Ley et al., 2006; Turnbaugh et al., 2006), insulin resistance (Cani et al., 2008) and non-alcoholic fatty liver disease (NAFLD) (Dumas et al., 2006). For example, conventionally raised mice have been shown to have 40% more body fat than their germ-free counterparts and colonisation with a normal gut microbiota induces hepatic lipogenesis and increases lipid storage in adipocytes (Backhed et al., 2004). Moreover, it has been reported that differences in the gut microbiota during the first year of life may precede the onset of obesity (Kalliomaki et al., 2008). In this study, the numbers of *Bifidobacterium* spp. were higher and the numbers of *Staphylococcus aureus* were lower in children who remained at normal weight than in children who became overweight. Thus, a microbiota profile in favour of a higher number of bifidobacteria and a lower number of *S. aureus* in infancy may provide protection against overweight and obesity development. Since antibiotic treatment and caesarean section result in lower numbers of bifidobacteria (Martin & Walker,

2008; Penders et al., 2006), this may lead to an increased risk for the development of obesity in later life. It has also been demonstrated that the gut microbiota does not only affect fat quantity in the host, but also fat composition. We reported that administration of a CLA-producing bifidobacteria strain in combination with linoleic acid resulted in modulation of the fatty acid composition of the host, including significantly elevated concentrations of *c*9, *t*11 CLA in the liver. Furthermore, changes in fatty acid composition were not only limited to CLA but included changes in the content of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), alterations that were coupled with an anti-inflammatory cytokine profile (Wall, Ross, Shanahan et al., 2009). Another recent study involving a comparison of conventionally raised and germ-free mice reported that the gut microbiota have the ability to affect lens and retinal lipid composition. The distinct lipid profiles suggest that conventional mice are exposed to more oxidative stress than germ-free mice, decreasing the lens lifespan. These findings open up a new field of research for modulating the fatty acid composition of distal tissues (Oresic et al., 2009).

1.1.5 METABOLITE PRODUCTION BY GUT BACTERIA

The human enteric microbiota can exert beneficial health effects through the production of bacterial metabolites or 'pharmabiotics', most often small molecules which interact with 'intelligent communication' systems in the body including those which are immune, endocrine and neuronal-based (Shanahan et al., 2009). Commensal bacteria have been shown to synthesise vitamins that are essential for human survival such as vitamins K_2 and B_{12} (Resta, 2009),

polyunsaturated fatty acids (PUFA) such as conjugated a-linolenic acid (CALA) and CLA, SCFA, neuroactive compounds such as GABA and histamine (Forsythe et al., 2010), PSA (Mazmanian et al., 2008) and a variety of other proteins, peptides and nucleotides with immunomodulatory and anti-inflammatory properties (Shanahan & Kiely, 2007). The effects of some of these compounds on human health are briefly reviewed below and summarised in Table 1.1.

- *SCFA* are the end products of the bacterial fermentation of carbohydrates in the gastrointestinal tract (Forsythe et al., 2010; Kovatcheva-Datchary et al., 2009). SCFA are important for human metabolism since they increase the amount of energy intake, stimulate water and sodium absorption, lower luminal pH and the bioavailability of toxic amines (Puccio et al., 2007). In a recent study, Maslowski et al. (2009) have shown that SCFA bind to the G-protein coupled receptor 43 (GPR43) and this interaction may affect immune and inflammatory responses. Moreover, butyrate is the primary energy source for colonocytes (Saulnier et al., 2009) and has been in the focus of studies because of its possible action in the prevention of colon cancer (Kovatcheva-Datchary et al., 2009).
- *Vitamin K_2* or menaquinone is a lipophilic vitamin that can be found in fermented foods and in the colon where it is synthesised by the gut microbiota (Greer, 2010; Schurgers et al., 2007). It is an essential cofactor for the enzyme responsible for the modification of specific glutamyl residues to gamma-carboxyglutamyl (Gla) residues in precursor proteins that possess the appropriate gamma-carboxylation recognition signal within the propeptide region (Hojo et al., 2007; Kaneda et al., 2008; Schurgers et al.,

2007; Van Summeren et al., 2009) such as vertebrate proteins involved in blood coagulation and bone mineralisation (Schurgers et al., 2007). Therefore, menaquinone is important for bone and vascular health, and deficiency has been associated with low bone mineral density, increased fracture risk, increased risk of cardiovascular diseases, melena neonatorum and intracranial hemorrhagic disorders in newborn infants (Greer, 2010; Hojo et al., 2007; Schurgers et al., 2007).

- *Vitamin B_{12}* or cobalamin is a compound required by humans but solely synthesised by some bacteria and archaea (Hay et al., 2008; Martens et al., 2002; Santos et al., 2008). During foetal life and infancy, this vitamin is important for rapid and normal growth and for the development of the nervous system (Dror & Allen, 2008; Hay et al., 2008). Its deficiency in infants is related to maternal deficiency and may cause failure to thrive, delay development or regression and lead to progressive or permanent neurological disorders and haematological abnormalities (Bjorke-Monsen et al., 2008; Dror & Allen, 2008; Hay et al., 2008).
- *CLA* is a mixture of conjugated isomers of the essential fatty acid linoleic acid that have been reported to be produced by some human strains from different bacterial groups such as *Lactobacillus, Propionibacterium, Bifidobacterium, Pediococcus, Enterococcus* and *Lactococcus* (Ross et al., 2010). CLA has been shown to exert such health properties as anticarcinogenic, anti-inflammatory and immunomodulatory effects (reviewed in Bhattacharya et al., 2006). Because of its anti-inflammatory

properties, CLA may be a potential therapeutic option for the prevention of necrotising enterocolitis in preterm infants (Rosberg-Cody et al., 2004).

- *GABA* is a non-protein amino acid that acts as a major inhibitory neurotransmitter in the central nervous system and exerts several other physiological functions such as induction of hypotension and diuresis (Huang et al., 2007; Komatsuzaki et al., 2008). GABA is produced by various microorganisms, especially lactobacilli (Forsythe et al., 2010) and has been used in the development of functional foods such as fermented meats, pickles, cheese (Huang et al., 2007; Komatsuzaki et al., 2008) and yogurt (Park & Oh, 2007).
- *PSA* is a polysaccharide produced by the human symbiotic *Bacteroides fragilis* (Mazmanian & Kasper, 2006). Animal studies have shown that PSA has several immunomodulatory activities and can ameliorate inflammatory diseases. Treatment with purified PSA protects mice against IBD, avoiding weight loss, decreasing levels of cytokines and inhibiting epithelial cell hyperplasia and neutrophil infiltration to the gut (Forsythe et al., 2010; Mshvildadze et al., 2008).

Table 1.1 Examples of metabolite-producing bacterial strains and their possible health effects

Metabolite	Bacterial strain	Health effects	References
Vitamin K_2	*Bacteroides fragilis*	Modulation of bone mineralization: ↑Bone mineral density ↓Fracture risk Modulation of blood coagulation: ↓Risk of cardiovascular disease ↓Risk of melena neonatarum ↓Risk of intracranial hemorrhagic disease	Greer, 2010 Kaneda et al., 2008 Parker et al., 2003 Schurgers et al, 2007 van Summeren et al, 2009
Vitamin B_{12}	*Lactobacillus reuteri*	Stimulation of nervous system development: Success to thrive ↓Risk of neurological disorders ↓Risk of hematological abnormalities	Dror & Allen, 2008 Hay et al., 2008 Martens et al., 2002 Santos et al., 2008
CLA	*Bifidobacterium breve* *B. longum*	Modulation of the immune system ↓Carcinogenesis ↓Atherosclerosis ↓Inflammation ↓Obesity ↓Diabetes	Barrett et al., 2007 Bhattacharya et al., 2006 Ross et al., 2010
GABA	*Lactobacillus brevis* *L. paracasei*	Central nervous system inhibition (inhibitory neurotransmitter): ↑Hypotension ↑Diuresis	Huang et al., 2007 Komatsuzaki et al.,2008
PSA	*Bacteroides fragilis*	↓Weight loss ↓Epithelial cell hyperplasia Modulation of the immune system: ↓Cytokines levels ↓Neutrophil infiltration	Mazmanian & Kasper, 2006

1.1.6 DIET AND THE ENTERIC MICROBIOTA

Diet can influence the composition of the intestinal microbiota, especially in the first days of life when the bacterial population is not yet established (Parracho et al., 2007). The infant microbiota is naturally shaped during breast-feeding but the colonisation pattern can also be manipulated towards a more beneficial community using dietary supplementation with probiotics and/or prebiotics.

Human breast milk is considered the best nutritional option for growth and health development of newborn infants, since it contains a wide range of protective compounds including carbohydrates, nucleotides, fatty acids, immunoglobulins, cytokines, intact immune cells and other immune-modulatory factors (Boehm & Moro et al., 2008; Martin et al., 2007; M'Rabet et al., 2008; Puccio et al.,2007; Singhal et al., 2008). Moreover, breast milk seems to be a continuous source of bacteria for the infant gut (Martin et al., 2008), with milk from healthy mothers containing up to 10^9 microbes/L from different bacterial groups (Morelli, 2008; Reinhardt et al, 2009). Commensal bacteria usually present in breast milk include staphylococci, streptococci, bifidobacteria and lactic acid bacteria (Martin et al., 2008; Morelli, 2008; Reinhardt et al, 2009). Bacteria with potential probiotic effects have been isolated from human milk such as *L. gasseri, L. rhamnosus, L. plantarum, L. fermentum, Enterococcus faecium,* (Martin et al., 2007) and some bifidobacterial species (*B. breve, B. adolescentis* and *B. bifidum*) (Martin et al., 2008).

Due to the positive effects of human breast milk especially in the maturation of the immune system of the newborn (Biasucci et al., 2008), many attempts have been made to develop artificial formula that stimulate gut colonisation similar to that of breast-fed infants, with high numbers of bifidobacteria (Boehm & Moro,

2008; Puccio et al., 2007). Strategies used in the supplementation of the basic infant formula include the incorporation of probiotics and/or prebiotics.

1.1.7 PROBIOTICS AND PREBIOTICS

Probiotics are defined as 'live microorganisms' which, when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2001). The most common groups of bacteria used as probiotics are bifidobacteria and lactobacilli (Parracho et al., 2007). Multiple mechanisms of probiotic action have been suggested; however mechanisms are strain-dependent. Probiotics may prevent the penetration of pathogens in the human gut by increasing the production of mucin, reducing the gut permeability, releasing antimicrobial compounds or modulating the immune system (Saulnier et al., 2009). Several studies have demonstrated that probiotic preparations are safe and effective when administered to children, aiding in the treatment and prevention of many types of diarrhoea (O'Hara & Shanahan, 2007; Parracho et al., 2007; Puccio et al., 2007), reducing the incidence and severity of necrotising enterocolitis (AlFaleh & Bassler, 2008; Deshpande et al., 2007; Martin & Walker, 2008; O'Hara & Shanahan, 2007), alleviating symptoms of lactose malabsorption and preventing atopic diseases and food allergies in infants (Parracho et al., 2007). However, probiotic concentration and viability can be affected during food processing, storage and ingestion (Parracho et al., 2007; Saulnier et al., 2009). Therefore, the possibilities of using prebiotic as a food supplement are much wider (Parracho et al., 2007).

Prebiotics are non-digestible food ingredients that selectively stimulate the growth of bifidobacteria and lactobacilli (Parracho et al., 2007; Saulnier et al.,

2009). The two prebiotic substrates commonly added to infant formulae are FOS and GOS (Parracho et al., 2007). Administration of prebiotics is often associated with a lower faecal pH and changes in the SCFA pattern (Boehm & Moro, 2008). Prebiotics increase the production of SCFA, improving the host absorption of minerals and facilitating host metabolism (Saulnier et al., 2009). Moreover, by changing the composition of the gut microbiota, prebiotics play a role in the development of the newborn immune system and protection against pathogens (Boehm & Moro, 2008; Saulnier et al., 2009). It has been demonstrated that prebiotics can increase the number of bifidobacteria in the gut, and are applicable in infant nutrition (Saulnier et al., 2009)

Another dietary strategy is the combination of probiotics and prebiotics, called synbiotic. Synbiotics positively affect the host by introducing new beneficial bacteria to the indigenous gut population whilst ensuring its growth and selectively stimulating the growth of the other endogenous health-promoting bacteria, such as bifidobacteria (Parracho et al., 2007; Puccio et al., 2007).

1.1.8 CONCLUSIONS

Early colonisation of the infant gut is undoubtedly an important factor for the overall health of the infant and may also have effects on the health status in later life. Indeed, the commensal microbiota have been implicated in many diseases that occur within the gastrointestinal tract and more recently have also been shown to be involved in disorders outside the gut such as obesity, diabetes and atopic allergies. Several factors have been shown to promote a greater microbial diversity in infants including breast milk feeding, vaginal delivery and decreased antibiotic

use. Moreover, by programming the infant gut microbiota using prebiotics, probiotics and/or synbiotics and thus accomplishing a more beneficial composition, several important diseases that develop in early life such as necrotising enterocolitis and atopic eczema may be prevented. Although current data support the use of probiotics in infants, adequately powered prospective studies that document changes in the microbiota in response to diet and antibiotics and incorporate long-term health status outcomes are keenly awaited. The intake limits (e.g. optimal dose, frequency, timing of administration), variability between different probiotic strains and specific effects for different populations (e.g. infants, elderly, pregnant women) are some aspects that have to be evaluated. For example, in a recent study by Mc Gee et al. (2010), a single oral dose of a probiotic led to colonisation of the neonatal intestinal tract in low birth weight infants for an extended period of time, which raises the possibility that less than daily dosing may be adequate to achieve colonisation of the moderately preterm infant gut with a microbiota resembling that of the healthy term infant. However, more research is necessary in order to evaluate how such a strategy will affect long-term health status. The incidence of childhood obesity has increased during the past years and recent studies have suggested that environmental factors such as mode of delivery, type of feeding and use of antibiotics which are known to affect the composition of the gut microbiota may lead to an increased risk for the development of obesity later in life (Backhed et al., 2004; Reinhardt et al., 2009). However, more well-designed clinical studies are required to confirm the impact of microbiota composition on the development of such conditions as obesity.

1.1.9 ACKNOWLEDGEMENTS

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Chapter 1.2

Gut microbiota modulation and implications for host health:

dietary strategies to influence the gut-brain axis

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1.2.1 ABSTRACT

The human intestinal microbiota evolves from an immature and unstable ecosystem during infancy into a more complex and stable ecosystem in adulthood. Diet is one of the main factors contributing to the composition and diversity of the human intestinal microbiota. From birth, breast milk offers the best nutritional regime for maturation of the gut, whereas the introduction of solid food selects the most adapted bacteria, converging towards an adult-like microbiota. The gut microbiota plays an important role in host health, influencing the maturation of the immune system and regulating energy metabolism. Moreover, it has become evident that the microbiota can affect brain function and behaviour. On this bidirectional communication between intestine and the central nervous system (CNS), the so called gut-brain axis, the gut influences brain development and biochemistry, whereas the brain affects gastrointestinal function. In this context, probiotics and prebiotics have been used as dietary strategies aimed at improving host health by modulating the gut ecosystem and, consequently, affecting host stress-responses, behaviour and cognition.

1.2.2 INTRODUCTION

Microbial colonisation of the infant intestine begins during birth, with the exposure of the newborn to the microbiota of the mother and surrounding environment (Adlerberth & Wold, 2009). In the weeks and months following birth, the gut microbiota of the infant intestine evolves ultimately into a diverse and relatively stable composition by approximately two years of age, remaining thus throughout adulthood (Palmer et al., 2007). The adult intestinal microbiota

comprises a microbial ecosystem of approximately 100 trillion organisms, i.e. 10 times more microbes than human cells, encoding 100-fold more genes than the human genome (Qin et al., 2010). The majority of these microorganisms are bacteria which cover all host mucosal surfaces within the gastrointestinal tract (GIT) (10^1 microbial cells/g of content in the stomach, 10^3 to 10^7 cells/g in the small intestine and up to 10^{12} cells/g in the colon) (Sommer & Backhed, 2013). The adult intestinal microbiota is subject-specific, highly variable (Palmer et al., 2007) and consists of approximately 500–1000 species, most of them belonging to the phyla *Firmicutes* and *Bacteroidetes* (Sommer & Backhed, 2013). The species diversity declines with the ageing process and shifts in the dominant species have been observed in the elderly; yet the total number of anaerobic bacteria reportedly remains relatively stable in individuals over 65 years old (Biagi et al., 2010). Altered gut microbiota composition is also associated with premature infancy and such medical conditions as obesity (Tremaroli & Backhed, 2012), diabetes (Brown et al., 2011), atherosclerosis (Koren et al., 2011), inflammatory bowel disease (IBD) (Maynard et al., 2012), and necrotizing enterocolitis (NEC) (AlFaleh et al., 2012).

While a stable core microbiome exists and is shared among individuals, numbers of some transient gut inhabitants fluctuate over time depending on diet, antibiotic treatment and other environmental factors (Rajilić-Stojanović et al., 2012). Dietary habits are among the main factors contributing to the diversity of human gut microbiota, as shown in a study by De Filippo et al. (2010) in which European children on a Western diet (high in animal protein and fat) were compared to children in rural Africa (high-carbohydrate, low animal protein diet). Increased gut microbial diversity, with reduced quantities of potentially pathogenic

organisms and higher numbers of short chain fatty acid (SCFA)-producing bacteria was observed in the African population, and diet seemed to contribute to these differences, over other possible variables such as ethnicity, sanitation, hygiene, geography and climate. The impact of diet in shaping the gut microbiota was also demonstrated in a recent controlled-feeding study by Wu *et al.* (2011) in which the long-term diet regimen was strongly associated with the enterotype partitioning suggested by Arumugam *et al.* (2011). While the microbiota seems to be modulated by different types of diet in a continuous mode, aiming to maximize the energy intake from fermentable substrates available in the colon (Arumugam et al., 2011; De Filippo et al., 2010), a more abrupt change is seen with the use of antibiotics. Indeed, antibiotic treatment results in major alterations in gut microbiota composition with severe disruption of microbial diversity, as shown in two studies where short-term antibiotic treatment significantly affected evolution of the infant gut microbiota, disturbing the colonisation pattern of *Bifidobacterium* in the first months of life (Hussey et al., 2011; Fouhy et al., 2012). The impact of commercial antibiotics and bacteriocins on microbiota composition has also been investigated *in vitro* (Rea et al., 2010), and *in vivo* in an animal model of obesity (Murphy et al., 2013). In both studies, treatment with broad-spectrum antibiotics led to drastically altered gut microbiota composition, while on the other hand, bacteriocins had a more specific effect, inducing more subtle changes in the microbiota, and in a less severe manner compared with antibiotics. These results suggest that the specificity of the antimicrobial agent employed is critical and that bacteria and their products may be a useful strategy to modulate the microbiota.

Probiotics and prebiotics are dietary components that may positively impact on microbiota composition and consequently, host health. Administration of probiotics and prebiotics has been shown to enhance the host's defence system against pathogens by stimulation of mucin production, and decreased gut permeability (Mennigen et al., 2009; Saulnier et al., 2009). Probiotics have also been successfully used to prevent gastrointestinal diseases, to reduce the risk of antibiotic-associated diarrhoea (AAD) (Hempel et al., 2012) and to improve irritable bowel syndrome (IBS) symptoms (Moayyedi et al., 2010). Moreover, several reports have shown that probiotics and prebiotics may modulate brain function and behaviour (Bravo et al., 2011; Silk et al., 2009), and in this respect, we recently coined the term 'psychobiotics' to describe a live organism that, when ingested in adequate amounts produces a health benefit in patients suffering from psychiatric illness (Dinan et al., 2013). Although more studies are necessary to understand the communication between the gut and the CNS, it is suggested that probiotics may affect the brain in a direct manner by producing neurotransmitters and neuromodulators (Lyte, 2011; Barrett, Dinan et al., 2013) or, by other mechanisms, such as activation of the vagus nerve, alteration of gut microbial composition and alteration of circulating levels of cytokines (Cryan & Dinan, 2012).

The interaction between gut microbiota and host is complex and dynamic and, although some commensal gut microbes have pathogenic potential (e.g. bacterial overgrowth following antibiotic treatment, translocation to other tissues/organs), in the majority of cases the symbiotic association offers mutual benefits. In a homeostatic state, the host provides protection and a nutrient-rich environment for the growth of microorganisms (Maynard et al., 2012), while the

microbiota inhibits the growth of potential pathogens, converts harmful compounds to less toxic substances and produces bioactive molecules that may play a role in host physiology (Marques et al., 2010). The gut microbiota is responsible for the development and maturation of the immune system (Maynard et al., 2012) and has a role in energy metabolism, thus regulating fat storage in the host (Tremaroli & Backhed, 2012). Moreover, the microbiota and its metabolites may influence the development and function of the brain (Collins et al., 2012; Cryan & Dinan, 2012).

Here, we review the changes in the microbiota from the colonisation of the newborn through adulthood to the elderly stage, with a focus on the importance of diet in shaping gut microbiota composition and diversity. The role of early infant nutrition on gut maturation is discussed and the use of probiotics and prebiotics as dietary strategies to maintain gut ecosystem homeostasis. Moreover, we assess the relationship between the gut microbiota and the CNS, exploring the concept of gut-brain axis, and how probiotics and prebiotics may impact on host brain function and behaviour.

1.2.3 GUT MICROBIOTA EVOLUTION IN THE HUMAN BODY

Gut colonisation and the development of the infant gut microbiota

Foetal development is believed to occur in a sterile intrauterine environment, with bacterial colonisation of the infant intestine beginning during birth (Penders et al., 2006; Koenig et al., 2010). The microbiota population that develops in the initial stages of colonisation is determined by the types of bacteria to which the infant is exposed in the first hours of life and the establishment of a

healthy microbiota is believed to have a profound impact on the future well-being of the individual (Palmer et al., 2007). During and following birth, the infant is exposed mainly to the maternal microbiota (vagina, anus and skin), but also to bacterial cells from the hospital environment (health-care workers, air, equipments, other newborns). In the earliest stage of community development, the newborn microbiota is less complex and stable than the adult microbiota (Adlerberth & Wold, 2009; Palmer et al., 2007) and is homogeneously distributed across the body (undifferentiated bacterial communities across skin, oral and nasopharyngeal cavities, and gut) (Dominguez-Bello et al., 2010). Although a remarkable degree of interindividual variation in microbiota population is observed, the microbial succession in the infant gut is expected to progress in a similar pattern – initially the gastrointestinal tract is colonised by facultative anaerobes such as *Staphylococcus, Streptococcus* and *Enterobacteria*. The bacterial population expands, and oxygen is gradually consumed, creating a more suitable environment for proliferation of strict anaerobes, including *Clostridium, Bacteroides and Bifidobacterium*. As the time passes, facultative bacteria cannot resist the competition and environmental changes and are eventually outnumbered by anaerobes, starting to resemble the adult mature gut (Adlerberth & Wold, 2009; Palmer et al., 2007).

A broad range of factors are known to influence the composition of the infant gut microbiota including mode of delivery, feeding regime, gestational age, and use of antibiotics and probiotics. In preterm infants, microbial colonisation is disrupted because of the gastrointestinal tract immaturity, frequent use of antibiotics, delayed nutrition and extended stay in hospital (Arboleya et al., 2011; Barrett, Kerr, et al., 2013). Although high inter-individual variability in bacterial

levels is also observed in preterm infants, their microbiota is characterised by reduced microbial diversity, with higher numbers of potentially pathogenic bacteria and lower numbers of commensal microorganisms such as *Bifidobacterium* and *Bacteroides* (Arboleya et al., 2011; Barrett, Kerr, et al., 2013), compared with full-term infants. Among several factors that may affect infant gut colonisation, mode of delivery and feeding regime have been extensively studied. Mode of delivery is the primary determinant of a newborn's bacterial community composition (Dominguez-Bello et al., 2010). Vaginally born infants are colonised by faecal and vaginal bacteria from the mother, whereas infants born via caesarean section are initially exposed to maternal skin microbiota and bacteria from the hospital environment (Adlerberth & Wold, 2009; Penders et al., 2006). Indeed, in a study conducted by Dominguez-Bello et al. (2010), the dominant taxa found in the infant communities were reflective of delivery mode, with higher numbers of *Lactobacillus, Prevotella, Atopobium,* or *Sneathia* spp. found in intestinal samples collected from vaginally delivered babies, and typical skin taxa, including *Staphylococcus* spp., in samples from infants born by caesarean section. Penders et al. (2006) also reported a significant impact of mode of delivery on infant's microbial population. In this study, infants born by caesarean section harboured lower numbers of *Bifidobacterium*, reduced levels of members of the *Bacteroides fragilis* group and higher amounts of *Clostridium difficile* when compared to infants born vaginally. Interestingly, a study by Aagaard et al. (2012) showed that in a pregnancy state, the mother's vaginal community shifts naturally to a less diverse and rich population, but with an increased number of *Lactobacillus* species that might be important for establishing a healthy neonatal microbiota during birth.

Diet is another factor that strikingly impacts gut microbiota composition (Adlerberth & Wold, 2009; Koenig et al., 2010). Before weaning, the infant's diet is a relatively constant supply of milk, either breast milk or formula. Breast milk contains a complex mixture of oligosaccharides that are believed to inhibit the binding of pathogenic bacteria and toxins and to stimulate the growth of beneficial bacteria such as *Bifidobacterium* (Zivkovic et al., 2011). Indeed, Penders et al. (2006) reported that breast feeding was associated with an infant microbiota dominated by *Bifidobacterium* and with lower levels of *Escherichia coli*, *C. difficile* and *B. fragilis* species, whereas *C. difficile*, *Bacteroides*, *Enterococcus* and *Enterobacteriaceae* were more commonly associated with infant formula-feeding (Adlerberth & Wold, 2009). However, recent technological advances in the design of infant formula have increased their similarity to breast milk, improving their bifidogenic effect and decreasing the differences previously seen between breast- and formula-fed infant microbiota (Adlerberth & Wold, 2009). While breast milk feeding offers the best nutrition for growth and maturation of the infant gut (Zivkovic et al., 2011), the change to a solid type of food during weaning is determinant for the transition of an immature, unstable infant microbiota to a more complex and stable adult ecosystem (Koenig et al., 2010). The introduction of solid food at weaning imposes increasingly rigorous selection for the most highly adapted bacteria (Palmer et al., 2007), inducing an increase in numbers of *Bacteroidetes* and *Firmicutes* and enrichment in functional genes characteristic of the adult gut microbiome (Koenig et al., 2010).

The individual-specific microbiota of an adult

Bacterial diversity increases with age (Yatsunenko et al., 2012). In the gut, interactions with the host and competition with other microorganisms select the most apt bacteria shaping the microbiota ecosystem (Arumugam et al., 2011). The adult gut ecosystem is dominated essentially by *Firmicutes* and *Bacteroidetes* phyla (Arumugam et al., 2011), but also by *Actinobacteria, Proteobacteria* and *Verrucomicrobia* (Salonen et al., 2012). Few highly adapted species form the stable core component of the microbiota consisting of primarily anaerobic bacteria, including *Bifidobacterium* spp., *Faecalibacterium* spp. and the majority of members of the *Bacteroidetes* phylum. On the other hand, oxygen-tolerant bacteria, such as *Lactobacillus* spp., and members of *Proteobacteria* phylum are transient gut inhabitants appearing at low numbers and fluctuating over time, depending on diet and other environmental factors (Arumugam et al., 2011; Rajilić-Stojanović et al., 2012; Salonen et al., 2012). The adult gut microbiota is individual-specific and relatively stable over time (Palmer et al., 2007). In a study conducted by Rajilić-Stojanović et al. (2012), the subject-specific microbiota pattern was preserved for longer than a decade, suggesting that a homeostatic ecosystem can resist several aggressive environmental factors. The study showed that the use of antibiotics, for example, permanently changes the microbiota composition, but its diversity is restored after the end of treatment.

Despite the individual-specific nature of the intestinal microbiota, and in agreement with the idea of a conserved core community that is not susceptible to environmental variation (Salonen et al., 2012), a study by Arumugan et al. (2011) suggested that the microbiota of all humans can be classified into one of three

enterotypes. Each enterotype is identifiable by the abundance of a specific bacterial genus – Enterotype 1 is enriched in *Bacteroides*, Enterotype 2 is enriched in *Prevotella* and Enterotype 3, the most frequent type, is enriched in *Ruminococcus*. Although each enterotype uses a different route to generate energy from fermentable substrates available in the gut, its composition is probably not influenced by nutritional habits and does not correlate with the host gender, age or body mass index (BMI).

The microbiota in the elderly

The ageing process can seriously affect the composition of the human gut microbiota. Besides the complex repertoire of medications used for the treatment of several types of illnesses (Claesson et al., 2011), natural changes in physiology such as masticatory dysfunction, swallowing difficulties, digestive problems and decreased intestinal motility can lead to ingestion of an imbalanced diet and malabsorption of nutrients, compromising intestinal microbiota composition in the elderly (Woodmansey, 2007). The decline in functionality of the immune system (immunosenescence) is another process that affects the homeostatic equilibrium of the gut microbiota in the elderly. Furthermore, the immunosenescence is accompanied by a chronic, low grade overall inflammatory state (inflamm-ageing) that favours the growth of pathobionts over symbiont bacteria (Biagi et al., 2010).

The composition of the intestinal microbiota in the elderly (>65 years) is extremely variable between individuals (Claesson et al., 2011), yet the biodiversity is reduced and the stability compromised (Biagi et al., 2013). Total numbers of

anaerobic bacteria reportedly remain relatively stable, but increases in facultative anaerobes and shifts in the dominant species are reported during ageing, with *Bacteroidetes* and *Firmicutes* still dominating the gut microbiota with smaller fractions of *Actinobacteria*, and *Proteobacteria* (Biagi et al., 2010). However, the effect of age on numbers and diversity of the components of the *Firmicutes* and *Bacteroidetes* phyla is controversial. The ELDERMET consortium reported a dominance of the phylum *Bacteroidetes* with lower proportion of the phylum *Firmicutes* (Claesson et al., 2011). The same age-related increase in *Bacteroidetes* was found in German, Austrian and Finnish elderly, but this was not confirmed in Italian elderly and centenarians (Biagi et al., 2013). Moreover, a clear shift to a more *Clostridium* cluster IV-dominated community was reported for the Irish elderly (Claesson et al., 2011), whereas the species *Faecalibacterium prausnitzii*, belonging to this same cluster, was markedly decreased in Italian elderly (Biagi et al., 2013). These variation in gut microbiota composition found among different nationalities may be attributed to differences in life style, and type of diet. Indeed, the important impact of nutrition on microbiota composition was revealed in a recent study by Claesson et al. (2012). Within the same ethnogeographic region (Ireland), it was possible to determine an individual segregation of microbiota depending on where the elderly lived, with location largely determining the diet. The results indicated that a healthy, diverse diet promotes a more diverse gut microbiota composition that is more beneficial to the health of the elderly.

1.2.4 IMPACT OF DIET ON HUMAN HEALTH

Breast milk and microbiota development in infants

Breast milk is an important source of nutrients for the healthy growth and development of infants. It contains a complex and diverse mixture of oligosaccharides (Barile & Rastall, 2013; Zivkovic et al., 2011), as well as several other factors such as cytokines, lysozyme and lactoferrin, which are essential for the optimum development and maturation of the gut (Cabrera-Rubio et al., 2012). Various oligosaccharides and glycoconjugates in milk have an anti-adhesive effect, inhibiting the binding of pathogenic bacteria and toxins. Breast milk helps to prevent infections by shaping the immune system and selectively supporting the establishment of the intestinal microbiota (Zivkovic et al., 2011). Breast milk is also a source of bioactive compounds as well as microorganisms and their growth factors (Cabrera-Rubio et al., 2012). Indeed, in a study conducted by Zivkovic et al. (2011), human milk oligosaccharides (HMO) were shown to act as prebiotic substrates stimulating the growth of beneficial bacteria such as bifidobacteria. HMO may selectively promote the growth of certain *Bifidobacterium* strains and enhance their persistence in the gut (Barile & Rastall, 2013). In a study by Cabrera-Rubio and colleagues (2012), the milk microbial community was characterised, showing that bacterial cells present in breast milk are not contaminants. The authors found that human breast milk is dominated by bacilli, with colostrum having higher diversity than mature milk. Interestingly, the results also suggested that several factors, such as mode of delivery and maternal BMI can significantly skew the breast-milk microbiome composition and diversity (Cabrera-Rubio et al., 2012).

Breastfeeding is believed to reduce the incidence of lower respiratory tract infections, NEC, sudden infant death syndrome (SIDS), childhood IBD and other diseases. An extensive review of the benefits of breastfeeding can be found in the American Academy of Pediatrics (AAP) policy statement published in 2012 (Eidelman et al., 2012).

The structural complexity of HMO is likely responsible for the several benefits attributed to breast milk and is an obstacle for the preparation of prebiotic formula with similar properties. Investigations into alternative sources have shown that milk from other mammals contains lower concentrations of these complex oligosaccharides with varying structures. Another alternative is the use of the prebiotic galacto-oligosaccharides (GOS). Despite the low similarity with HMO structure, commercial GOS in infant formula leads to significantly increased *Bifidobacterium* levels in human intestine following ingestion, and it has been shown to exert a positive impact on the immune system (Barile & Rastall, 2013).

Dietary interventions using probiotics and prebiotics

Maintenance of the homeostasis in the gut ecosystem is essential for health, and, in this perspective, dietary manipulation may represent a strategy to preserve a healthy gastrointestinal microbial community and contribute to the well being of the host. Several food products containing probiotics and prebiotics have been developed for specific age groups such as infants and elderly. These are important target groups because of the immaturity of the infant gut immune system and the elderly immunosenescence, respectively. Infant formula containing specific oligosaccharides have been shown to be good replicas of breast milk, leading to

enhanced levels of *Bifidobacterium* (Adlerberth & Wold, 2009), whereas both probiotics and prebiotics have been reported to successfully improve intestinal microbial composition and immune function in the elderly (Saulnier et al., 2009).

Probiotics are defined as "live micro-organisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). *Lactobacillus* and *Bifidobacterium* are the main genera of microorganisms used as probiotics in food and are most commonly sold in dairy products (FAO/WHO, 2001). Although they belong to a group of bacteria regarded as "Generally Recognized as Safe" (GRAS), potential probiotic strains must be isolated as pure microorganisms (Preidis & Versalovic, 2009) and must be tested for the presence of transmissible antibiotic resistance genes (EFSA, 2004; FAO/WHO, 2001). Several reports suggest that probiotics improve intestinal barrier function, modulate the immune system and compete for nutrients and binding sites, enhancing the host defence system against pathogens (Saulnier et al., 2009). In addition, probiotics can synthesize organic acids and other metabolites such as proteins or peptides which may have a direct antimicrobial action or which may induce the host to produce a beneficial action (Marques et al., 2010; Preidis & Versalovic, 2009; Saulnier et al., 2009). Probiotic interventions have been shown to have a role in the prevention or improvement of some gastrointestinal diseases such as AAD (Hempel et al., 2012; Hickson, 2011), NEC (AlFaleh et al., 2012) and IBS (Moayyedi et al., 2010) (see Sanders et al., 2013 for a recent review on the use of probiotics). Moreover, probiotic therapy has been reported as an alternative in the treatment of neurological and psychiatric disorders, affecting stress-responses, behaviour and cognition (Cryan & Dinan, 2012). Probiotics act through diverse mechanisms that

may vary from one strain to another or may be a combination of events. Probiotic concentrations in foods can be affected in many ways from food processing to ingestion since most of them are delivered orally as food or dietary supplements. Thus, the strain selected must not only be capable of surviving passage through the digestive tract and have the capability to proliferate in the gut, but must also remain viable independent of the food matrix or delivery strategy used (FAO/WHO, 2001; Saulnier et al., 2009).

Prebiotics are non-viable food components that confer health benefits on the host associated with modulation of the microbiota (Pineiro et al., 2008). Thus, prebiotics are a dietary approach that target the indigenous bacterial community already established in the gut. Prebiotics are typically oligosaccharides or more complex saccharides and the majority of studies have so far focused on inulin, fructooligosaccharides (FOS) and GOS because of their safety and efficacy (Pineiro et al., 2008; Preidis & Versalovic, 2009). A prebiotic must resist host digestion, absorption, and adsorption, reaching the intestine unaltered to be fermented by the gut microbiota. Products of fermentation by *Lactobacillus* and *Bifidobacterium*, such as lactic and acetic acids, are used by other bacteria to generate more SCFA and all these compounds have an effect on host metabolism (Preidis & Versalovic, 2009; Saulnier et al., 2009). In the gut, prebiotics not only enhance the colonisation of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*, but also indirectly affect the growth of other important bacteria such as *F. prausnitzii* (Saulnier et al., 2009). Moreover, these compounds may directly stimulate immunity, prevent the adhesion of pathogens, decrease faecal transit time and facilitate host mineral absorption (Pineiro et al., 2008; Saulnier et al., 2009).

In addition to the modulation of the gut microbiota composition with the stimulation of beneficial gut commensals, prebiotics may help in the colonisation, survival, and function of probiotics. Synbiotics refer to synergistic beneficial effects derived from a mixture of probiotics and prebiotics. Careful selection of probiotic strains and compatible prebiotics will maximize the potential effect of these strategies (Preidis & Versalovic, 2009). There are numerous studies showing the beneficial effects of probiotics and prebiotics in health and nutrition. However, the lack of standardization prevents the comparison of these claims (the probiotic effect is strain-specific and the results cannot be extrapolated without new experiments (FAO/WHO, 2001)). Both probiotic and prebiotic effects are dependent on frequent ingestion, highlighting the importance of optimal dose and duration of intake (Saulnier et al., 2009). Therefore, well-designed and carefully conducted randomised controlled trials (with relevant inclusion/exclusion criteria, adequate sample sizes and information about long safe use) are necessary to evaluate the variety of biological effects of probiotics and prebiotics, and their implications for the human microbiome.

1.2.5 THE GUT-BRAIN AXIS

In recent years, interdisciplinary investigation has unveiled strong evidence for the existence of a bidirectional signalling between the intestine and the brain, the so called “gut-brain axis”. This communication system integrates neural, hormonal and immunological signalling between the gut and the brain (Collins et al., 2012). Moreover, the axis concept can be expanded to a “microbiota-gut-brain axis”, since not only the intestinal tract itself can affect the brain, but also its

microbial inhabitants (Fig. 1.2). While the gut microbiota and its metabolites may modulate the peripheral and CNS, influencing brain development and function (Forsythe et al., 2010), the brain may affect gastrointestinal functions such as motility, secretion, blood flow and mucin production as well as the immune system and microbiota composition (Collins et al., 2012; Sudo et al., 2004).

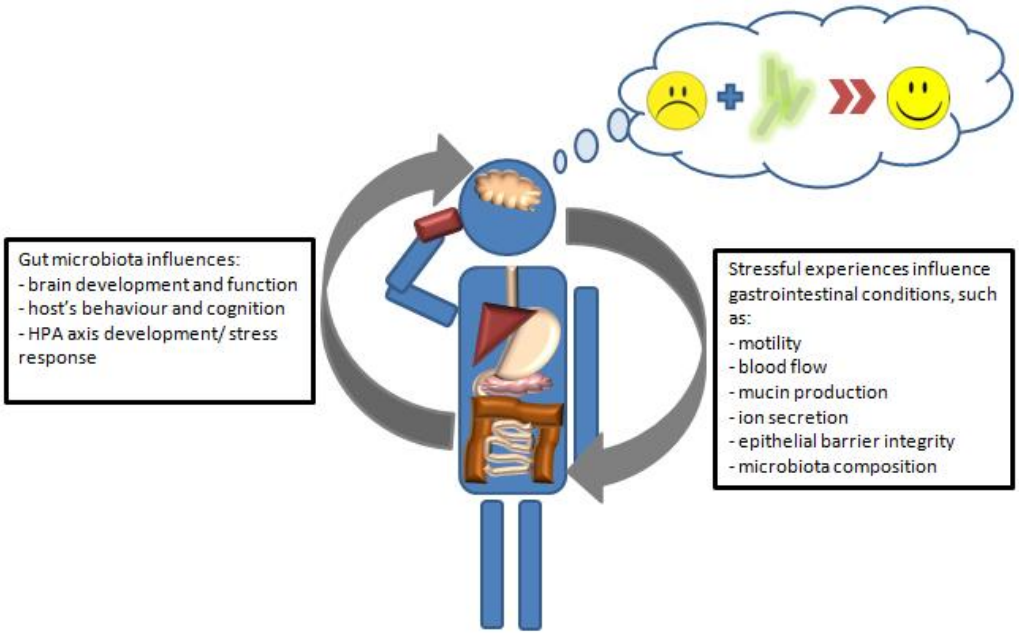


Figure 1.2 The microbiota-gut-brain axis. The communication system is bidirectional and integrates neural, hormonal and immunological signalling. Dietary strategies, such as probiotics and prebiotics, aim to modulate the gut microbiota and may affect host's stress-responses, behaviour and cognition.

Different strategies have been used to elucidate the role of the gut microbiota on behaviour and cognition, including the use of germ-free (GF) animals, animals with pathogenic bacterial infections, and animals exposed to probiotic agents and antibiotics (Cryan & Dinan, 2012). Studies using germ-free animals have shown that the complete absence of gut bacteria can affect behavioural functions and CNS neurotransmission. Heijtz and colleagues (2011) demonstrated that GF mice display increased motor activity and reduced anxiety-like behaviour, compared with mice with a normal gut microbiota. Supporting these results, a study by Clarke *et al.* (2012) showed the same reduction in anxiety on male GF animals, a significant elevation in the hippocampal concentration of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) and an increased concentration of plasma tryptophan, the precursor of 5-HT. These alterations on the serotonergic system, which is particularly relevant to stress, anxiety and depression, suggest that CNS signalling may be modulated by microbiota manipulation (Clarke et al., 2012). Another study, conducted by Sudo *et al.* (2004), used a GF animal model to evaluate how the commensal microbiota regulates the development of the hypothalamus-pituitary-adrenal (HPA) axis response. The HPA axis is a neuroendocrine system that controls reactions to stress and is subjected to programming by early life events. The data showed an enhanced HPA response to stress in GF mice with an exaggerated release of adrenocorticotropic hormone and corticosterone. Interestingly, the increase in the HPA response was partially reversed by early colonisation of the GF group with faeces from a control-specific pathogen free group or completely reversed by monoassociation with *Bifidobacterium infantis.*

Bercik, Denou, *et al.* (2011) used an antibiotic (ATB) treatment approach to provide evidence for a microbiota–gut–brain axis. Treatment with antibiotics for one week affected murine gut bacterial composition, increasing the levels of *Firmicutes* and *Actinobacteria*, and decreasing g-proteobacteria and *Bacteroidetes*. The authors hypothesized that the changes in the microbiota were responsible for the increased exploratory behaviour on ATB mice, since the same antibiotic treatment did not affect the GF group. Furthermore, it was suggested that the altered behaviour was consistent with an increase in brain-derived neurotrophic factor (BDNF) levels in the hippocampus and decreased levels in the amygdala. Alterations in BDNF levels have been implicated in changes in anxiety behaviour, and its regulation probably involves multiple mechanisms (Duman, 2004). An exaggerated HPA axis response to stress with release of corticosterone decreases BDNF expression, as was observed in a study by Sudo and colleagues (2004). However, removal of the adrenal glands where corticosterone is synthesized did not block BDNF downregulation, indicating that other factors are required for the effects of stress (Duman, 2004). Furthermore, high levels of hippocampal BDNF are associated with anxiolytic and antidepressant behaviour and increased BDNF expression in the amygdala are observed during fear learning (Bercik, Park, et al., 2011). BDNF levels are decreased in patients who are depressed and administration of one of the several different classes of antidepressants, 5-HT or noradrenaline (NA) selective reuptake inhibitors, has been shown to increase neurogenesis in adult hippocampus (Duman, 2004). However, in contrast, both Clark *et al.* (2012) and Heitz *et al.* (2011) have shown significantly lower BDNF mRNA expression in the hippocampus of GF mice and this was accompanied by a decrease in anxiety-like

behaviour. Intriguingly, the higher levels of 5-HT and tryptophan in the male GF mice did not increase BDNF levels as observed for antidepressants treatment. As BDNF reduction was sex-dependent, and only associated with male animals, it may be that sexual hormones are involved in the regulation. Stress is also reported to increase levels of interleukin (IL)-1β and it is possible that BDNF downregulation is mediated by this cytokine (Duman, 2004). A study conducted by Gareau et al., (2010) demonstrated a correlation between hippocampal BDNF and memory, reinforcing the concept of the gut-brain axis. Mice infected with *Citrobacter rodentium* displayed stress-induced memory impairment, with reduced BDNF levels in the hippocampus. These changes were accompanied by an increase in serum corticosterone and changes in microbiota composition. Administration of a probiotic mixture containing *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052 restored the hippocampal-dependent memory, ameliorated stress-induced increases in serum corticosterone, normalized the microbiota and recovered BDNF expression. Moreover, the experiment showed that, although GF animals are not anxious, they lack non-spatial and working memory and exhibit decreased BDNF, demonstrating the importance of the microbiota for the development of hippocampal-dependent memory.

Several other studies have used probiotic administration in an attempt to better understand the mechanisms involved in the gut-brain axis signalling (Table 1.2). In a study conducted by Zareie et al. (2006), the effects of psychological stress on intestinal mucosa integrity and the ability of a probiotic strain to prevent the damage were evaluated. Chronic stress induced ion secretion and barrier dysfunction with increased bacterial adhesion and penetration into surface

epithelial cells. Pre-treatment with *Lactobacillus* species prevented the bacterial adherence induced by stress and, more importantly, abolished the bacterial translocation to mesenteric lymph nodes. The authors concluded that administration of the probiotic during chronic psychological stress enhanced mucosal defence against luminal bacteria, a desirable feature for the treatment of patients with IBD. IBD and IBS are two different conditions that cause significant gastrointestinal and psychological discomfort and are both characterised by impaired gut barrier function and changes in gut microbiota diversity (Spiller & Lam, 2011). IBS is the most common gastrointestinal dysfunction, yet little is known about its aetiology (Silk et al., 2009). Given the few options for treatment and the evidence that modulation of gut microbiota may ameliorate inflammation and reduce psychological distress, a human trial was conducted to investigate efficacy of a trans-galactooligosaccharide in IBS (Silk et al., 2009). Treatment with the prebiotic affected intestinal microbiota composition and stimulated growth of bifidobacteria. Moreover, IBS symptoms were alleviated, with changes in stool consistency, improvement of flatulence and bloating, and improved composite symptom score (abdominal pain/discomfort, bloating⁄distension and bowel movement difficulty). Anxiety scores were significantly reduced in diarrhoea predominant IBS (IBS-D) patients during treatment with the highest concentration of prebiotic. In general, administration of prebiotic improved the quality of life of patients suffering with IBS, supporting the concept of dietary interventions as an alternative treatment for gastrointestinal disorders. A reduction in anxiety scores was also reported in two other human trials in which healthy subjects (Messaoudi et al., 2011) and patients suffering from chronic fatigue syndrome (CFS) (Rao et al.,

2009) were treated with probiotics. It is possible that the decreased anxiety was a consequence of improved bowel function, especially for the conditions of IBS and CFS. Desbonnet et al. (2008) investigated the impact of chronic administration of *B. infantis* 35624 on behaviour and neuronal signalling. The probiotic administration had no effect on swim behaviours of naive rats, while lower levels of pro-inflammatory cytokines and higher concentrations of serotonin precursor tryptophan were observed in probiotic-treated rats compared to the control group. These findings suggest that the *B. infantis* strain may possess antidepressant properties and, in a second study, the same authors used a maternal separation (MS) model to evaluate this hypothesis (Desbonnet et al., 2010). In contrast to the first study, postnatal MS induced a state of behavioural despair that was attenuated by the treatment with the probiotic. However, probiotic treatment had no effect on tryptophan levels as previously demonstrated , indicating that maybe MS compromises other biological systems involved in the synthesis of this metabolite. Both studies provided evidence of a probiotic beneficially affecting the neuronal system and behaviour, but also highlighted the complexity of the role of the microbiota in the regulation of mood. Bercik, Park, et al. (2011) used a murine model of chemical colitis induced by administration of dextran sodium sulfate (DSS) to explore how the strain *B. longum* NCC 3001 would affect behaviour. Administration of the probiotic reversed the anxiety-like behaviour in DSS-treated mice but did not affect BDNF mRNA expression or gut inflammation. The behavioural change was lost when the animals were vagotomised. The authors concluded from these results that the anxiolytic effect of *B. longum* involves vagal integrity but is independent of gut immuno-modulation or production of BDNF by

neuronal cells. Bravo et al. (2011) reported similar findings when exploring the activation of the vagus nerve using the probiotic *Lactobacillus rhamnosus* JB-1. Mice receiving the probiotic were less anxious and displayed antidepressant-like behaviours in comparison with broth-fed controls. There were also alterations in the mRNA expression of gamma-aminobutyric acid (GABA) receptors associated with anxiety and depression in several brain regions. All these changes were abolished when the animals were vagotomised, demonstrating the role of the vagus nerve in communicating visceral changes to the CNS. However, other mechanisms independent of the vagus nerve activation must be also considered as, for example, autonomic pathways are not involved in the behavioural alterations that accompanied antibiotic treatments (Bercik, Denou, et al., 2011). We have also recently reported that the administration of a *Bifidobacterium* strain to mice had a significant impact on the fatty acid composition of the brain (Wall et al., 2012). Mice that received the bacteria for 8 weeks exhibited significantly higher concentrations of arachidonic acid (ARA) and docosahexaenoic acid (DHA) when compared to unsupplemented control mice. ARA and DHA are critical in the development of the brain, having a role in neurotransmission and protection against oxidative stress (Innis, 2007; Maekawa et al., 2009). These fatty acids are also neuroprotective agents, involved in the improvement of cognitive function, including memory and learning (Henriksen et al., 2008; Yurko-Mauro et al., 2010). Accordingly, these data indicate that probiotic ingestion may be effective for achieving optimal brain health, leading to enhanced cognitive function.

In a study in which probiotics and prebiotics were not used, a beef-containing diet significantly changed bacterial diversity and improved working and

reference memory in mice (Li et al., 2009). The beef-enriched diet contained a higher content of fat and taurine in comparison to the standard diet and these components could potentially be responsible for the differences observed during the experiment. The dietary components could act directly on the brain, or induce changes in the microbiota composition stimulating the production of microbial metabolites that would affect behaviour. Bioactive compounds produced by the host microbiota may induce local changes in the gut epithelium and the enteric nervous system, as well as the immune system, with an impact in CNS signalling (Cryan & Dinan, 2012; Forsythe et al., 2010; Lyte, 2011). Different species of bacteria have been shown to produce neuroactive molecules such as GABA (Barrett et al., 2012), vitamins such as cobalamin (Santos et al., 2008) and biogenic amines such as histamine, serotonin and dopamine (Özogul, 2011), molecules that may directly affect the brain. Indeed, the production of histamine by lactic acid bacteria isolated from wine is well documented, and is perceived as problematic, as it can cause undesirable symptoms such as headaches and nausea when ingested (Landete et al., 2007). Other bacterial metabolites may impact the CNS indirectly by changing the microbiota composition (antimicrobial bacteriocins) (Rea et al., 2010) or by modulating the immune response (polysaccharide A, SCFA) (Maslowski et al., 2009; Mazmanian et al., 2008). The broad range of metabolites produced by bacteria may exert beneficial effects on the host or disturb its homeostasis (Forsythe et al., 2010; Lyte, 2011). However, the mechanisms of action are diverse and not well understood, and more studies are necessary to understand the highly complex pathways that link the gut to the brain.

Table 1.2 Effects of probiotics and prebiotics on the immune system, gut microbiota and the peripheral and central nervous system

Probiotic strain	Model	Trial duration	Effects	Reference
Lb. helveticus R0052 *and* *Lb. rhamnosus* R0011	Brown Norway rats	17 days	Prevented stress induced bacterial adherence to enterocytes Prevented bacterial translocation to mesenteric lymph nodes Inhibited chronic stress induced elevated intestinal ion secretion	Zareie et al., 2006
B. infantis 35624	Sprague Dawley rats	2 weeks	Decreased concentration of pro-inflammatory cytokines Increased levels of tryptophan in plasma	Desbonnet et al., 2008
Lb. casei strain Shirota	Humans with chronic fatigue syndrome	8 weeks	Increased *Bifidobacterium* and *Lactobacillus* numbers Decreased anxiety symptoms	Rao et al., 2009
Lb. rhamnosus R0011 and *Lb. helveticus* R0052	C57BL/6 mice infected with *Citrobacter rodentium*	10/30 days	Prevented memory dysfunction after exposition to acute stress	Gareau et al., 2010
B. infantis 35624	SD rats (maternal separation model)	45 days	Normalized the immune response Reversed the behavioural deficits Restored the basal noradrenaline concentrations in the brainstem	Desbonnet et al., 2010
B. longum NCC3001	AKR mice colitis model	14 days	Reduced anxiety-like behaviour in DSS-treated mice	Bercik, Park, et al., 2011
Lb. helveticus R0052 and *B. longum* R0175	Wistar rats and humans	14 days/ 30 days	Reduced anxiety-like behaviour in rats Alleviated psychological distress in humans	Messaoudi et al., 2011
Lb. rhamnosus JB-1	BALB/c mice	28 days	Reduced stress-induced corticosterone Reduced anxiety- and depression-related behaviour Altered GABA receptors expression in different brain regions	Bravo et al., 2011
B. breve NCIMB702258	C57BL/6 mice	8 weeks	Increased arachidonic acid and docosahexaenoic acid in the brain	Wall et al., 2012
Prebiotic: trans-galactooligosaccharide mixture	Humans with IBS	12 weeks	Increased *Bifidobacterium* numbers Improved quality of life Decreased anxiety scores in the IBS-D patients group (diarrhoea predominant)	Silk et al., 2009

1.2.6 CONCLUSIONS

The gut microbiota evolves from birth, changing from an immature and unstable state during infancy to a more complex, diverse and stable ecosystem in adulthood. Disturbance of its homeostatic state may have a negative impact on host health, leading to gastrointestinal, immunological and even neurological disorders. In this context, the use of dietary approaches such as probiotics, prebiotics and diets enriched with fatty acids or proteins to modulate the microbiota may beneficially affect host health. While these strategies have been shown to be effective in several different studies, the lack of standardization in terms of optimal dosage and treatment duration limits comparison between studies and makes it difficult to draw solid conclusions. Moreover, the mechanisms of action by which probiotics and prebiotics may affect host health are not well understood. These limitations highlight the need for well designed human intervention studies employing multidisciplinary approaches, aiming to better understand the interactions between gut microbiota and brain, and the identification of specific mechanisms of action, leading to new dietary strategies targeting the gut-brain axis for enhanced mental health.

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Chapter 2

Gas chromatography method optimization for analysis of SCFA levels in intestinal samples

2.1 ABSTRACT

Background: Short chain fatty acids (SCFA), the main products of bacterial fermentation in the gut, have been shown to exert a wide range of beneficial effects to the host.

Objective: To optimize a gas chromatography method described by Tangerman & Nagengast (1996) in order to obtain good peak resolution and assay sensitivity when analyzing SCFA levels in faecal and caecal samples from animal and human studies.

Method: SCFA was extracted from faecal or caecal samples with Milli-Q water, centrifuged and filtrate to eliminate bacteria and solids and injected into the GC-FID system fitted with a capillary GC column instead of a packed glass column. High injector and detector temperatures (240^{o}C and 250^{o}C, respectively), programmed oven temperature and a narrow glass liner to simulate an on-column injection were used. Internal and external standards were used for calibration purpose. Recovery and reproducibility test were done to validate the method.

Results: SCFA level detected in caecal sample was as expected, with higher concentration of acetate (82.56 umol/g), followed by n-butyrate (34.55 umol/g), propionate (11.4 umol/g) and isobutyrate (1.29 umol/g). Recovery rates were around 100% for all the SCFA tested. The intra-assay reproducibility test showed consistent results after multiple repetitions (RSD < 5%), whereas the inter-assay variation was higher than 10% after 3 wks storage.

Conclusion: The optimized method applying direct aqueous injection of samples into the GC-FID proved to be efficient and accurate for the detection of acetate, propionate and butyrate in faecal/caecal contents, provided that sample storage at

-20^{o}C did not exceed 2 weeks and the GC system was maintained in good operating conditions.

2.2 INTRODUCTION

The gut microbiota synthesizes many enzymes for processing of ingested food substrates, yielding energy for microbial growth and metabolic end products such as short chain fatty acids (SCFA). Approximately 100-200 mM of SCFA are produced daily by humans consuming a Western diet, with the major ones being acetate, propionate and butyrate (Cook & Sellin, 1998). Total SCFA concentration in the human proximal colon is around 70–140 mM falling to 20–70 mM in the distal colon, although alterations in production and absorption may occur with dietary changes (Topping & Clifton, 2001). Several factors may affect bacterial metabolism and SCFA production in the gut, such as substrate type and availability, the composition of the gut microbiota and the length of time that partially digested food takes to pass through the bowel (Cook & Sellin, 1998; Macfarlane & Macfarlane, 2003). Moreover, drugs, ageing, diseases and stress may affect SCFA concentrations due to their effects on microbiota population and host health (Macfarlane & Macfarlane, 2003; Reilly & Rombeau, 1993).

Butyrate is almost completely used as energy source by colonocytes (Cook & Sellin, 1998), whereas acetate is used for lipogenesis and most of the propionate is consumed for hepatic gluconeogenesis (Reilly & Rombeau, 1993; Wolever et al., 1991). SCFA also affect various physiological and pathophysiological functions in the organism by stimulating gut motility and intestinal transit (Fukumoto et al., 2003;

Grider & Piland, 2007), regulating gut hormone release and suppressing food intake (Lin et al., 2012) and lowering colon cancer risk (O'Keefe et al., 2009; Ou et al., 2013; Waldecker et al., 2008). SCFA activate GPR41 and GPR43 receptors present in immune cells, adipocytes and enteroendocrine cells (Brown et al., 2003; Lee et al., 2008; Le Poul et al., 2003), decreasing gut inflammation (Maslowski et al., 2009), controlling cancer cell proliferation in tissues outside the gut (Bindels et al., 2012), reducing intestinal transit time and increasing SCFA absorption (Samuel et al., 2008), inhibiting lipolysis in adipocytes (Ge *et al*., 2008; Hong et al., 2005), increasing circulating levels of hormones (e.g. peptide YY, leptin) and affecting appetite (Karaki et al., 2008; Samuel et al., 2008).

Considering the effects of SCFA on health and disease, their accurate analysis in intestinal samples is vitally important, and different analytical techniques have been developed and applied in the measurement of SCFA. SCFA have been indentified and quantified by high performance liquid chromatography (HPLC) (Chen & Lifschitz, 1989; Kotani et al., 2009), ion-exclusion chromatography (Dias et al., 2009), gas-chromatography with flame ionization detector (GC-FID) (Brinkworth et al., 2009; Chen & Lifschitz, 1989; Schwiertz et al., 2009; Schafer, 1995; Zhao et al., 2006), gas-chromatography with mass spectrometer (GC-MS) (Garcia-Villalba et al., 2012; Mills et al., 2000) and NMR spectroscopy (Monleon et al., 2008). GC-MS and NMR techniques are greatly limited by high cost of instruments and GC-MS by the complexity of sample preparation. GC-MS may improve the sensitivity and selectivity of the analysis, but for an effective use of the system, it is desirable to avoid the use of water in the sample (Garcia-Villalba et al., 2012). Among all these

techniques, GC-FID analysis is the most frequently used due to SCFA volatile nature and equipment high resolution, sensitivity and inexpensive costs.

Several published methods have applied pre-treatments that are laborious, requiring complex sample preparations such as extractions with organic solvents (Henningsson et al., 2001; Garcia-Villalba et al., 2012), extraction with acidic and basic solutions (Claus et al., 2003; Kruse et al., 1999; Scheppach et al., 1987; Schwiertz et al., 2009; Zhao et al., 2006), steam distillation (Chen & Lifschitz, 1989), vacuum distillation (Brinkworth et al., 2009; McOrist et al., 2008), ultrafiltration/ultracentifugation (Chen & Lifschitz, 1989; Schafer, 1995) and solid phase micro-extraction (SPME) (Garcia-Villalba et al., 2012; Mills et al., 2000). Moreover, some protocols also performed derivatisation of SCFA with n-(tert-butyldimethylsilyl)-n-methyltrifluoro-acetamide (MTBSTFA) (Henningsson et al., 2001; Walker et al., 2005) or boron trifluoride (BF_3) (Collin et al., 1974). Although pre-treatments may generate good results with higher performance, they are relatively time-consuming and may reduce recovery rates due to the loss of more volatile SCFA. Moreover, some of these methods require large amount of samples and use large volumes of solvents and hazardous reagents that should be avoided.

The extraction of SCFA with water is simple, rapid and efficient, decreasing the loss of volatile compounds. In the intestines, at physiological pH around 6-7, more than 90% of SCFA exist in the anionic, dissociated form. SCFA are weak acids (pKa ~ 4.8) and, therefore, are more soluble in their anionic than their protonated forms (Velazquez et al., 1996). pH is the most significant variable affecting the partition coefficient for SCFA and, at pH around 6-7 (Milli-Q water pH ~7), most SCFA will be dissolved in the aqueous phase (Reinsel et al., 1994) (Fig 2.1). To

analyze SCFA diluted in aqueous solution a specific fused silica capillary column has to be used. FFAP (nitroterephthalic acid modified polyethylene glycol) columns are made of high polarity stationary phase and are modified with acid to provide a very inert column that is not decomposed in the presence of strong acids and water used as solvents.

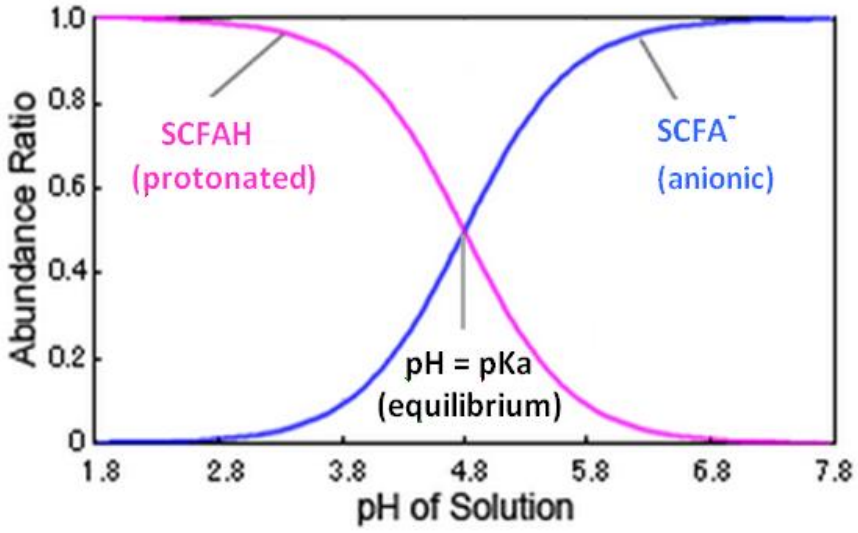


Figure 2.1 Relationship between solution pH and dissociation status of SCFA. Extraction with Milli-Q water generates a solution with pH ~ 7, when most of SCFA are in their anionic, hydrophilic form.

In this study, a simple and reliable method previously described by Tangerman & Nagengast (1996) was optimized with some adaptations, as follows – capillary GC column was used instead of a packed glass column, programmed oven temperature instead of isothermal, higher injector and detector temperatures (observing the column limits given by the manufacturer) and narrow glass liner to simulate an on-column injection. Capillary columns are thermally and chemically stable and produce more precise results when compared to packed glass columns, especially for active molecules such as free fatty acids. Moreover, due to its thermal strength capillary columns allow the use of higher temperatures leading to better peak resolution. A programmed oven temperature reduces analysis time and produces sharper peaks, whereas, on column injection increases the assay reproducibility. However, on column injection can just be applied to clean samples, as non-volatile compounds may accumulate into the GC column. Using a narrow glass liner we simulated an on column injection focusing the analytes of interest into the head of the column, but trapping the non-volatile compounds in the liner instead of the column. Liners are cheap and can be easily replaced, avoiding interferences in the assay reproducibility.

The method applied involved the direct injection of faecal/caecal supernatants into the GC system without any pre-treatment. The faecal or caecal sample was extracted with water, the solid pellet discarded after centrifugation and the supernatant injected into the GC-FID system after the addition of an internal standard. The method and the instrument system were validated by recovery and reproducibility tests (intra-assay and inter-assay). Reproducibility tests were repeated between batches of samples. The internal standard, 2-ethylbutyric acid,

and an external standard mixture containing acetate, propionate, isobutyrate and n-butyrate were used for quantitative purposes.

2.3 MATERIALS AND METHODS

Sample preparation

Fresh mice faecal or caecal contents were snap-frozen in liquid nitrogen or dry ice immediately after collection and stored at -80^{o}C until analysis. Samples (approximately 100 mg) were vortex-mixed with 1.0 mL Milli-Q water and the mixture was kept at room temperature for 10 min and centrifuged at 10000 x g for 5 min to pellet bacteria and other solids. Fresh internal standard solution of 2-ethylbutyric acid in formic acid was prepared and added to the supernatant to a final concentration of 3.0 mM. After homogenization, samples were filtered using a 0.2 µm syringe filter, and the light-brown supernatant transferred to a glass GC vial.

Gas chromatography

Analyses were carried out using a Varian 3500 GC system equipped with a flame-ionisation detector (FID) and fitted with a FFAP column (30 m x 0.32 mm coated with 0.5 µm film thickness). Helium was supplied as the carrier gas at a flow rate of 1.3 mL/min. The initial oven temperature was set at 100^{o}C for 0.5 min, raised to 180^{o}C at 8^{o}C/min and held for 1.0 min, then increased to 200^{o}C at 20^{o}C/min and kept for 7.5 min. The injector temperature was set to 240^{o}C and the detector to 250^{o}C. The injected sample volume was 0.5 µL, and the total running time was 20 min. Calibration curves were built with a standard mixture containing acetate, propionate, isobutyrate and n-butyrate at concentrations of 0.5, 1.0, 2.0,

4.0, 6.0, 8.0 and 10.0 mM. Injections of 10% formic acid in water were made to clear the column of unknown impurities. Data analysis was performed using the Varian Star Chromatography Workstation Software version 6.0.

All chemicals used were of analytical grade, from Sigma-Aldrich, and the columns used for SCFA analysis were made of the same stationary phase (FFAP) and no significant differences were observed between different manufacturers (TRB-FFAP, Teknokroma, Spain; ZB-FFAP, Phenomenex, UK; Nukol-FFAP, Sigma-Aldrich, St. Louis, MO) (data not shown).

Recovery and reproducibility tests

The recovery test was conducted by spiking caecal samples with three different concentrations of the external standard mix and recovered compounds were quantified by GC as described above. To test for intra-assay variation, the same caecal sample and 2.0 mM standard were run repeatedly at the same day and for the inter-assay variation, these same samples were tested on different days over 3 weeks (stored at -20^{o}C between tests). Data are presented as mean values ± standard deviation (SD), and relative standard deviation (RSD).

2.4 RESULTS AND DISCUSSION

In the present study, a GC method was optimised for determining SCFA levels in faecal and caecal samples. SCFA produced in the colon are absorbed while passing through the large intestine, and as expected, highest SCFA concentrations were measured in mice caecal samples, while in faecal samples only acetate could be detected at significant concentrations (data not shown).

The chromatogram peaks obtained were sharp, symmetric and well resolved. The baseline was stable and there were no problems with split peaks. A good linear correlation was found between the peak area ratio and the corresponding standard SCFA ($r^2 > 0.99$ for all SCFA).

In the recovery test, the original amount of each SCFA detected in the caecal sample was as expected with higher concentration of acetate (82.56 umol/g), followed by n-butyrate (34.55 umol/g), propionate (11.4 umol/g) and lastly, the branched chain SCFA, isobutyrate (1.29 umol/g) (Table 2.1). Moreover, the recovery of each compound was around 100% for all the SCFA tested and the chromatograms overlaid perfectly with a very similar retention time (Fig. 2.2). Some protocols use acidified water to extract SCFA from samples in order to obtain better recovery (Claus et al., 2003; Kruse et al., 1999; Scheppach et al., 1987; Schwiertz et al., 2009; Zhao et al., 2006). However, in a study conducted by Claus et al. (2003), the adaptation of the method by Tangeman & Nagengast (1996) using a FFAP column and sample extraction with sulphuric acid solution yielded recovery rates lower than 100% (82%, 79%, and 80% for acetate, propionate, and butyrate, respectively). In this study, no difference was observed between extractions with acidified water or Milli-Q water (data not shown).

Two small peaks were detected between the n-butyrate and 2-ethylbutyric acid peaks (Fig. 2.2). They corresponded to the isovalerate and valerate respectively; however their values were not calculated in this study.

The reproducibility test showed consistent results after multiple repetitions, with the relative standard deviation (RSD) being lower than 5% in the intra-assay test. However the variation of the concentration during the inter-assay, especially

for acetate (RSD > 10% after 3 weeks storage), suggest that samples should not be stored at -20^{o}C during periods longer than 2 weeks (Tables 2.3 and 2.4).

Table 2.1 Recovery of SCFA from caecal samples spiked with different amounts of SCFA standard mix

	Acetate	Propionate	Isobutyrate	N-butyrate
Original amount (umol/g)	82.56 ± 7.4	11.4 ± 0.74	1.29 ± 0.03	34.55 ± 1.43
Amount added (umol/g)	23.68	23.68	23.68	23.68
Amount recovered (umol/g)	109.7 ± 4.11	36.42 ± 0.8	24.94 ± 0.24	59.70 ± 1.46
Recovery (%)	103	104	100	102
Amount added (umol/g)	71.04	71.04	71.04	71.04
Amount recovered (umol/g)	154.71 ± 0.71	82.76 ± 0.93	71.63 ± 0.61	106.48 ± 0.04
Recovery (%)	101	100	99	101
Amount added (umol/g)	118.40	118.40	118.40	118.40
Amount recovered (umol/g)	207.37 ± 2.36	133.20 ± 0.76	122.11 ± 1.89	155.90 ± 1.10
Recovery (%)	103	103	102	102

Values are means ± SDs.

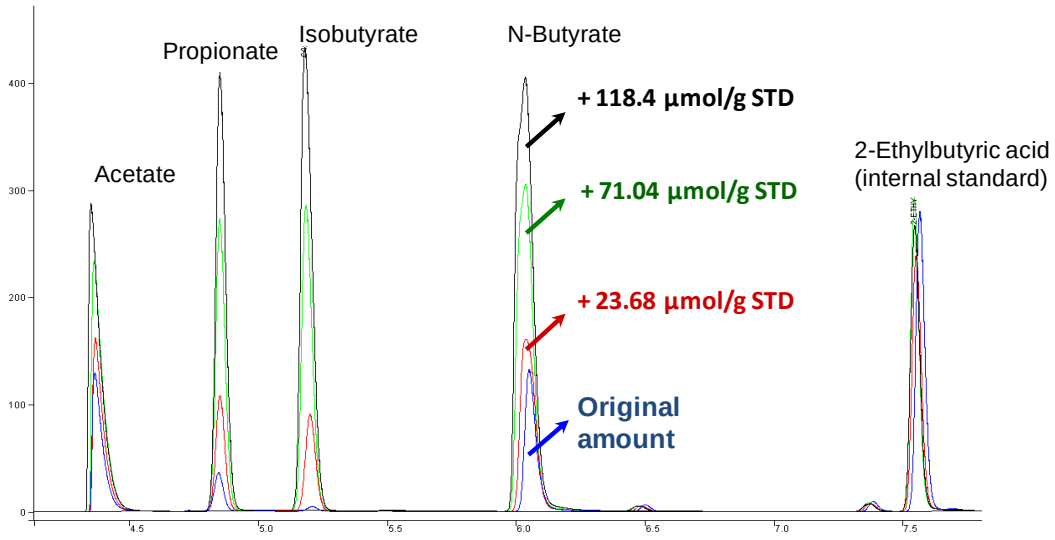


Figure 2.2 Recovery test overlaid chromatograms

Table 2.2 Intra-assay reproducibility test

	Acetate	Propionate	Isobutyrate	N-butyrate
Caecal sample (umol/g) (n=14)	87.34 ± 3.48 (4.0%)	11.79 ± 0.55 (4.7%)	1.21 ± 0.07 (5.7%)	35.40 ± 1.18 (3.3%)
2.0 mM Standard (umol/g) (n=6)	2.13 ± 0.07 (3.5%)	2.00 ± 0.03 (1.5%)	2.03 ± 0.03 (1.2%)	2.09 ± 0.03 (1.3%)
Retention time (min)	4.38	4.87	5.22	6.05

Values are means ± SDs. Relative standard deviation (RSD%) represented between brackets

Table 2.3 Inter-assay reproducibility test

		Acetate	Propionate	Isobutyrate	N-butyrate
Caecal sample (umol/g) (n=14)	*After 1 week*	83.54 ± 5.65 (6.8%)	11.56 ± 0.74 (6.4%)	1.18 ± 0.06 (5.2%)	34.79 ± 1.63 (4.7%)
	After 2 weeks	90.14 ± 5.29 (5.9%)	11.80 ± 0.81 (6.9%)	1.19 ± 0.12 (9.8%)	35.77 ± 1.17 (3.3%)
	After 3 weeks	84.3 ± 9.72 (11.5%)	11.38 ± 1.49 (13.1%)	1.21 ± 0.1 (8.1%)	33.89 ± 1.13 (3.3%)
2.0mM Standard (umol/g) (n=6)	*After 1 week*	2.19 ± 0.13 (6.2%)	2.09 ± 0.09 (4.1%)	2.05 ± 0.02 (1.2%)	2.10 ± 0.05 (2.4%)
	After 2 weeks	2.54 ± 0.28 (10.8%)	2.26 ± 0.12 (5.2%)	2.07 ± 0.07 (3.3%)	2.16 ± 0.05 (2.4%)
	After 3 weeks	2.81 ± 0.35 (12.6%)	2.40 ± 0.09 (3.9%)	2.11 ± 0.03 (1.4%)	2.29 ± 0.06 (2.7%)

Values are means ± SDs. Relative standard deviation (RSD%) represented between brackets

The extraction of SCFA with water was fast, efficient and appropriate for analysis of small samples such as mice intestinal contents. However, supernatant filtration did not eliminate all impurities present in the samples and brown non-volatile faecal material adhered onto the wall of the glass liner after multiple runs. Intestinal samples are complex and contain a variety of non-volatile compounds such as proteins and carbohydrates and, therefore, accumulation of a brown coloured layer on the glass liner surface is a common problem in this type of analysis. In a study conducted by Schafer (1995), samples were subjected to ultrafiltration or ultracentrifugation, but the precision of the SCFA determination was not improved by the application of this separation step and the same brown deposits of non-volatile materials were seen in the glass liners. Moreover, Chen & Lifschitz (1989) determined that multiple-step distillation for sample clean-up was as effective as one-step ultrafiltration of faecal aqueous extract, indicating that complex pre-treatments do not necessarily yield superior results. Therefore, in an effort to obtain accurate SCFA analysis, glass liners were changed routinely, each sample injection was followed by a cleaning procedure, involving injection of 10% (v/v) formic acid solution in water, and samples were always run in triplicate. Furthermore, repetition vials were run in separate batches, fresh set of standards were used with each run and reproducibility tests were carried out frequently.

2.5 CONCLUSIONS

SCFA are among the most important gut microbial products, affecting a range of host processes from gut motility to modulation of inflammation, fat metabolism and appetite. Further research is required on the study of SCFA flux

between intestines and other sites of the body, their role in health and disease, and how the interactions between gut microorganisms may affect their production in the gut. Therefore, simple and rapid analytical methods are required in order to identify and quantify SCFA in biological samples efficiently.

The method tested in this study involved a direct aqueous injection of faecal/caecal samples into GC/FID fitted with a FFAP capillary column and proved to be rapid and efficient for the detection of acetate, propionate, isobutyrate and butyrate. Other SCFA, such as isovalerate and valerate were also detected, and, therefore, the method can be expanded for the detection of these compounds if using appropriate standard mix. The optimized method produced high resolution chromatograms with sharp, well separated peaks (no coelutions), and no peak distortions (split peaks). Moreover, the good recovery of SCFA from spiked samples and the consistent results obtained after multiple runs during the intra-assay reproducibility test demonstrated the accuracy of the assay. Provided that sample storage at -20^{o}C did not exceed 2 weeks and the GC system was maintained in good operating conditions, the method could be used in the analysis of large number of faecal/caecal samples as a routine assay. However, other tests are necessary for the analyses of SCFA in other kind of matrices, such as serum and tissues.

Considering the results obtained, the method was applied in the studies described in the following Chapters 3, 4 and 6 and in the published papers annexed at the end of this thesis.

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Chapter 3

Dietary intake of *trans*-10, *cis*-12 conjugated linoleic acid alters fatty acid metabolism and microbiota composition in mice

3.1 ABSTRACT

Background: A number of studies in rodent have revealed that dietary t*rans*10, *cis*12 conjugated linoleic acid (CLA) supplementation is associated with reduced visceral fat mass, accompanied by severe hepatic steatosis (Clément et al., 2002; Liu et al., 2012; Vyas et al., 2012).

Objective: To investigate the effects of dietary *t*10, *c*12 CLA supplementation on intestinal microbiota composition and production of short chain fatty acids (SCFA) in the large intestine.

Methods: C57BL/6 mice (n=8/group) were fed standard diet supplemented with either *t*10, *c*12 CLA (0.5% w/w) or no supplementation (control), daily, for 8 weeks. Metabolic markers (serum glucose, leptin, insulin and triglycerides and liver triglycerides) were assessed by ELISA commercial kits, tissue long chain fatty acids (LCFA) and caecal SCFA by gas chromatography and microbial composition by 16S rRNA pyrosequencing and bioinformatics analysis.

Results: Animals receiving *t*10, *c*12 CLA exhibited lower visceral fat mass ($p<0.001$) and higher liver mass ($p<0.01$) than unsupplemented controls. Serum triglycerides ($p<0.01$) and leptin ($p<0.05$) were reduced, whereas glucose ($p<0.01$) and insulin ($p<0.05$) were increased in the *t*10, *c*12 CLA group compared with the control group. Moreover, dietary *t*10, *c*12 CLA affected lipid mass and composition with higher n-6/n-3 ratio in brain, liver and epididymal adipose tissue, and lower MUFA/SFA ratio in brain, kidney and epididymal adipose tissue. Caecal concentrations of total SCFA, acetate, propionate and isobutyrate were higher ($p<0.05$) in the *t*10, *c*12 CLA–supplemented group compared with the control group. Analysis of microbiota composition following 8 weeks of *t*10, *c*12 CLA

supplementation revealed lower proportions of *Firmicutes* (p=0.003) and greater proportions of *Bacteroidetes* (p=0.027) compared with no supplementation. Furthermore, the CLA supplemented group had lower proportions of *Desulfovibrionaceae, Lachnospiraceae*, Family XIII Incertae Sedis and *Peptococcaceae* (p<0.05), and higher proportions of *Porphyromonadaceae* (p=0.002) compared with unsupplemented control group.

Conclusions: Dietary *t*10, *c*12 CLA supplementation for 8 wk was associated with significantly altered gut microbiota composition, harbouring significantly higher proportions of *Bacteroidetes,* including *Porphyromonadaceae* bacteria previously linked with negative effects on lipid metabolism and induction of hepatic steatosis. The data indicate that the mechanism of dietary *t*10, *c*12 CLA on lipid metabolism in mice may be at least partially mediated by alterations in gut microbiota composition and functionality.

3.2 INTRODUCTION

Conjugated linoleic acid (CLA) is a class of isomers of linoleic acid that occur naturally in dairy products and meat from ruminant animals due to bacterial biohydrogenation of ingested polyunsaturated fatty acids (PUFA) in the rumen (Bhattacharya et al., 2006). CLA has been shown to be produced *in vitro* and *in vivo* by different species of bacteria (Coakley et al, 2003; Barrett et al, 2007; Lee et al, 2007; Wall et al., 2009). Although *c*9, *t*11 CLA (rumenic acid) is the major natural form/isomer of CLA in foods, accounting for more than 90% of CLA intake in the diet, mixtures containing equal amounts of *c*9, *t*11 and *t*10, *c*12 CLA isomers are produced industrially and sold as supplements. Both these isomers exhibit

significant biological activities, which may exert synergistic or antagonistic effects (Bhattacharya et al., 2006). CLA has been shown to inhibit carcinogenesis (Kelley et al., 2007), prevent atherosclerosis in different animal models (Kritchevsky et al., 2004; Wilson et al., 2000; Toomey et al., 2006), modulate the immune system (Yang & Cook, 2003; Yu et al., 2002), and affect body composition, by reducing body fat and increasing lean body mass (Clément et al., 2002; Liu et al., 2012). *T*10, *c*12 CLA has been shown to be the isomer responsible for the anti-obesity effect attributed to CLA and over the past years its impact on body fat modulation have been largely studied in different animal models and in humans (Clément et al., 2002; Liu et al., 2012; Salas-Salvado et al., 2006). There is evidence to suggest that fat mass reduction is the result of multiple interactions of *t*10, *c*12 CLA with numerous metabolic signalling pathways leading to decreased energy intake and increased energy expenditure, inhibition of adipogenesis and lipogenesis, modulation of adipokines and cytokines and increased fatty acid β-oxidation in skeletal muscle (Park and Pariza, 2007).

The response to CLA supplementation appears to be highly species-specific, with mice generally being more sensitive than other animal models and humans. These differences are attributed to the dose levels used (human trials use lower doses), age (animal trials usually use young subjects), rate of body fat turnover (small animals have faster metabolism) and dietary regimes (*ad libitum* in animal models vs. calorie restriction in human trials) (Park & Pariza, 2007; Wang & Jones, 2004). In most studies using mice, body fat reduction induced by *t*10, *c*12 CLA supplementation is accompanied by such adverse effects as hepatic steatosis and hyperinsulinemia (Clément et al. 2002; Liu et al., 2009). These are features

frequently associated with metabolic syndrome and commonly observed in obese and diabetic individuals (Le Roy et al., 2012; Tamura & Shimomura, 2005). Hepatic steatosis is characterized by an increase in liver mass with accumulation of intracellular lipids, primarily in the form of triglycerides. Increased uptake of circulating fatty acids (FA), increased hepatic *de novo* lipogenesis (DNL), reduced rate of fatty acid oxidation and reduced FA secretion are the multiple mechanisms leading to increased accumulation of lipids in the liver (Vyas et al., 2012). Moreover, recent studies have indentified the gut microbiota as an environmental factor with an important role in host fat metabolism and the development of hepatic steatosis (Le Roy et al., 2012). In a continuous bidirectional communication between gut and liver, hepatic products can directly influence microbiota composition, whereas bacterial metabolites may have both direct and indirect effects on liver function and physiology (Bajaj, Hylemon, et al, 2012; Quigley et al., 2013). Thus, in this study we investigated the impact of dietary *t*10, *c*12 CLA on intestinal microbiota composition and the production of SCFA.

3.3 MATERIALS AND METHODS

Animals and diets

Experiments involving animal were approved by the University College Cork Animal Ethics Committee, and experimental procedures were conducted under the appropriate license from the Irish Government. Male C57BL/6 mice, 7-8 weeks of age were obtained from Charles River and housed under barrier-maintained conditions within the Biological Services Unit, University College Cork. After one week of acclimatization, animals were divided into 2 groups (n=8/group). Both

groups were fed *ad libitum* with Teklad Global rodent standard diet (#2018S; Harlan Laboratories) and allowed free access to water. The fatty acids present in the diet included palmitic acid (0.7%), stearic acid (0.2%), oleic acid (1.2%), linoleic acid (3.1%), and linolenic acid (0.3%). The treatment group received 0.5% (w/w) *t*10, *c*12 CLA incorporated into the diet. Body weight was assessed weekly. After 8 weeks of dietary intervention, fasted animals were sacrificed by decapitation and blood samples were collected, allowed to clot for 2 h at 4^{o}C and centrifuged at 2000 x g for 20 min. Liver, brain, fat pads (epididymal, perirenal and mesenteric), heart, kidney and intestines were removed, blotted dry on filter paper, weighed and flash-frozen in liquid nitrogen. Caecal content was collected for pyrosequencing and SCFA analyses. All samples were stored at -80^{o}C prior to analyses.

Lipid extraction and fatty acid analysis

Fatty acid profiles were determined for liver, brain, epididymal adipose tissue, heart and kidney. Lipids were extracted with chloroform:methanol (Fisher Scientific, UK) according to the method by Folch et al. (1957). After extraction, samples were methylated by using 0.5 N NaOH (Sigma) in methanol for 10 min at 90°C followed by 10 min incubation at 90°C with 14% boron trifluoride (BF_3) in methanol (Sigma) (Park and Goins, 1994). Fatty acid methyl esters (FAME) were recovered with hexane (Fisher Scientific). Before gas-liquid chromatographic analysis, samples were dried over anhydrous sodium sulfate (Sigma) for 1 h and stored at -20°C. FAME were separated using a Varian 3800 GC flame-ionization system, fitted with a Chrompack CP Sil 88 column (Chrompack; 100 m x 0.25 mm internal diameter, 0.20 um film thickness) and helium as carrier gas. The column

oven was programmed initially at 80°C for 8 min and then increased 8.5°C/min to a final column temperature of 200°C. The injection volume used was 0.6 uL, with automatic sample injection on a SPI 1093 splitless on-column temperature-programmable injector. Peaks were integrated using the Varian Star Chromatography Workstation version 6.0 software, and peaks were identified by comparison of retention times with pure FAME standards (Nu-Chek Prep Inc., Elysian, MN). The percentage of individual fatty acids was calculated according to the peak areas relative to the total area (total fatty acids were set at 100%). All fatty acid results are shown as mean ± standard error of the mean (SEM) in g/100 g FAME.

SCFA analysis

Caecal content was vortex-mixed with Milli-Q water, incubated at room temperature for 10 min and centrifuged at 10000 x g to pellet bacteria and other solids. The supernatant was filtered and transferred to a clear GC vial. 2-ethylbutyric acid (Sigma) was used as internal standard. The concentration of SCFA was measured by using a Varian 3500 GC flame-ionization system, fitted with a Nukol-FFAP column (30 m x 0.32 mm x 0.25 um; Sigma). The initial oven temperature was set at 100^{o}C for 0.5 min, raised to 180^{o}C at 8^{o}C/min and held for 1 min, then increased to 200^{o}C at 20^{o}C/min, and finally held at 200^{o}C for 5 min. The temperature of the injector and the detector were set at 240^{o}C and 250^{o}C, respectively. Helium was used as carrier gas at a flow rate of 1.3 mL/min. A standard curve was built with different concentrations of a standard mix containing

acetate, propionate, isobutyrate and n-butyrate (Sigma). Peaks were integrated by using the Varian Star Chromatography Workstation version 6.0 software.

Measurement of hepatic triglycerides

Hepatic lipids were extracted according to the method by Folch et al. (1957). After extraction, samples were dried under a stream of nitrogen and resuspended in 5% (v/v) solution of Triton X-100 in distilled water. Triglyceride concentrations were determined using a commercial kit (EnzyChrom Triglyceride Assay, BioAssay Systems, Hayward, CA).

Serum analyses

Commercial kits were used for measurement of metabolic markers in serum. Glucose was determined using the QuantiChrom glucose assay (BioAssay Systems), triglycerides by using EnzyChrom Triglyceride Assay kit (BioAssay Systems), insulin using the Ultra Sensitive Mouse ELISA kit (Crystal Chem Inc, Downers Grove, IL), and leptin was determined using the Mouse Leptin ELISA kit (Crystal Chem Inc).

Gut microbiota composition

Amplicon Sequencing

DNA extraction and high-throughput amplicon sequencing DNA was purified from faecal samples using the QIAmp DNA Stool Mini Kit (Qiagen, Crawley, West Sussex, UK) according to manufacturer's instructions with addition of a bead-beating step (30s x 3) and stored at -20^{o}C. The microbiota composition of the

samples was established by amplicon sequencing of the 16S rRNA gene V4; universal 16S rRNA primers estimated to bind to 94.6% of all 16S genes (i.e. the forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAAAGNG), R2 (TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP'S Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp) were employed for PCR amplification. Molecular identifier tags were attached between the 454 adaptor sequence and the target-specific primer sequence, allowing for identification of individual sequences from the pooled amplicons. Ampure purification system (Beckman Coulter, Takeley, UK) was used to clean the amplicons before being sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) in line with 454 protocols at the Teagasc high throughput sequencing centre.

Sequence Analysis

Raw sequences were quality trimmed and filtered using the Qiime Suite of programs (Caporaso et al 2010). Resulting trimmed fasta sequences were assigned to taxa through BLAST analysis against the SILVA database (version 106) for 16S reads. BLAST outputs were parsed using MEGAN (Huson et al., 2007) with a bit-score of 86; taxonomy was assigned to phylum, family and genus level. Sequence reads were clustered, aligned and chimera checked with Qiime and phylogenetic analysis was implemented with FastTreeMP (Price et al., 2010). Alpha and beta diversities were calculated again using Qiime and subsequently principal coordinate

analysis (PCoA) was performed on the distance matrices. PCoA plots were visualised with KiNG viewer (Chen et al., 2009).

Statistical analysis

To assess whether differences between treatment groups were significant, statistical analysis was performed by Student *t* test (Graph-Pad Software, San Diego, CA, USA). Treatment effects with $p<0.05$ were considered significant. Kruskal–Wallis and Mann–Whitney tests, implement in SPSS statistical software package were used to find significant differences in microbial taxa and alpha diversity. Significance was taken as $p<0.05$. Data in the text, tables, and figures are presented as mean values ± SEM.

3.4 RESULTS

Dietary t10, c12 CLA decreases fat storage, increases liver weight and impact on host metabolism, but does not alter body weight

After 8 weeks of dietary supplementation with *t*10, *c*12 CLA, a 2-fold decrease in visceral body fat (sum of epididymal, mesenteric and perirenal fat pads; $p<0.001$) and a significant increase in liver mass ($p<0.01$; Table 3.1) was obtained, compared with unsupplemented mice. Body weight gain and final body weight did not differ between the groups (Table 3.1). The greater liver weight observed in mice supplemented with *t*10, *c*12 CLA was accompanied by a 7-fold increase in hepatic triglycerides ($p<0.001$; Table 3.1). In contrast, serum triglycerides were lower in mice fed *t*10, *c*12 CLA when compared with unsupplemented controls ($p<0.01$). *T*10, *c*12 CLA supplementation was also associated with higher serum glucose

concentrations ($p<0.01$) and serum insulin ($p<0.05$). The concentration of leptin in serum, which is proportional to the amount of fat in the body, was lower in CLA supplemented animals compared with unsupplemented controls ($p<0.05$) (Table 3.1).

Table 3.1 Effect of *t*10, *c*12 CLA on body mass, liver mass and visceral fat mass, and on metabolic markers

	***T*10,*c*12 CLA**	**Unsupplemented**
Final body weight (g)	31.3 ± 1.0	30.6 ± 0.9
Body weight gain (%)	16.0 ± 3.0	20.5 ± 3.1
Liver weight (g)	2.2 ± 0.1**	1.5 ± 0.1
Visceral fat weight (g)	0.6 ± 0.06***	1.2 ± 0.1
Leptin (ng/mL)	0.7 ± 0.2*	2.4 ± 0.7
Glucose (mg/dL)	200.4 ± 4.8**	183.3 ± 2.8
Insulin (ng/mL)	4.0 ± 0.9*	1.7 ± 0.4
Serum triglycerides (mg/dL)	28.6 ± 4.3**	61.3 ± 8.2
Liver triglycerides (mg/g)	20.6 ± 2.9***	2.9 ± 0.2

All values are means ± SEMs; n=8 mice per group. Numbers with (*) are significantly different (Student *t* test; *p<0.05; **p<0.01; ***p<0.001). Visceral fat= sum of epididymal, perirenal, and mesenteric fat pads.

Host fatty acid composition is affected by dietary t10, c12 CLA

Fatty acid profiles were determined in brain, epididymal adipose tissue, liver, heart and kidney, following 8 weeks of dietary *t*10, *c*12 CLA supplementation. *T*10, *c*12 CLA was incorporated into epididymal adipose tissue, liver, heart and kidney tissues, but was not detected in the brain (Table 3.2). *T*10, *c*12 CLA was not detected in any tissues in the unsupplemented group. Fatty acid composition of all tissues tested was altered in the *t*10, *c*12 CLA group compared with unsupplemented controls, with the greatest impact on brain, epididymal adipose tissue and liver (Table 3.2, Fig. 3.1). Mice supplemented with *t*10, *c*12 CLA had significantly higher amounts of total saturated fatty acids (SFA) in brain and epididymal adipose tissue, compared with unsupplemented animals ($p<0.001$, Fig. 3.1), including 1.6-fold increase in myristic acid (C14:0; $p<0.05$) and 1.2-fold increase in palmitic acid (C16:0; $p<0.001$) in brain and 2-fold increases in myristic ($p<0.01$) and palmitic acids ($p<0.001$) in epididymal adipose tissue (Table 3.2). Moreover, mice that received *t*10, *c*12 CLA had significantly lower myristic acid in the heart (57%; $p<0.05$), and lower stearic acid (C18:0) in epididymal adipose tissue (17%; $p<0.05$) and liver (41%; $p<0.001$), whereas palmitic acid was higher in the liver (13%; $p<0.001$). No differences were observed in the levels of SFA in the kidney of the *t*10, *c*12 CLA group compared with the unsupplemented group. *T*10, *c*12 CLA supplementation was associated with reduced monounsaturated fatty acids (MUFA) in both epididymal adipose tissue and kidney, while increased levels were found in liver and brain (Table 3.2, Fig 3.1). Palmitoleic acid (C16:1c9) was detected in higher amounts in brain (11%; $p<0.05$), and lower concentrations in liver (31%; $p<0.05$), heart (74%; $p<0.05$) and kidney (56%; $p<0.001$) of mice

supplemented with *t*10, *c*12 CLA compared with unsupplemented controls. CLA supplementation also led to increased concentrations of oleic acid (C18:1c9) in liver (50%; $p<0.001$) and brain (8%; $p<0.01$), and decreased oleic acid concentration in epididymal adipose tissue (30%; $p<0.01$) and kidney (19%; $p<0.05$). Omega-3 polyunsaturated fatty acids (n-3 PUFA) were also affected by *t*10, *c*12 CLA supplementation, with the brain of mice receiving *t*10, *c*12 CLA having 24% less docosahexaenoic acid (DHA) (22:6n-3) than unsupplemented controls ($p<0.001$) and epididymal adipose tissue having 58% less linolenic acid (C18:3n-3; $p<0.001$), 32% less docosapentaenoic acid (DPA) (C22:5n-3; $p<0.05$) and 67% less DHA ($p<0.001$) than unsupplemented mice. Eicosapentaenoic acid (EPA) was 50% lower ($p<0.001$), DHA 55% lower ($p<0.001$) and DPA 33% higher ($p<0.05$) in liver of the *t*10, *c*12 CLA group compared with unsupplemented controls. Moreover, the heart of mice fed *t*10, *c*12 CLA had 57% less linolenic acid ($p<0.05$) and 60% more DPA ($p<0.001$), whereas the kidney had 43% less linolenic acid ($p<0.01$) and 45% more DPA ($p<0.01$) when compared to the same tissues from the unsupplemented group. Significant differences in n-6 PUFA composition were also observed after supplementation with *t*10, *c*12 CLA in all tissues analysed. In the brain of mice fed *t*10, *c*12 CLA, arachidonic acid (AA; C20:4n-6) content was decreased by 21% ($p<0.001$) while dihomo-*γ*-linolenic acid (C20:3n-6) content was increased by 67% ($p<0.001$). Linoleic acid (C18:2n-6), dihomo-*γ*-linolenic acid and AA concentrations were decreased by 32% ($p<0.001$), 50% ($p<0.001$) and 29% ($p<0.01$), respectively, in the epididymal adipose tissue of animals receiving *t*10, *c*12 CLA, compared with unsupplemented controls. Significantly lower amounts of *γ*-linolenic acid (33%; $p<0.001$), dihomo-*γ*-linolenic acid (22%; $p<0.001$) and AA (49%; $p<0.001$) were also

detected in liver of mice receiving *t*10, *c*12 CLA, compared with unsupplemented controls. Less linoleic acid was detected in the heart (14%; $p<0.05$) and kidney (17%; $p<0.05$) of the *t*10, *c*12 CLA-fed group, whereas dihomo-*c*-linolenic acid was detected in higher amounts in the kidney (50%; $p<0.01$) of mice receiving CLA supplementation when compared with unsupplemented controls. Furthermore, the relative proportion of saturated to monounsaturated fatty acids, an important aspect of phospholipid compositions and membrane fluidity, was altered in all tissues of mice receiving *t*10, *c*12 CLA except the heart, and the ratio of n-6 PUFA to n-3 PUFA was also changed, with higher ($p<0.05$) proportions of n-6 PUFA in the brain, epididymal adipose tissue and liver (Table 3.2) of CLA supplemented animals, compared with unsupplemented controls.

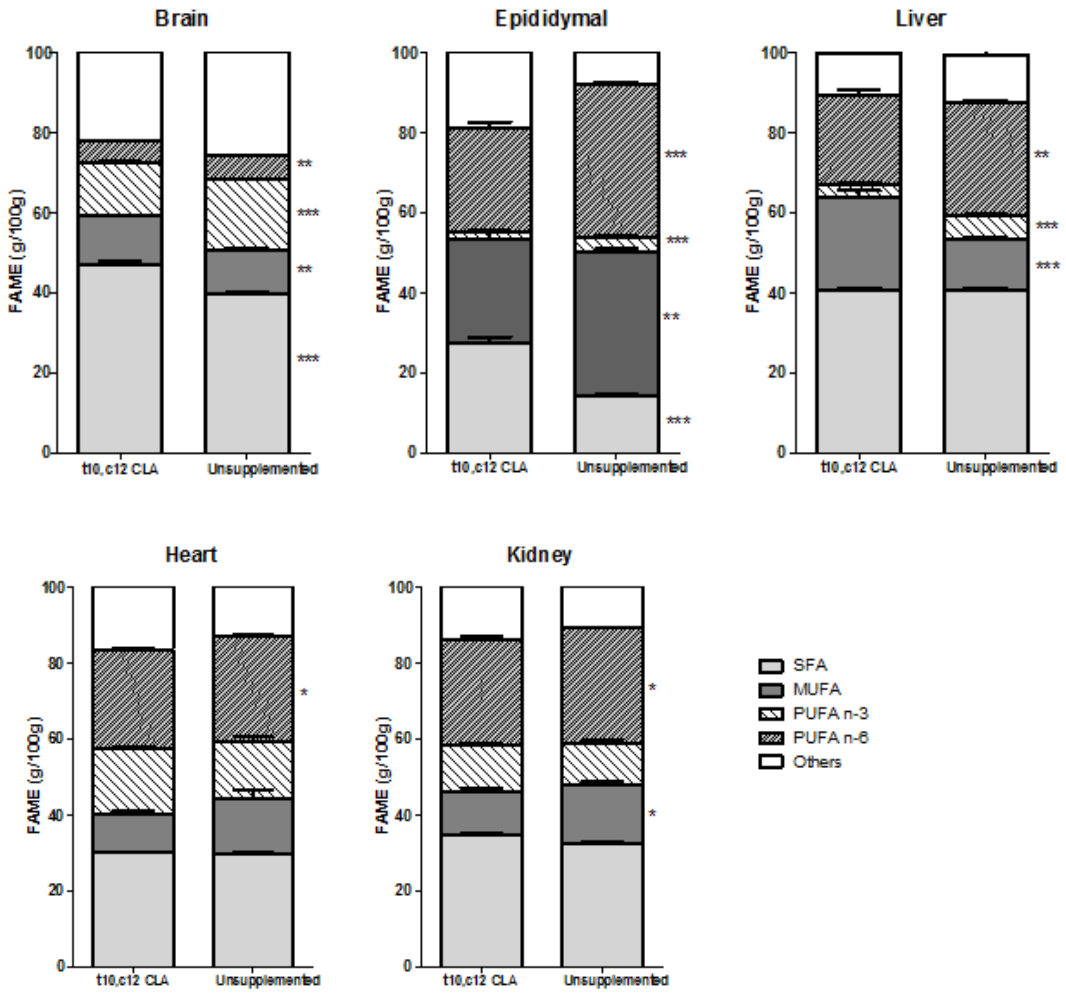


Figure 3.1 Tissue fatty acid composition is altered by *t*10, *c*12 CLA supplementation, with a greater impact on brain, epididymal adipose tissue and liver. Columns with (*) are significantly different (Student *t* test; *p<0.05; **p<0.01; ***p<0.001).

Table 3.2 Effect of dietary *t*10, *c*12 CLA or unsupplemented diet on fatty acid composition (g/100g FAME) of mouse tissues

FAME	Brain		Epididymal adipose tissue		Liver		Heart		Kidney	
	t10, c12 CLA	Unsupplemented	t10, c12 CLA	Unsupplemented	t10, c12 CLA	Unsupplemented	t10, c12 CLA	Unsupplemented	t10, c12 CLA	Unsupplemented
Saturated fatty acids(SFA)										
C14:0	3.1 ± 0.4*	1.9 ± 0.1	2.5 ± 0.3**	1.1 ± 0.2	0.7 ± 0.0	0.7 ± 0.0	0.3 ± 0.0*	0.7 ± 0.1	0.8 ± 0.2	0.7 ± 0.1
C16:0	32.9 ± 0.6***	26.7 ± 0.4	22.9 ± 1.6***	10.7 ± 0.5	34.2 ± 0.5***	30.4 ± 0.4	14.9 ± 0.4	15.7 ± 0.9	19.9 ± 0.4	19.0 ± 0.3
C18:0	11.2 ± 0.1	11.1 ± 0.2	1.9 ± 0.1*	2.3 ± 0.0	5.7 ± 0.6***	9.6 ± 0.5	15.0 ± 0.5	13.4 ± 1.1	13.7 ± 0.7	12.7 ± 0.4
Monounsaturated fatty acids (MUFA)										
C16:1c9	1.0 ± 0.0*	0.9 ± 0.0	2.9 ± 0.2	2.8 ± 0.1	1.8 ± 0.2*	2.6 ± 0.2	0.5 ± 0.1*	1.9 ± 0.5	0.7 ± 0.1***	1.6 ± 0.1
C18:1c9	11.1 ± 0.2**	10.2 ± 0.1	23.4 ± 2.2**	33.5 ± 0.5	21.5 ± 1.5***	10.8 ± 0.4	9.3 ± 0.8	12.8 ± 1.8	11.1 ± 0.9*	13.7 ± 0.7
Polyunsaturated fatty acids n-3										
C18:3n-3	ND	ND	1.1 ± 0.1***	2.6 ± 0.1	0.6 ± 0.0	0.7 ± 0.0	0.3 ± 0.0*	0.7 ± 0.1	0.4 ± 0.1**	0.7 ± 0.1
C 20:5n-3	ND	ND	ND	0.1 ± 0.0	0.1 ± 0.0***	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
C22:5n-3	0.2 ± 0.0	0.2 ± 0.1	0.17 ± 0.02*	0.25 ± 0.02	0.6 ± 0.1*	0.4 ± 0.0	2.5 ± 0.1***	1.0 ± 0.1	1.1 ± 0.1**	0.6 ± 0.1
C 22:6n-3	13.0 ± 0.5***	17.2 ± 0.1	0.2 ± 0.0***	0.6 ± 0.1	2.0 ± 0.2***	4.5 ± 0.2	14.4 ± 0.6	13.2 ± 1.4	10.2 ± 0.7	9.3 ± 0.8
Polyunsaturated fatty acids n-6										
C18:2n-6	0.5 ± 0.0	0.5 ± 0.0	25.1 ± 1.6***	36.7 ± 0.7	17.3 ± 0.9	19.1 ± 0.3	19.0 ± 0.6*	22.0 ± 0.8	15.6 ± 1.1*	18.9 ± 0.7
C18:3n-6	0.3 ± 0.0	ND	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0***	0.3 ± 0.0	ND	ND	ND	ND
C20:3n-6	0.5 ± 0.0***	0.3 ± 0.0	0.2 ± 0.0***	0.4 ± 0.0	0.7 ± 0.0***	0.9 ± 0.0	0.9 ± 0.1**	0.6 ± 0.0	1.0 0.1	1.0 ± 0.0
C 20:4n-6	4.2 ± 0.1***	5.3 ± 0.1	0.5 ± 0.0**	0.7 ± 0.0	4.0 ± 0.4***	7.8 ± 0.4	6.3 ± 0.3	5.2 ± 0.4	11.5 ± 0.9	10.4 ± 0.5
t10,c12 CLA	ND	ND	2.2 ± 0.2	ND	0.4 ± 0.0	ND	0.8 ± 0.1	ND	0.6 ± 0.1	ND
Ratios										
MUFA/SFA	0.26 ± 0.0*	0.28 ± 0.0	1.00 ± 0.13*	2.60 ± 0.1	0.58 ± 0.05*	0.33 ± 0.02	0.33 ± 0.03	0.50 ± 0.08	0.34 ± 0.03*	0.48 ± 0.03
n-6/n-3	0.42 ± 0.01*	0.34 ± 0.0	17.61 ± 0.72*	10.86 ± 0.35	6.86 ± 0.24*	4.84 ± 0.09	1.52 ± 0.07	2.00 ± 0.21	2.43 ± 0.19	2.92 ± 0.23

All values are means ± SEM; n = 8 mice per group. Numbers with (*) are significantly different (Student *t* test; * p<0.05; ** p<0.01; *** p<0.001) from corresponding numbers in unsupplemented control group. FAME= fatty acid methyl esters; ND= not detected.

T10, c12 CLA intake stimulates the production of SCFA

Microbial fermentation in caecum was enhanced by dietary supplementation with *t*10, *c*12 CLA. Acetate, propionate and isobutyrate levels were significantly higher ($p<0.05$; Table 3.3) in mice fed *t*10, *c*12 CLA compared with unsupplemented controls, whereas no difference between groups was observed for n-butyrate. Moreover, total SCFA concentration (sum of acetate, propionate, isobutyrate and n-butyrate) was 34% higher in the caecal content of mice that received *t*10, *c*12 CLA compared with unsupplemented group ($p<0.05$) (Fig. 3.2).

Table 3.3 Short chain fatty acids in the caecum content of mice fed *t*10, *c*12 CLA or an unsupplemented diet for 8 weeks

	***T*10, *c*12 CLA**	**Unsupplemented**
Acetate	54.5 ± 4.2*	40.7 ± 2.4
Propionate	10.7 ± 1.1*	7.1 ± 0.4
N-butyrate	17.1 ± 2.1	13.7 ± 1.6
Isobutyrate	0.9 ± 0.09*	0.7 ± 0.01
Total SCFA	83.2 ± 7.1*	62.2 ± 4.1

All values are means ± SEMs; n=8 mice per group. Numbers with (*) are significantly different (P<0.05; Student *t* test).

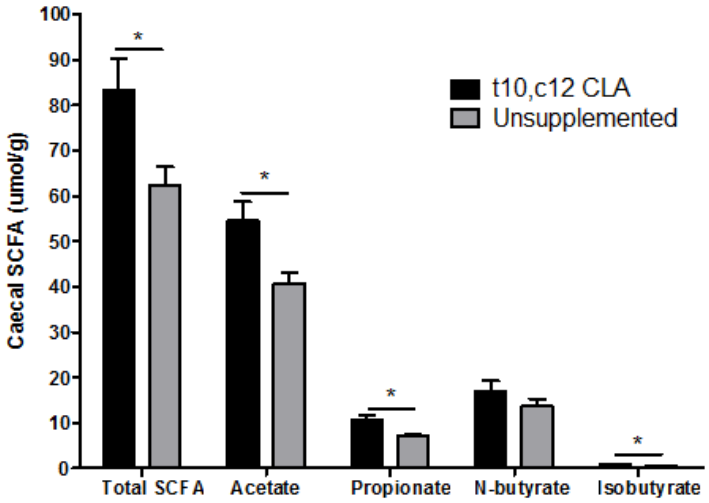


Figure 3.2 Caecal SCFA concentration in mice receiving a diet supplemented with *t*10, *c*12 CLA or with no supplementation; n=8 per group (Student *t* test; *p<0.05)

Dietary t10, c12 CLA significantly alters gut microbiota composition

A total of 416,309 V4 16S amplicon sequence reads were generated; corresponding to a mean of 23,200 reads per mouse post quality checking. All rarefaction curves were approaching parallel (data not shown) indicating sufficient depth of sequencing. Reads were clustered into operational taxonomical units (OTUs) of 97% identity and diversity metrics estimated. Of the five alpha diversity metrics used (Shannon, Simpson, chao1, phylogenetic diversity and observed species), no significant differences were observed between CLA-supplemented and unsupplemented groups. Beta-diversity was calculated with both weighted and un-weighted Unifrac distance matrices. Subsequent PCoA revealed a distinct separation of the two groups for both measures (Fig. 3.3).

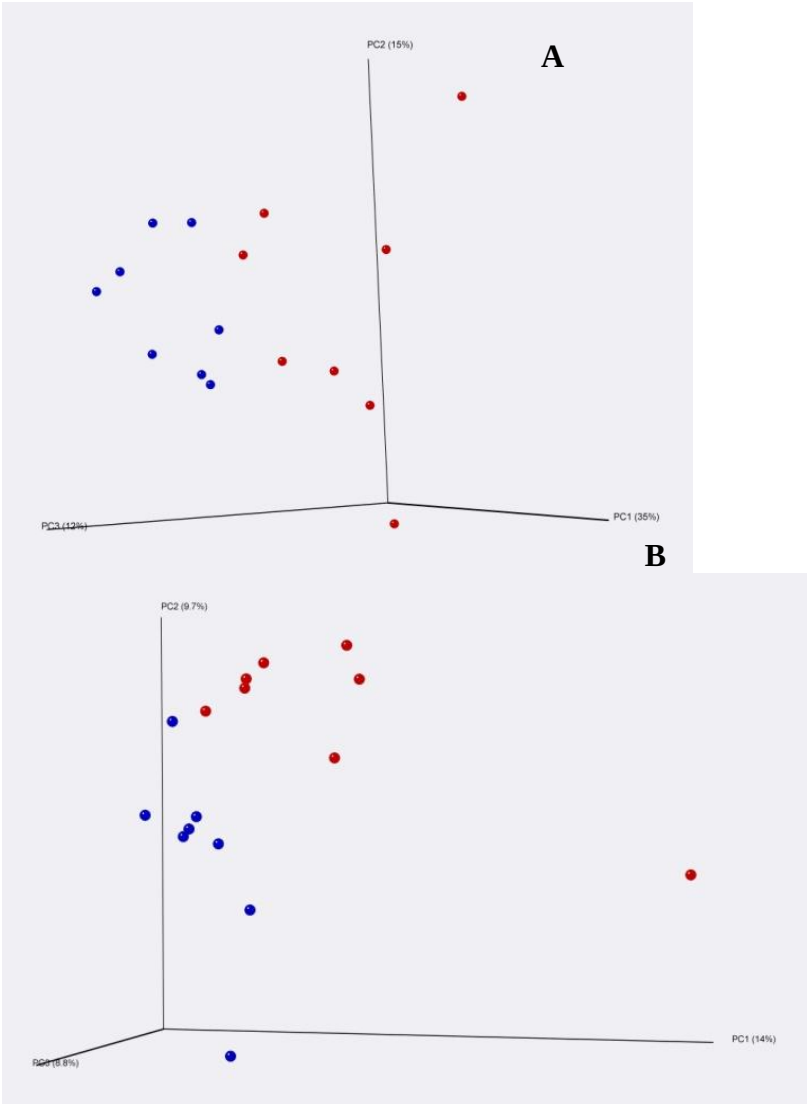


Figure 3.3 PCoA analysis based on weighted Unifrac (A) and unweighted Unifrac (B) distances; *t*10, *c*12 CLA supplemented group (red circles) and unsupplemented group (blue circles); n=8 per group.

The mouse gut microbiota was dominated by members of the *Firmicutes* and *Bacteroidetes* phyla. Microbial composition assignment revealed several significant differences between CLA-supplemented and unsupplemented groups. At the phylum level, *t*10, *c*12 CLA supplementation was associated with significantly reduced proportions of *Firmicutes* (p=0.003) and increased amounts of *Bacteroidetes* (p=0.027) when compared with unsupplemented controls. At the family level, members of *Desulfovibrionaceae* (p=0.027), *Lachnospiraceae* (p=0.006), Family XIII Incertae Sedis (p=0.016) and *Peptococcaceae* (p=0.009) were all significantly decreased in *t*10, *c*12 CLA group compared with unsupplemented controls, whilst members of *Porphyromonadaceae* (p=0.002) were significantly increased in *t*10, *c*12 CLA group (Fig. 3.4). At the genus level, populations of *Desulfovibrio* (p=0.021), *Lachnospiraceae* Incertae Sedis (p=0.006) and *Ruminococcus* Incertae Sedis (p=0.027) were significantly decreased and *Odoribacter* (p=0.002) populations were significantly increased in *t*10, *c*12 CLA group when compared with the unsupplemented group.

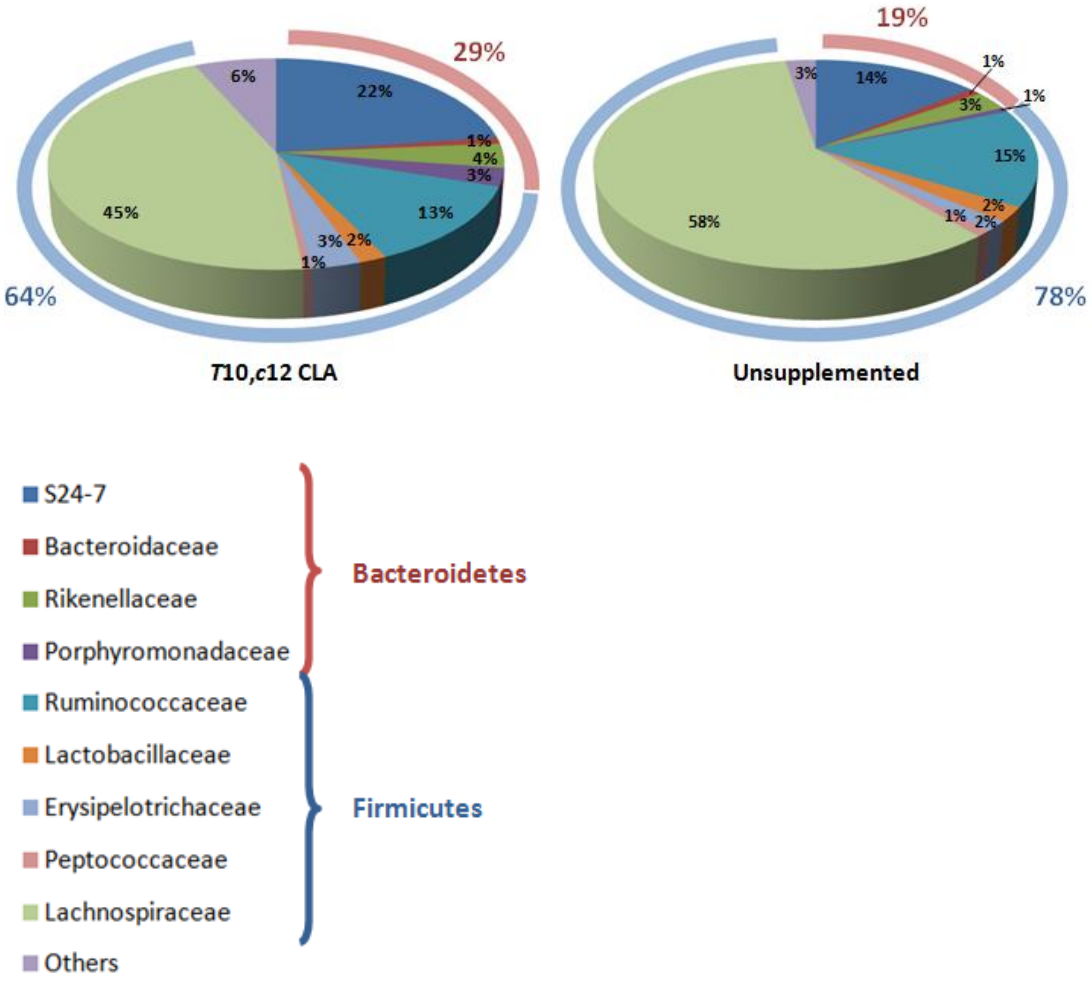


Figure 3.4 The gut microbiota composition is altered in mice receiving *t*10, *c*12 CLA supplementation, as determined by pyrosequencing of 16S rRNA (n=8 per group). Pie charts represent the mean percentage read number for the corresponding colour coded family (only reads ≥1% are shown).

3.5 DISCUSSION

The effects of dietary CLA on body fat have been largely studied in mice and, although *t*10, *c*12 CLA effects seems to be independent of genetic strain (House et al, 2005), the C57BL/6 mouse has been shown to be very sensitive and constitutes an interesting model for studying lipid metabolism dysfunctions (Degrace, 2006). In this study, C57BL/6 mice developed severe lipoatrophy with concomitant liver steatosis after 8 weeks of dietary supplementation with 0.5% (w/w) *t*10, *c*12 CLA. Previous studies have shown that feeding *t*10, *c*12 CLA triggers changes in the pattern of gene expression, reducing fatty acid uptake and storage in the adipocytes and favouring lipid accumulation in the liver of mice (Clément, 2002; Jourdan 2009; Vyas et al., 2012).

The fat lowering effect of *t*10, *c*12 CLA is complex, involving multiple mechanisms and seems to be related to the development of hepatic steatosis in mice. In the adipose tissue, *t*10, *c*12 CLA has been reported to increase (pre)adipocyte apoptosis and reduce adipogenesis and lipogenesis by inhibition of key transcription factors such as peroxisome proliferator-activated receptor γ (PPAR-γ), CAAT/enhancer binding protein (C/EBP), sterol regulatory element binding protein 1c (SREBP-1c), liver X receptor α (LXR-α) and adipocyte-specific fatty acid binding protein (aP2) (Kennedy et al, 2010). With the nearly complete absence of adipose tissue, leptin is produced at very low rates by adipocytes, and the release of free FA is decreased, reducing lipid flux to the liver and subsequent VLDL secretion rates (Degrace et al., 2006). Indeed, in the current study, lower triglycerides and leptin serum levels were observed in the *t*10, *c*12 CLA-supplemented group compared with unsupplemented controls. Another

modification induced by *t*10, *c*12 CLA supplementation was the markedly increased serum glucose concentration, compared with no supplementation. *T*10, *c*12 CLA supplementation may impair insulin signalling and glucose uptake via inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), affecting the expression of lipogenic proteins, including lipoprotein lipase (LPL), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD1) and the insulin-dependent glucose transporter 4 (GLUT4) (Kennedy et al, 2010). Thus, impaired glucose uptake by adipocytes is expected to be a consequence of adipose tissue atrophy and lower expression of GLUT4. Moreover, higher serum insulin levels were found in the *t*10, *c*12 CLA group compared with unsupplemented controls. High levels of circulating insulin and glucose have been reported to induce the expression of SREBP1c and carbohydrate response element-binding protein (ChREBP) in the liver and therefore stimulate hepatic *de novo* lipogenesis (Shimomura, et al., 1999; Yamashita et al., 2001). Accordingly, a study conducted by Clément et al. (2002) suggested that liver steatosis induced by *t*10, *c*12-CLA is secondary to hyperinsulinemia, which causes high levels of FA uptake and synthesis in the liver. They observed an increase in PPARγ mRNA levels and its target genes fatty acid transporter (FAT/CD36) and aP2, both involved in FA uptake. Moreover, FAS and SREBP-1 genes were upregulated by *t*10, *c*12 CLA feeding. Another study using diabetic mice demonstrated that *t*10, *c*12 CLA induces liver steatosis in the absence of insulin (Jourdan, 2009). Apparently, *t*10,*c*12 CLA may exert its effects through a concomitant induction of hepatic expression of VLDLR, LPL and FAT/CD36, improving FA utilization by the liver and increasing plasma lipoprotein clearance (Degrace et al., 2006; Jourdan et al.,2009) (Fig. 3.5).

Another factor that may contribute to the development of liver steatosis is the profound change in LCFA composition of liver, caused by *t*10, *c*12 CLA supplementation. As seen in this study, *t*10, *c*12 CLA supplementation was associated with increased n-6 PUFA/n-3 PUFA ratio in the liver. Reduced availability of n-3 PUFA may increase SREBP-1 expression and reduce PPARα expression, stimulating lipogenesis over lipid oxidation, with accumulation of TG in the liver (El-Badry et al., 2007). Furthermore, besides changing liver FA profiles, *t*10, *c*12 CLA supplementation was associated with altered FA composition of other tissues. Our observations confirmed previous reports demonstrating that *t*10, *c*12 CLA supplementation decreased AA and altered the ratio of SFA to MUFA, especially increasing the amount of palmitic acid over palmitoleic acid (Evans et al., 2002; House et al., 2005; Kelley et al., 2006). Although the exact mechanism of CLA action on tissue FA composition has not been elucidated, some studies suggest that the reduction in SCD-1 activity may impair the conversion of SFA to MUFA (Evans et al., 2002; House et al., 2005), whereas the inhibition of linoleic acid elongation and desaturation may impact on n-6 PUFA synthesis (Eder et al., 2002; Lin et al., 2011).

The current study also showed that while there were no significant differences in diversity between groups (data not shown), supplementation with *t*10, *c*12 CLA had an effect on the composition of the murine gut microbiota when compared to no supplementation, as demonstrated by divergent clustering pattern in the PCoA analysis (Fig. 3.3). Perturbations of gut microbiota composition may play an important role in the development of diseases associated with altered metabolism such as obesity, diabetes and cardiovascular diseases (Tremaroli and Bäckhed, 2012). For example, studies using germ free animals have shown that the

absence of microbiota is accompanied by increased fatty acid oxidation and decreased lipogenesis, making these animals resistant to diet-induced obesity, steatosis and insulin resistance (Bäckhed et al., 2004; Bäckhed et al., 2007). Furthermore, some evidence suggest that body weight gain is associated with higher proportions of *Firmicutes* and lower *Bacteroidetes* among gut microbiota (Bäckhed et al., 2004; Ley et al., 2005; Turnbaugh et al., 2009), while body weight loss has been correlated with increased abundance of *Bacteroidetes* (Ley et al., 2006). In this study, we observed that decreased body fat in mice receiving *t*10, *c*12 CLA was associated with higher proportions of *Bacteroidetes* and lower abundance of bacteria from the *Firmicutes* phylum. Interestingly, a study by Larsen et al. (2010) demonstrated the same compositional changes in the intestinal microbiota of humans with type-2 diabetes. Moreover, they showed that higher *Bacteroidetes* to *Firmicutes* ratio correlates positively with higher blood glucose levels and lower body mass. As type 2 diabetes is usually associated with increased body weight, these findings led to the suggestion that overweight and diabetes are associated with different groups of intestinal microbiota and that levels of glucose tolerance should be considered when linking microbiota with obesity and other metabolic diseases. Furthermore, Membrez et al. (2008) demonstrated that modulation of gut microbiota with antibiotics influences whole body glucose homeostasis, independent of body weight/body fat mass. In antibiotic-treated mice, reduced liver triglycerides correlated with improved insulin resistance, suggesting that the influence of gut microbiota on glucose and liver metabolism may have similar mechanisms. The mechanism suggested for these changes was an increase in lipopolysaccharides (LPS), the main compound of the outer membrane of Gram-

negative bacteria, such as bacteria from the *Bacteroidetes* phylum. LPS have been shown to cause acute whole body insulin resistance (Virkamaki et al., 1994) and is a potent stimulator of inflammation. In the gut, LPS activate Toll-like receptor 4 (TLR4), leading to the expression of TNF-α (Membrez 2008). Increased TNF-α has been shown to exert potent antiadipogenic effects (Petruschke et al., 1993) and induce hepatic steatosis (Yang et al, 1997). In a study by Le Roy et al. (2012), it was further confirmed that insulin resistance develops separately from obesity. However, they did not observe differences in TNF-α levels and suggested that the gut microbiota may affect hepatic metabolism through other mechanisms, independent of the immune system (Fig. 3.5). As an example of a different mechanism, in a lipidomic study, Velagapudi et al. (2010) suggested that the increase in phosphatidylcholine (16:0/18:1) induced by the microbiota may activate LPL, resulting in reduced serum triglyceride levels together with increased storage of lipids in adipose tissue and the liver. Although we did not examine single lipid classes in this study, we observed an increase in C16:0 and C18:1 fatty acids in the liver of mice fed *t*10, *c*12 CLA and therefore, there is a possibility that these animals could have more 16:0/18:1 phospholipds.

We also observed other important changes in gut microbiota composition as a result of dietary supplementation with *t*10, *c*12 CLA including increased proportions of *Porphyromonadaceae* and decreased abundance of *Lachnospiraceae* and *Desulfovibrionaceae*. *Porphyromonadaceae* have previously been associated with non-alcoholic fatty liver disease (NAFLD), atherosclerosis and diabetes (Henao-Mejia et al., 2012). Members of the family *Lachnospiraceae* have been shown to protect mice against colonisation by *Clostridium difficile* (Reeves et al., 2012),

whereas enhanced levels of bacteria from the *Desulfovibrionaceae* family was associated to impaired glucose tolerance and serious metabolic syndrome phenotype (Zhang et al., 2010). Moreover, in a study by Bajaj, Ridlon, et al. (2012), *Lachnospiraceae* abundance was reported to be lower in patients with cirrhosis, while *Porphyromonadaceae* abundance was positively correlated with cognitive impairment and inflammation in patients with hepatic encephalopathy.

SCFA are the end-products of bacterial fermentation, acetate, propionate and butyrate being the major SCFA in the mammalian gut. The type and amount of SCFA produced depends on diet, intestinal transit and microbiota composition (Macfarlane and MacFarlane, 2012). In the current study, SCFA levels were altered by *t*10, *c*12 CLA supplementation even though both groups received similar amount of carbohydrates and proteins, with higher levels of acetate, propionate and isobutyrate detected in the caecal content of mice receiving *t*10, *c*12 CLA supplementation. Thus, the higher amount of SCFA detected in the *t*10, *c*12 CLA fed group was probably due to the marked changes in microbiota composition induced by dietary *t*10, *c*12 CLA. SCFA are considered important energy sources for the host and some studies have suggested a link between increased levels of SCFA and obesity (Turnbaugh et al, 2006; Turnbaugh et al, 2008). However, in our study the higher levels of SCFA were detected in mice with lower fat mass. The precise mechanism involved in SCFA modulation of host metabolism is not clear and the results in literature are conflicting. Thus, for this study we propose three explanations to the role of SCFA in *t*10, *c*12 CLA-induced lipoatrophy/liver steatosis: 1) increased propionate induces gluconeogenesis (Wolever et al., 1991); 2) SCFA activate GPR41 receptor, stimulating hepatic lipogenesis (Samuel et al., 2008); and

3) SCFA activate GPR43 receptor that regulates energy uptake by adipose tissue and promotes the utilization of excess energy in other tissues instead of storage in the adipocytes (Kimura et al., 2013) (Fig. 3.5).

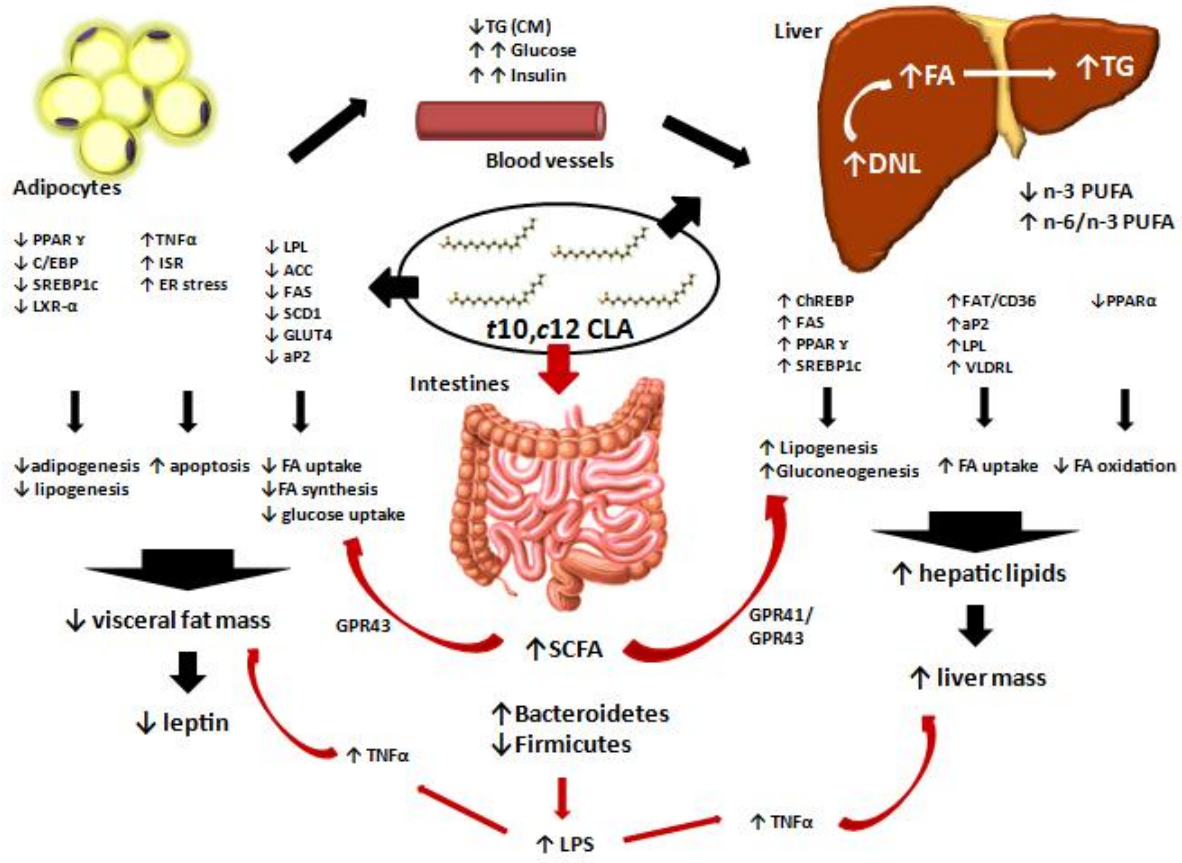


Figure 3.5 Schematic summary of all the mechanisms of action of *t*10, *c*12 CLA, as proposed by the different studies cited in this paper. There are a variety of proposed mechanisms through which *t*10, *c*12 CLA supplementation may cause hepatic steatosis and lipoatrophy in mice. *T*10, *c*12 CLA up- and down-regulate genes involved in fatty acid synthesis, uptake and oxidation in adipose tissue and liver in a direct and indirect manner (black arrows). In this study we propose that the gut microbiota and its products are extra environmental factors affecting host lipid metabolism (red arrows).

3.6 CONCLUSIONS

Several studies investigating the anti-obesity effect of CLA supplements have studied gene regulation in the liver and adipose tissue but, to our knowledge, this is the first study to show the impact of dietary *t*10, *c*12 CLA on gut microbiota composition and SCFA production. Long dietary exposure to *t*10, *c*12 CLA transformed the gut microbiota, favouring the growth of harmful bacteria, thus increasing host susceptibility to a variety of diseases. The greater proportions of *Bacteroidetes* and *Porphyromonadaceae* bacteria found in the *t*10, *c*12 CLA supplemented mice most likely had an influence on lipid metabolism and induction of hepatic steatosis, with higher levels of SCFA contributing to enhanced lipogenesis and gluconeogenesis in the liver.

Diet plays an important role in modulating the gut microbiota composition, and, as seen in this study, a single dietary fatty acid is capable of inducing a systemic effect on the host. Therefore, dietary approaches targeting beneficial bacteria and suppressing harmful species may be a new strategy to prevent or treat hepatic steatosis and associated metabolic disorders. The use of fatty acid mixtures with equal proportions of CLA isomers or alternatively, the use of probiotics and prebiotics to balance the potentially negative effects of *t*10, *c*12 CLA on microbiota composition may be safer alternatives for individuals looking for anti-obesity dietary solutions. Future studies comparing the effects of dietary *t*10, *c*12 CLA with dietary *c*9, *t*11 CLA and other trans-fatty acids on human intestinal microbiota composition would provide important information about the mechanisms of action of these fatty acids on metabolic pathways. Moreover, future studies using germ-

free animals would help further our understanding of the impact of microbiota on lipid metabolism.

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Chapter 4

Contrasting effects of *Bifidobacterium breve* NCIMB 702258 and *Bifidobacterium breve* DPC 6330 on the composition of murine brain fatty acids and gut microbiota

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Tatiana M. Marques is second author of this paper. She contributed for animal feeding, culling, and dissection, along with fatty acid analyses by gas chromatography.

4.1 ABSTRACT

Background: We previously showed that microbial metabolism in the gut influences the composition of bioactive fatty acids in host adipose tissue.

Objective: This study compared the effect of dietary supplementation for 8 wk with human-derived *Bifidobacterium breve* strains on fat distribution and composition and the composition of the gut microbiota in mice.

Methods: C57BL/6 mice (n=8 per group) received *B. breve* DPC 6330 or *B. breve* NCIMB 702258 (10^9 microorganisms) daily for 8wk or no supplement (controls). Tissue fatty acid composition was assessed by gas-liquid chromatography while 16S rRNA pyrosequencing was used to investigate microbiota composition.

Results: Visceral fat mass and brain stearic acid, arachidonic acid (AA), and docosahexaenoic acid (DHA) were higher in mice supplemented with *B. breve* NCIMB 702258 than in mice in the other 2 groups ($p<0.05$). In addition, both *B. breve* DPC 6330 and *B. breve* NCIMB 702258 supplementation resulted in higher propionate concentrations in the caecum than did no supplementation ($p<0.05$). Compositional sequencing of the gut microbiota showed a tendency for greater proportions of *Clostridiaceae* (25%, 12%, and 18%; p=0.08) and lower proportions of *Eubacteriaceae* (3%, 12%, and 13%; p=0.06) in mice supplemented with *B. breve* DPC 6330 than in mice supplemented with *B. breve* NCIMB 702258 and unsupplemented controls, respectively.

Conclusion: The response of fatty acid metabolism to administration of bifidobacteria is strain-dependent, and strain-strain differences are important factors that influence modulation of the gut microbial community by ingested microorganisms.

4.2 INTRODUCTION

The human gut microbiota comprises trillions of microorganisms, reaching cell numbers that outnumber that of host cells, and contains ≥100-fold more genes than the human genome (Qin et al., 2010). The collective genome of these microorganisms (the metagenome) contributes to a broad range of metabolic and biochemical functions that the host could not otherwise perform (Ley et al., 2006). Recent research has shown an interaction between the gut microbiota and host metabolism, energy utilization, and fat storage, which suggests that intestinal microbes play a direct role in the development of obesity (Backhed et al., 2004; Ley et al., 2005; Ley et al., 2006; Turnbaugh et al., 2006). Metagenomic analyses have also shown that the caecal microbiota of ob/ob mice and obese individuals are more efficient at energy extraction from the diet and at producing short-chain fatty acids (SCFA) than are those of normal phenotype (Schwiertz et al., 2010; Turnbaugh et al., 2006). These SCFA can then be used for *de novo* synthesis of lipids and glucose (Wolever at al., 1989), thus providing an additional source of energy for the host. Whereas some studies of bacterial communities in the gut microbiota in both mouse models and in humans, using compositional sequencing, have shown an increase in the ratio of *Firmicutes* to *Bacteroidetes* in obese subjects (Ley et al., 2006; Turnbaugh et al., 2009), the identity of the microbial populations that are associated with obesity continues to be the subject of much debate, with others reporting a decreased *Firmicutes* to *Bacteroidetes* ratio in overweight and obese individuals (Murphy et al., 2010; Schwiertz et al., 2010). Another study reported no link between the proportion of *Firmicutes* and *Bacteroidetes* and human obesity (Duncan et al., 2008). Whether alterations in the microbiota are a cause or

consequence of obesity is furthermore controversial, and the role of the gut microbiota in fat metabolism and obesity is more complex than first considered (Duncan et al., 2008; Hildebrandt et al., 2009; Murphy et al., 2010). Thus, the interrelation between the gut microbial composition, diet, and host adiposity has to be further investigated. Knowledge of the interactions between energy intake and specific microbial populations, and their influence on body weight, is limited to small-scale clinical trials (Ley et al., 2006). Among the gastrointestinal bacteria, *Bifidobacterium* is an important commensal group, accounting for an estimated 3% of the intestinal microbiota from an average adult (Turroni, Foroni, et al., 2009; Turroni, Marchesi, et al., 2009). Because of their well-documented beneficial health effects (Leahy et al., 2005), bifidobacteria have attracted significant interest for probiotic applications in pharmaceutical and dairy products. In relation to host energy metabolism, higher numbers of bifidobacteria have been documented in normal-weight adults and adolescents than in their overweight counterparts (Collado et al., 2008; Kalliomaki et al., 2008). However, some recent studies suggest that the role of bifidobacteria in weight management may be species-specific. Thus, Santacruz et al. (2009) reported that weight loss was associated with a reduction in the numbers of *Bifidobacterium breve* and *Bifidobacterium bifidum* and an increase in the numbers of *Bifidobacterium catenulatum*.

A promising mechanism by which the manipulation of the gut microbiota can affect host metabolism and fat storage is the modulation of fatty acid composition of host cellular membranes. We previously showed that dietary supplementation with a conjugated linoleic acid (CLA)–producing *Bifidobacterium* strain of human origin (*B. breve* NCIMB 702258) influenced the composition of

bioactive fatty acids in host liver and adipose tissue in different animal species (Wall et al., 2009; Wall at al., 2010). Thus, in this study, we compared the effect of administering 2 different CLA-producing strains of the same species (i.e., *B. breve* DPC 6330 and *B. breve* NCIMB 702258) on host fat distribution and composition. Furthermore, we performed a high-throughput pyrosequence based assessment of the effect of oral administration of these *B. breve* strains on the diversity of the resident gut microbiota.

4.3 MATERIALS AND METHODS

Animals and treatment

Wild-type C57BL/6 male mice aged 7–8 wk were obtained from Charles River and housed under barrier-maintained conditions within the Biological Services Unit, University College Cork. All animal experiments were approved by the University College Cork Animal Ethics Committee, and experimental procedures were conducted under the appropriate license from the Irish Government. Mice were allowed to acclimatize for 1 wk before the start of the study and were fed *ad libitum* with Teklad Global rodent standard diet (#2018S; Harlan Laboratories) and allowed free access to water at all times. Mice were housed in groups of 4 per cage and kept in a controlled environment at 25^{o}C under a 12-h-light/12-h-dark cycle. After 1 wk of acclimatization, the mice were divided into 3 groups (n=8 per group): a control group fed with standard diet and placebo freeze-dried powder (15% wt:vol trehalose in dH2O), a group fed with standard diet and *B. breve* DPC 6330 (approximate daily dose of 10^9 microorganisms), and a group fed a standard diet and *B. breve* NCIMB 702258 (approximate daily dose of 10^9 microorganisms). The

diet contained the following nutrient composition: crude protein (18.6%), carbohydrate (44.2%), fat (6.2%), crude fiber (3.5%), neutral detergent fiber (14.7%), and ash (5.3%). The fatty acids present in the diet included palmitic acid (16:0, 0.7%), stearic acid (18:0, 0.2%), oleic acid (18:1n29, 1.2%), linoleic acid (18:2n26, 3.1%), and linolenic acid (18:3n23, 0.3%). Body weight and food intake were assessed weekly. After 8 wk on the experimental diets, the animals were killed by decapitation. Liver, brain, fat pads (epididymal, perirenal, and mesenteric), gastrointestinal tract from stomach to anus, and caecal contents were removed, blotted dry on filter paper, weighed, and flash-frozen immediately in liquid nitrogen. All samples were stored at -80^{o}C until processed. Blood samples were collected from starved animals and allowed to clot for 2 h at 4^{o}C before centrifugation for 20 min at 2000 x g.

Preparation and administration of *B. breve* NCIMB 702258 and *B. breve* DPC 6330

We previously showed that *B. breve* NCIMB 702258 and *B. breve* DPC 6330 are efficient CLA producers, converting up to 65% and 76%, respectively, of linoleic acid to *c*9, *t*11 CLA when grown in 0.5 mg linoleic acid/mL *in vitro* (Barrett et al., 2007; Coakley et al., 2003). Rifampicin resistant variants of *B. breve* NCIMB 702258 and *B. breve* DPC 6330 were isolated by spread-plating ~10^{9} colony forming units (CFU) from an overnight culture onto mMRS agar (Difco Laboratories) supplemented with 0.05% (wt:vol) L-cysteine hydrochloride (98% pure; Sigma Chemical Co) containing 500 ug rifampicin/mL (Sigma). After anaerobic incubation (anaerobic jars with Anaerocult A gas packs; Merck) at 37^{o}C for 3 d, colonies were stocked in de Man, Rogosa, and Sharpe (MRS) broth containing 40% (vol:vol)

glycerol and stored at -80^{o}C. To confirm that the rifampicin-resistant variant was identical to the parent strain, pulse-field gel electrophoresis was used for molecular fingerprinting. Before freeze-drying, *B. breve* NCIMB 702258 and *B. breve* DPC 6330 were grown in MRS by incubating overnight at 37^{o}C under anaerobic conditions. The culture was washed twice in phosphate-buffered saline and resuspended at a concentration of ~2 x 10^{10} cells/mL in 15% (wt:vol) trehalose (Sigma) in dH2O. One-milliliter aliquots were freeze-dried by using a 24-h program (freeze temperature, -40^{o}C; condenser set point, -60^{o}C; vacuum set point, 600 mTorr). Each mouse that received the bacterial strains consumed ~1 x 10^{9} live microorganisms/d. This was achieved by resuspending appropriate quantities of freeze-dried powder in water, which mice consumed *ad libitum*. Mice that did not receive the bacterial strains received placebo freeze-dried powder [15% (wt:vol) trehalose in dH2O]. Water containing either the bacterial strains or placebo freeze-dried powder was the only water supply provided to the animals throughout the trial. Freeze-dried powders with the bacterial strains underwent continuous quality control of cell counts for the duration of the trial by plating serial dilutions on MRS agar supplemented with 100 ug mupirocin/mL (Oxoid) and 100 ug rifampicin/mL (Sigma) and incubating plates anaerobically for 72 h at 37^{o}C.

Culture-dependent microbial analysis

Fresh faecal samples were taken from C57BL/6 mice every week for microbial analysis. Microbial analysis of the faecal samples involved enumeration of the *B. breve* strains by plating serial dilutions on MRS agar supplemented with 100 ug mupirocin (Oxoid)/mL, 100 ug rifampicin/mL (Sigma), and 50 U nystatin/mL

(Sigma). Agar plates were incubated anaerobically for 72 h at 37°C. In addition, proximal colonic contents were sampled at the time the mice were killed for enumeration of the administered *B. breve* strains.

Lipid extraction and fatty acid analysis

Lipids were extracted with chloroform:methanol (2:1, vol:vol; Fisher Scientific) according to the method of Folch et al. (1957). Fatty acid methyl esters (FAMEs) were prepared by using first 10 mL 0.5 N NaOH (Sigma) in methanol for 10 min at 90°C followed by 10 mL 14% BF3 in methanol (Sigma) for 10 min at 90°C (Park & Gois, 1994). FAMEs were recovered with hexane (Fisher Scientific). Before gas-liquid chromatographic analysis, samples were dried over 0.5 g anhydrous sodium sulfate (Sigma) for 1 h and stored at -20°C. FAMEs were separated by gas-liquid chromatography (Varian 3800; Varian) fitted with a flame ionization detector by using a Chrompack CP Sil 88 column (Chrompack; 100 m x 0.25 mm internal diameter, 0.20 um film thickness) and helium as carrier gas. The column oven was programmed to be held initially at 80°C for 8 min and then increased by 8.5°C/min to a final column temperature of 200°C. The injection volume used was 0.6 uL, with automatic sample injection on a SPI 1093 splitless on-column temperature-programmable injector. Peaks were integrated by using the Varian Star Chromatography Workstation version 6.0 software, and peaks were identified by comparison of retention times with pure FAME standards (Nu-Chek Prep). The percentage of individual fatty acids was calculated according to the peak areas relative to the total area (total fatty acids were set at 100%). All fatty acid results are shown as means ± SEMs in g/100 g FAMEs.

SCFA analysis

Approximately 100 mg caecal content was vortex-mixed with 1.0 mL Milli-Q water and, after standing for 10 min at room temperature, centrifuged at 10000 x g for 5 min to pellet bacteria and other solids. The supernatant fluid was collected, 3.0 mM 2-ethylbutyric acid (Sigma) was added as internal standard, and samples were filtered before being transferred to clean vials. Standard solutions containing 10.0 mmol/L, 8.0 mmol/L, 6.0 mmol/L, 4.0 mmol/L, 2.0 mmol/L, 1.0 mmol/L, and 0.5 mmol/L of acetic acid, propionic acid, isobutyric acid, and butyric acid (Sigma), were used for calibration. The concentration of SCFA was measured by using a Varian 3500 GC flame-ionization system, fitted with a Nukol-FFAP column (30 m x 0.32 mm x 0.25 um; Sigma). Helium was used as the carrier gas at a flow rate of 1.3 mL/min. The initial oven temperature was 100^{o}C for 0.5 min, raised to 180^{o}C at 8^{o}C/min and held for 1 min, then increased to 200^{o}C at 20^{o}C/min, and finally held at 200^{o}C for 5 min. The temperature of the detector and the injector were set at 250^{o}C and 240^{o}C, respectively. Peaks were integrated by using the Varian Star Chromatography Workstation version 6.0 software. Standards were included in each run to maintain the calibration.

Measurement of triglycerides in liver

Liver lipids were extracted and purified according to the method of Folch et al. (1957). After being mixed thoroughly, the samples were dried under nitrogen and resolubilized in 5% (vol:vol) Triton X-100 in dH2O. The concentration of triglycerides was measured by using a commercial kit (EnzyChrom Triglyceride

Assay kit; BioAssay Systems). The results were normalized to the weight of the samples.

Measurements of serum variables

Serum variables were measured with commercial kits. Serum glucose was measured by using a QuantiChrom glucose assay kit (BioAssay Systems), serum insulin was measured by using the Ultra Sensitive Mouse ELISA kit (Crystal Chem Inc), serum leptin was measured by using the Mouse Leptin ELISA kit (Crystal Chem Inc), and serum triglycerides were measured by using EnzyChrom Triglyceride Assay kit (BioAssay Systems).

Culture-independent microbial analysis

For analysis of the microbial community composition of the caecal contents, total DNA was extracted from the caecal contents of all mice by using the QIAamp DNA stool mini kit according to the manufacturer's instructions (Qiagen) coupled with an initial bead-beating step. Universal 16s rRNA primers, designed to amplify from highly conserved regions corresponding to those flanking the V4 region, i.e., the forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of 4 reverse primers— R1 (5' TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp)—were used for Taq-based polymerase chain reaction amplification. Sequencing was performed on a Roche 454 GS-FLX by using Titanium chemistry with the Teagasc 454 Sequencing Platform. Resulting raw sequences reads were quality trimmed as previously

described (Claesson et al., 2009). Trimmed FASTA sequences were then BLASTed (Altschul et al., 1997) against a previously published 16S-specific database (Urich et al., 2008) by using default parameters. The resulting BLAST output was parsed by using MEGAN (Huson et al., 2007). MEGAN assigns reads to NCBI taxonomies by using the Lowest Common Ancestor algorithm. Bit scores were used from within MEGAN for filtering the results before tree construction and summarization. A bit score of 86 was selected as previously used for 16S ribosomal sequence data (Urich et al., 2008). Phylum and family counts for each subject were extracted from MEGAN. Clustering and alpha diversities were generated with the MOTHUR software package (Schloss & Handelsman, 2008). Beta diversities and principal coordinate analysis (PCoA) of sequence reads were calculated by using the Qiime suite of tools (Caporaso et al., 2010).

Statistical analysis

Results in the text, tables, and figures are presented as means ± SEMs (per group). To assess whether differences between treatment groups were significant, data were analyzed by using one-factor ANOVA followed by post hoc Tukey's multiple comparisons test with the use of GraphPad Prism version 4.0 for Windows (GraphPad Software). Compositional data were statistically analyzed by using Minitab release 15.1.1.0 (www.minitab.com). The nonparametric Kruskal-Wallis test was used to estimate the relations between different groups. Statistical significances were accepted at $p<0.05$, and trends for statistically significant differences were recognized at $p<0.10$.

4.4 RESULTS

Survival and transit of *B. breve* DPC 6330 and *B. breve* NCIMB 702258 in C57BL/6 mice

Quantification of the numbers of the administered *B. breve* strains in the faeces of mice confirmed their gastrointestinal transit and survival. Stool recovery of *B. breve* DPC 6330 and *B. breve* NCIMB 702258 were ~1.1 x 10^6 CFU/g faeces and 8.2 x 10^6 CFU/g faeces after 2 wk of feeding the respective strain and remained at similar numbers for weeks 4 and 6. At week 8, there was a decline in the numbers of excreted *B. breve* strains, with stool recovery of *B. breve* DPC 6330 and *B. breve* NCIMB 702258 being 1.2 x 10^5 CFU/g faeces and 3.4 x 10^5 CFU/g faeces in the respective groups.

At sacrifice, *B. breve* DPC 6330 and *B. breve* NCIMB 702258 were detected in the large intestine at 7 x 10^5 CFU/g content and 1 x 10^6 CFU/g content, respectively, in the mice administered the strains.

Dietary supplementation with *B. breve* NCIMB 702258, but not *B. breve* DPC 6330, increases visceral host fat storage

The weight of visceral body fat—the sum of epididymal, mesenteric, and perirenal fat pads—was significantly higher in mice fed *B. breve* NCIMB 702258 than in unsupplemented mice (1.71 ± 0.13 and 1.20 ± 0.10 g; $p<0.05$; Table 4.1). This increase in visceral fat mass correlated with a tendency for greater circulating leptin in mice supplemented with *B. breve* NCIMB 702258 than in unsupplemented controls (5.2 ± 0.9 and 2.4 ± 0.7 ng/mL; $p=0.06$; Table 4.2). No significant difference in body mass was found between the groups over the 8-wk feeding period (Table

4.1). No effect on liver mass or liver triglycerides was observed after administration of *B. breve* NCIMB 702258. In contrast, administration of *B. breve* DPC 6330 was associated with a higher concentration of liver triglycerides than was no supplementation ($p<0.05$; Table 4.1). Neither of the administered *B. breve* strains affected the concentrations of circulating insulin, glucose, or triglycerides relative to no supplementation (Table 4.2). In addition, no difference in food intake was observed between the groups (data not shown).

Table 4.1 Body mass, fat mass, liver mass and liver triglyceride levels of mice fed *B. breve* DPC 6330, *B. breve* NCIMB 702258 and unsupplemented diet for 8 weeks

	***B. breve* DPC 6330 fed mice**	***B. breve* NCIMB 702258 fed mice**	**Unsupplemented mice**
Initial weight (g)	26.5 ± 0.6	26.7 ± 0.6	25.4 ± 0.5
Final weight (g)	31.7 ± 0.8	33.4 ± 0.7	30.6 ± 0.8
Weight gain (%)	20.3 ± 4.5	25.1 ± 3.7	20.5 ± 3.1
Visceral fat (g)	$1.51 \pm 0.16^{A,B}$	1.71 ± 0.13^{A}	1.20 ± 0.10^{B}
Liver mass (g)	1.64 ± 0.05	1.55 ± 0.06	1.47 ± 0.05
Liver triglycerides (mg/g)	4.64 ± 0.73^{A}	$3.44 \pm 0.29^{A,B}$	2.93 ± 0.14^{B}

Visceral fat mass include epididymal, perirenal, and mesenteric fat pads. Results are expressed as Means ± SEM, n=8 mice/group. Means not sharing a common superscript letter are significantly different at $p<0.05$ based on ANOVA followed by post hoc Tukey's multiple comparison tests.

Table 4.2 Serum parameters in mice fed *B. breve* DPC 6330, *B. breve* NCIMB 702258 and unsupplemented diet for 8 weeks

Serum parameter	*B. breve* DPC 6330 fed mice	*B. breve* NCIMB 702258 fed mice	Unsupplemented mice
Triglycerides (mg/dL)	78.1 ± 12.8	56.2 ± 11.8	61.3 ± 8.2
Glucose (mg/dL)	217.6 ± 17.6	186.0 ± 6.4	183.3 ± 2.8
Insulin (ng/mL)	1.4 ± 0.2	2.5 ± 0.8	1.7 ± 0.3
Leptin (ng/mL)	3.9 ± 0.8	5.2 ± 0.9[1]	2.4 ± 0.7[1]

Blood samples were collected after fasting. Results are expressed as Means ± SEM, n=8 mice/group. No significant differences were observed. [1]p=0.06, based on ANOVA followed by post hoc Tukey's multiple comparison tests.

Higher gut concentrations of SCFA after administration of *B. breve* DPC 6330 and *B. breve* NCIMB 702258

Analysis of caecal SCFA, the major fermentation end products and source of energy for the host, showed that propionate was significantly higher in mice fed *B. breve* DPC 6330 and *B. breve* NCIMB 702258 than in nonsupplemented mice ($p<0.05$; Table 4.3). Higher concentrations of isobutyrate were also observed in mice supplemented with *B. breve* NCIMB 702258 than in unsupplemented controls ($p<0.05$; Table 4.3). In addition, the mean total SCFA concentration (acetate, propionate, butyrate, and isobutyrate) detected in the caecal contents was 24% higher in mice fed *B. breve* DPC 6330 than in unsupplemented controls ($p=0.07$; Table 4.3).

Table 4.3 SCFA concentrations (μmol/g) of caecum content

SCFA	*B. breve* DPC 6330 fed mice	*B. breve* NCIMB 702258 fed mice	Unsupplemented mice
Acetate	48.05 ± 3.58	45.05 ± 2.73	40.71 ± 2.43
Propionate	9.80 ± 0.51^{A}	9.28 ± 0.49^{A}	7.14 ± 0.40^{B}
Butyrate	18.24 ± 2.67	13.44 ± 1.45	13.71 ± 1.59
Isobutyrate	$0.80 \pm 0.09^{A,B}$	0.88 ± 0.05^{A}	0.67 ± 0.02^{B}
Total acids	76.88 ± 6.39	68.65 ± 4.03	62.23 ± 4.14

Results are expressed as Means ± SEM; *B. breve* DPC 6330 (n=8), *B. breve* NCIMB 702258 (n=7), unsupplemented mice (n=8). Means not sharing a common superscript letter are significantly different at $p<0.05$ based on ANOVA followed by post hoc Tukey's multiple comparison tests.

Contrasting effects on host tissue fatty acid composition of dietary supplementation with *B. breve* DPC 6330 and *B. breve* NCIMB 702258

To investigate the effects of dietary supplementation with *B. breve* DPC 6330 and *B. breve* NCIMB 702258 on the fatty acid composition of host tissues, fatty acid profiling was performed on brain, epididymal adipose tissue, and liver. Significant differences in tissue fatty acid composition were observed after supplementation with the *B. breve* strains (Tables 4.4–4.6). Mice fed *B. breve* NCIMB 702258 had significantly higher stearic acid (18:0), AA (20:4n26), and DHA (22:6n23) contents in the brain than did the unsupplemented mice ($p<0.05$) and the mice supplemented with *B. breve* DPC 6330 ($p<0.05$; Table 4.4), whereas dietary supplementation with *B. breve* DPC 6330 resulted in higher myristic acid (14:0) in the brain compared with both unsupplemented mice and mice supplemented with *B. breve* NCIMB 702258 ($p<0.05$; Table 4.4). However, both groups of *B. breve*–supplemented mice had significantly lower palmitic acid (16:0), palmitoleic acid (16:1c9), and dihomo-c-linolenic acid (20:3n26) in the brain than did the unsupplemented controls ($p<0.05$; Table 4.4).

Mice that received *B. breve* DPC 6330 had significantly higher myristic acid, palmitic acid, palmitoleic acid, and DHA in epididymal adipose tissue than did unsupplemented mice ($p<0.05$; Table 4.5). In contrast, these fatty acids were not higher in the epididymal adipose tissue of the mice supplemented with *B. breve* NCIMB 702258. Mice that received *B. breve* DPC 6330 also had significantly lower oleic acid (18:1c9) in epididymal adipose tissue than did both the unsupplemented mice and mice supplemented with *B. breve* NCIMB 702258 ($p<0.05$; Table 4.5). In contrast to a higher concentration of DHA in epididymal adipose tissue of mice fed

B. breve DPC 6330, these mice had a lower concentration of DHA in liver than did unsupplemented mice ($p<0.05$; Table 4.6). There was a tendency for higher concentrations ($p=0.06$) of *c*9, *t*11 CLA in the livers of mice receiving *B. breve* NCIMB 702258 (0.031 ± 0.006g/100g FAME) than in mice supplemented with *B. breve* DPC 6330 (0.015 ± 0.007g/100g FAME) and in unsupplemented controls (0.013 ± 0.006 g/100 g FAME).

Table 4.4 Fatty acid profile in brain of mice fed *B. breve* DPC 6330, *B. breve* NCIMB 702258 or unsupplemented diet for 8 wk[1]

Fatty acid	Mice fed *B. breve* DPC 6330	Mice fed *B. breve* NCIMB 702258	Unsupplemented mice
	g/100g FAME	*g/100g FAME*	*g/100g FAME*
Myristic acid, 14:0	3.66 ± 0.21^{A}	1.36 ± 0.05^{B}	1.90 ± 0.08^{C}
Palmitic acid, 16:0	22.34 ± 0.80^{A}	21.46 ± 0.18^{A}	26.75 ± 0.45^{B}
Palmitoleic acid, 16:1c9	0.69 ± 0.03^{A}	0.72 ± 0.03^{A}	0.90 ± 0.04^{B}
Stearic acid, 18:0	10.80 ± 0.18^{A}	11.99 ± 0.15^{B}	11.08 ± 0.16^{A}
Oleic acid, 18:1c9	9.83 ± 0.17^{A}	10.48 ± 0.07^{B}	10.22 ± 0.13^{A,B}
Linoleic acid, 18:2n-6	0.49 ± 0.02^{A}	0.58 ± 0.02^{B}	0.49 ± 0.01^{A}
Linolenic acid, 18:3n-3	ND	ND	ND
ɣ-Linolenic acid, 18:3n-6	ND	ND	ND
Dihomo-c-linolenic acid, 20:3n-6	0.33 ± 0.01^{A}	0.37 ± 0.01^{A}	0.27 ± 0.01^{B}
Arachidonic acid, 20:4n-6	5.70 ± 0.31^{A}	6.55 ± 0.07^{B}	5.27 ± 0.07^{A}
EPA, 20:5n-3	ND	ND	ND
Docosapentaenoic acid, 22:5n-3	0.24 ± 0.04	0.21 ± 0.04	0.20 ± 0.09
DHA, 22:6n-3	17.22 ± 0.35^{A}	18.58 ± 0.32^{B}	17.19 ± 0.10^{A}

[1] All values are means ± SEMs; n=8 mice per group. Values in the same row with different superscript letters are significantly different, $p<0.05$ (ANOVA followed by post hoc Tukey's multiple comparisons tests). FAME, fatty acid methyl ester; ND, not detected.

Table 4.5 Fatty acid profile in epididymal adipose tissue of mice fed *B. breve* DPC 6330, *B. breve* NCIMB 702258 or unsupplemented diet for 8 wk[1]

Fatty acid	Mice fed *B. breve* DPC 6330	Mice fed *B. breve* NCIMB 702258	Unsupplemented mice
	g/100g FAME	*g/100g FAME*	*g/100g FAME*
Myristic acid, 14:0	1.96 ± 0.24^{A}	1.44 ± 0.27^{A,B}	1.10 ± 0.15^{B}
Palmitic acid, 16:0	15.19 ± 0.94^{A}	12.78 ± 0.68^{A,B}	10.68 ± 0.52^{B}
Palmitoleic acid, 16:1c9	4.31 ± 0.39^{A}	3.75 ± 0.53^{A,B}	2.85 ± 0.12^{B}
Stearic acid, 18:0	2.04 ± 0.13	2.26 ± 0.12	2.31 ± 0.03
Oleic acid, 18:1c9	28.71 ± 0.92^{A}	32.22 ± 0.89^{B}	33.47 ± 0.48^{B}
Linoleic acid, 18:2n-6	33.57 ± 1.18	34.93 ± 1.13	36.66 ± 0.68
Linolenic acid, 18:3n-3	2.44 ± 0.16	2.42 ± 0.10	2.57 ± 0.10
ɤ-Linolenic acid, 18:3n-6	0.16 ± 0.01	0.15 ± 0.01	0.17 ± 0.005
Dihomo-c-linolenic acid, 20:3n-6	0.42 ± 0.02	0.42 ± 0.02	0.42 ± 0.01
Arachidonic acid, 20:4n-6	0.77 ± 0.04	0.71 ± 0.05	0.72 ± 0.03
EPA, 20:5n-3	0.093 ± 0.01	0.072 ± 0.01	0.085 ± 0.01
Docosapentaenoic acid, 22:5n-3	0.29 ± 0.02	0.26 ± 0.03	0.25 ± 0.02
DHA, 22:6n-3	0.77 ± 0.04^{A}	0.66 ± 0.05^{A,B}	0.62 ± 0.05^{B}

[1] All values are means ± SEMs; n=8 mice per group. Values in the same row with different superscript letters are significantly different, $p<0.05$ (ANOVA followed by post hoc Tukey's multiple comparisons tests). FAME, fatty acid methyl ester; ND, not detected.

Table 4.6 Fatty acid profile in liver of mice fed *B. breve* DPC 6330, *B. breve* NCIMB 702258 or unsupplemented diet for 8 wk[1]

Fatty acid	Mice fed *B. breve* DPC 6330	Mice fed *B. breve* NCIMB 702258	Unsupplemented mice
	g/100g FAME	*g/100g FAME*	*g/100g FAME*
Myristic acid, 14:0	0.65 ± 0.08	0.67 ± 0.03	0.67 ± 0.05
Palmitic acid, 16:0	31.87 ± 0.33	30.53 ± 0.66	30.37 ± 0.40
Palmitoleic acid, 16:1c9	2.81 ± 0.26	2.91 ± 0.24	2.60 ± 0.23
Stearic acid, 18:0	9.32 ± 0.48	9.03 ± 0.30	9.61 ± 0.45
Oleic acid, 18:1c9	13.48 ± 0.82	13.05 ± 0.92	10.85 ± 0.35
Linoleic acid, 18:2n-6	21.55 ± 0.56^{A}	19.40 ± 0.84^{A,B}	19.07 ± 0.31^{B}
Linolenic acid, 18:3n-3	0.83 ± 0.05	0.71 ± 0.05	0.71 ± 0.03
ɤ-Linolenic acid, 18:3n-6	0.30 ± 0.01	0.26 ± 0.02	0.28 ± 0.01
Dihomo-c-linolenic acid, 20:3n-6	0.91 ± 0.05	0.96 ± 0.06	0.94 ± 0.02
Arachidonic acid, 20:4n-6	7.07 ± 0.48	7.25 ± 0.24	7.81 ± 0.35
EPA, 20:5n-3	0.22 ± 0.01	0.22 ± 0.02	0.22 ± 0.01
Docosapentaenoic acid, 22:5n-3	0.38 ± 0.03	0.42 ± 0.03	0.41 ± 0.02
DHA, 22:6n-3	3.57 ± 0.27^{A}	3.82 ± 0.12^{A,B}	4.48 ± 0.16^{B}

[1] All values are means ± SEMs; n=8 mice per group. Values in the same row with different superscript letters are significantly different, $p<0.05$ (ANOVA followed by post hoc Tukey's multiple comparisons tests). FAME, fatty acid methyl ester; ND, not detected.

Contrasting effects on gut microbiota composition of dietary supplementation with *B. breve* NCIMB 702258 and *B. breve* DPC 6330

At the end of the 8-wk study, the microbial composition of the gut microbiota of individual mice was elucidated through high throughput pyrosequencing (Roche-454 Titanium) of 16S rRNA (V4) amplicons generated from DNA extracted from the caecal content. A total of 103,711 reads were sequenced, averaging at 4509 reads per animal. Species-richness, coverage, and diversity estimations were calculated for each data set (data not shown). At the 97% similarity level, the Shannon index—a metric for community diversity showed a high level of overall biodiversity within all samples with values exceeding 5.1. The Good's coverage at the 97% similarity level ranged between 88% and 95% for all the data sets. Chao1 richness also indicated a sufficient level of overall diversity (data not shown). Rarefaction curves for each data set were parallel or approaching parallel with the x axis, which indicated that the total bacterial diversity present within these was well represented and that additional sampling would yield a limited increase in species richness (Supplementary Figure 4.1).

In agreement with previous work (Ley et al., 2005; Ley et al., 2006; Turnbaugh et al., 2006), taxonomy-based analysis showed that, at the phylum level, the mouse gut microbiota was dominated by *Firmicutes* and *Bacteroidetes* (together harboring on average 94% of sequences; Figure 4.1). At the family level, the most dominant groups were *Bacteroidaceae, Clostridiaceae, Eubacteriaceae*, and *Lactobacillaceae* (Figure 4.2).

A comparison of the composition of the microbiota of mice supplemented with *B. breve* NCIMB 702258, mice supplemented with *B. breve* DPC 6330, and

unsupplemented mice showed that administration of these strains altered the composition of the gut microbiota differently at the phylum, family, and genus levels. There was a tendency for a reduction in the *Firmicutes* population from 74% in the unsupplemented mice to 68% in the mice supplemented with *B. breve* NCIMB 702258 and 67% in mice supplemented with *B. breve* DPC 6330 ($p=0.08$; Figure 4.1). All other phyla remained at relatively similar proportions across the groups. At the family level, administration of both *B. breve* DPC 6330 and *B. breve* NCIMB 702258 resulted in a significantly lower proportion of *Lachnospiraceae* than did no supplementation ($p<0.05$; Figure 4.2). Furthermore, *Eubacteriaceae* tended to be less abundant in the caecum of mice supplemented with *B. breve* DPC 6330 than in mice supplemented with *B. breve* NCIMB 702258 and unsupplemented controls (3%, 12%, and 13%, respectively; $p=0.06$; Figure 4.2). Other observed differences at the family level included a tendency for an apparent enrichment in the *Clostridiaceae* population in mice supplemented with *B. breve* DPC 6330 (25%) than in mice supplemented with *B. breve* NCIMB 702258 (12%) and unsupplemented mice (18%; $p=0.08$; Figure 4.2). At the genus level, *Eubacterium* tended to be lower after dietary supplementation with *B. breve* DPC 6330 than after no supplementation or supplementation with *B. breve* NCIMB 702258 ($p=0.06$; Figure 4.3).

PCoA generated using unweighted UNIFRAC distances showed that mice clustered into relatively distinct groups based on whether they received *B. breve* DPC 6330, *B. breve* NCIMB 702258, or no supplementation (Figure 4.4). Mice fed *B. breve* DPC 6330 clustered closely together and showed a clear separation from the unsupplemented mice and the mice supplemented with *B. breve* NCIMB 702258.

This suggests that supplementation with *B. breve* DPC 6330 had a greater effect on the gut microbiota than did supplementation with *B. breve* NCIMB 702258.

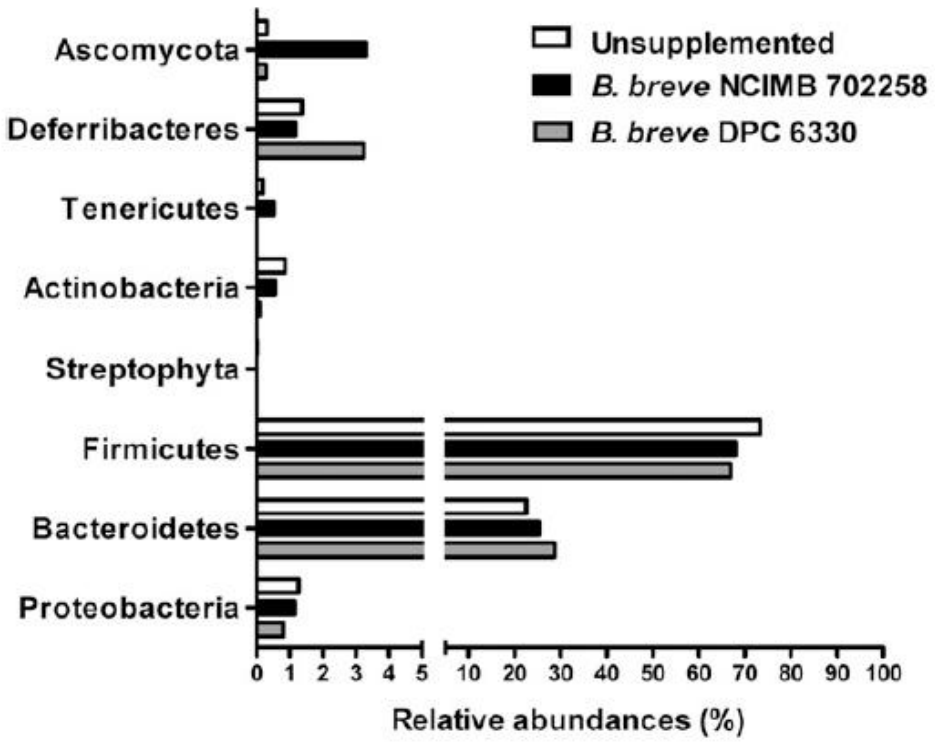


Figure 4.1 Phylum-level distributions of the microbial communities in caecal contents, expressed as a percentage of the total population of assignable tags, in mice supplemented with *B. breve* DPC 6330 (n=7) or *B. breve* NCIMB 702258 (n=7) and in unsupplemented mice (n=8). No significant differences were observed.

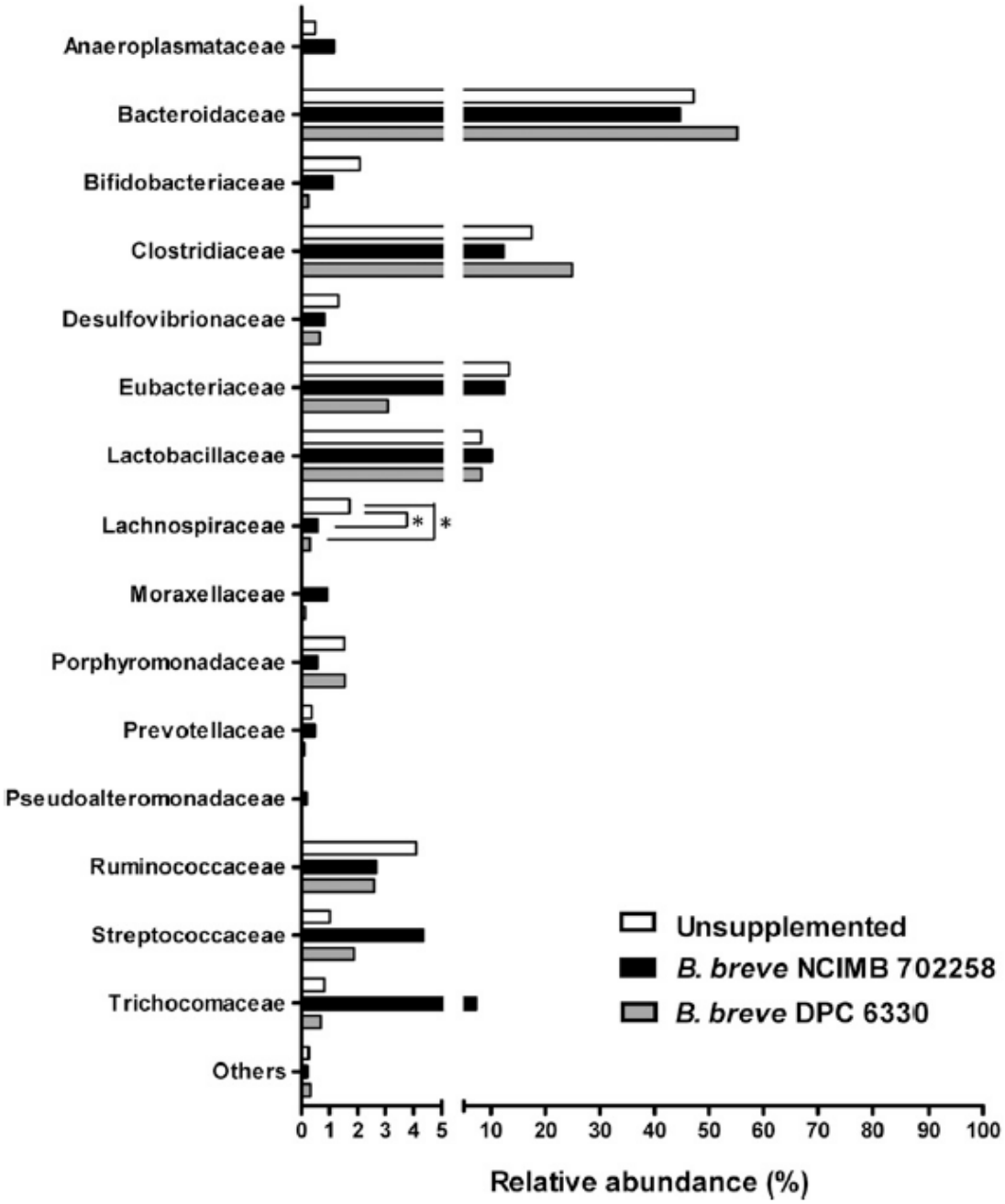


Figure 4.2 Family-level taxonomic distributions of the microbial communities in caecal contents, expressed as percentage of total tags assignable at family level, in mice supplemented with *B. breve* DPC 6330 (n=7) or *B. breve* NCIMB 702258 (n=7) and in unsupplemented mice (n=8). *p<0.05 (Kruskal-Wallis test).

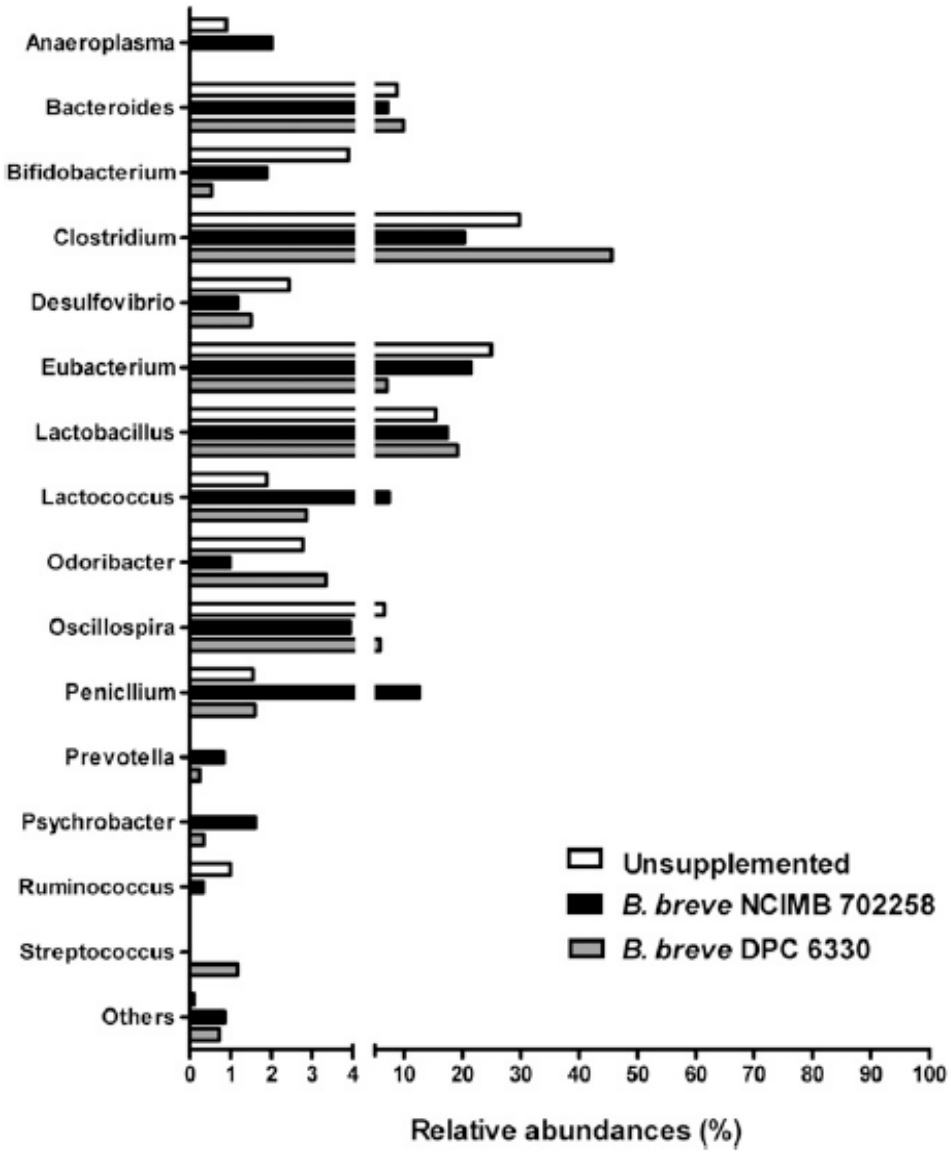


Figure 4.3 Genus-level taxonomic distributions of the microbial communities present in caecal contents, expressed as a percentage of total tags assignable at the genus level, in mice supplemented with *B. breve* DPC 6330 (n=7) or *B. breve* NCIMB 702258 (n=7) and in unsupplemented mice (n=8). No significant differences were observed.

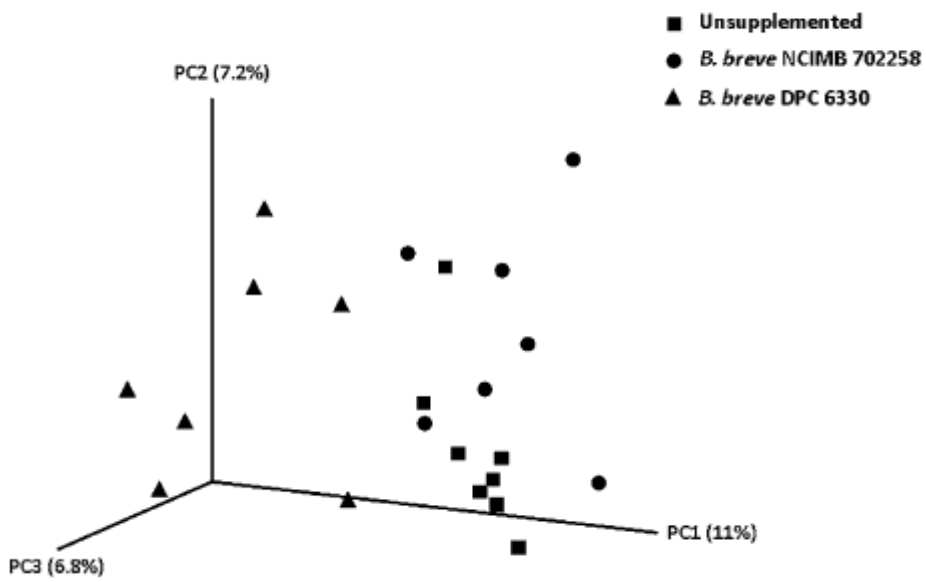


Figure 4.4 Principal coordinate analysis using unweighted UniFrac distances in mice supplemented with *Bifidobacterium breve* DPC 6330 (n=7) or *B. breve* NCIMB 702258 (n=7) and in unsupplemented mice (n=8).

4.5 DISCUSSION

This study showed that the response of fatty acid metabolism to administration of bifidobacteria is strain-dependent and furthermore highlights the importance of strain-strain differences in modifying the gut microbiota composition of the host. Administration of *B. breve* NCIMB 702258 increased visceral fat mass and weight gain in C57BL/6 mice, whereas administration of *B. breve* DPC 6330 did not. In general, beneficial effects have been attributed to *Bifidobacterium* in connection with obesity and weight management, and some reports support the preventative role of bifidobacteria in promoting fat mass development and body weight gain (Cani et al., 2007; Collado et al., 2008; Kalliomaki et al., 2008). However, other studies contradict the preventative role of *Bifidobacterium* in body weight gain (Furet et al., 2010; Santacruz et al., 2009) and question the role of specific *Bifidobacterium* species in obesity and weight management (Santacruz et al., 2009). Because the *Bifidobacterium* genus is complex, it is possible that certain bifidobacterial species, or even strains, as observed in the current study, promote fat mass storage and body weight more efficiently than others through diverse regulatory roles in fat absorption and distribution. Notably, strains of *B. breve* were previously shown to improve weight gain in very-low–birthweight infants (Kitajima et al., 1997). However, it must be acknowledged that this study was limited to 2 strains of *B. breve*; therefore, further studies are required to draw conclusions about the role of specific *Bifidobacterium* species and strains in fat/energy absorption and their effect on weight gain in animal models of obesity and in humans.

Previous studies have shown that the gut microbiota affect the composition and quality of fat in the host as well as the quantity of fat (Velagapudi et al., 2010). We showed that manipulation of the gut microbiota by administering a single metabolically active strain, *B. breve* NCIMB 702258, to different animal species influenced the composition of bioactive fatty acids in host liver and adipose tissue (Wall et al., 2009). In the current study, we compared the effect of *B. breve* NCIMB 702258 and *B. breve* DPC 6330 on fatty acid composition of different host tissues in mice. We observed that administration of these *B. breve* strains altered the fatty acid composition in distinct ways. Whereas administration of *B. breve* DPC 6330 had a greater influence on the fatty acid composition of epididymal adipose tissue, with higher palmitic acid, palmitoleic acid, and DHA, administration of *B. breve* NCIMB 702258 had a greater effect on the fatty acid composition of the brain. Intriguingly, mice supplemented with *B. breve* NCIMB 702258 had significantly higher concentrations of ARA and DHA in brain than did both mice supplemented with *B. breve* DPC 6330 and unsupplemented mice. This observation of increased ARA and DHA in the brain of mice administered *B. breve* NCIMB 702258 is consistent with our previous findings in a different model (Wall et al., 2009). ARA and DHA play important roles in neurogenesis, neurotransmission, and protection against oxidative stress (Innis, 2007; Maekawa et al., 2009), and their concentrations in the brain influence cognitive processes such as learning and memory (Henriksen et al., 2008; Yurko-Mauro et al., 2010). The importance of these fatty acids in neurodevelopment has led to their supplementation in infant formula (EFSA, 2009). The current study supports our previous observations (Wall et al., 2009; Wall et al., 2010), and findings by others (Kankaanpaa et al., 2002; Kaplas et al., 2007), in which

manipulation of the gut microbiota altered the composition of fat in the host. Interactions between fatty acids and members of the gut microbiota might affect the biological roles of both, and such interactions may therefore result in physiologic consequences for the host.

The mechanism by which these ingested strains mediate the changes in fatty acid composition observed in the current study is unclear and remains to be elucidated. Possible explanations include modulations of fat-absorption processes in the small intestine and/or desaturase activities involved in the metabolism of fatty acids to the longer-chain unsaturated derivatives caused either directly by the strains administered or by alterations in the gut microbiota. Interestingly, a previous study in lactating goats showed that administration of a *Lactobacillus plantarum* strain resulted in changes in the faecal microbiota and modulated the milk fatty acid composition with a higher content of PUFAs (Maragkoudakis et al., 2010). Furthermore, a recent study by Hoppu et al. (2011) reported that dietary supplementation of lactating women with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12, in combination with rapeseed oil, resulted in higher ɤ-linolenic acid concentrations in breast milk than did fatty acid supplementation alone.

The current study showed not only significant differences in the composition of the gut microbiota between mice fed or not fed *B. breve*, but also differences between mice fed different strains of *B. breve*. At the phylum level, the *Firmicutes* population tended to be more abundant in the caecum of mice supplemented with *B. breve* DPC 6330 and *B. breve* NCIMB 702258 than in unsupplemented mice. At the family level, the proportions of *Eubacteriaceae* were reduced in the animals

receiving *B. breve* DPC 6330, but not in those receiving *B. breve* NCIMB 702258, compared with unsupplemented controls. In addition, administration of *B. breve* DPC 6330 led to an apparent enrichment in *Clostridiaceae* compared with the unsupplemented mice, whereas this enrichment was absent in mice supplemented with *B. breve* NCIMB 702258. *Penicillium* was found in the mice supplemented with *B. breve* NCIMB 702258; however, this genus is not considered to be a typical member of the gut microbiota, and factors contributing to its presence in the gut, as found in this instance, require further investigation. PCoA analysis showed that mice fed *B. breve* DPC 6330 had a more divergent clustering pattern and were separated from the unsupplemented mice and from mice fed *B. breve* NCIMB 702258. This suggests that supplementation with *B. breve* DPC 6330 has a greater effect on the composition of the murine gut microbiota than does supplementation with *B. breve* NCIMB 702258. Our observations suggest that administration of a single strain can play a role in determining the composition of gut bacterial populations in vivo and furthermore that strain-strain differences are important factors with respect to modulation of the gut microbial community. Although changing the intestinal microbiota may be more difficult in free-living individuals than in laboratory models, it is important to perform extensive microbiota related studies on bifidobacteria-associated probiotics in human trials, especially in groups in which shifts in the composition have been observed due to the state of physiology (i.e., obesity and diabetes), particularly because an alteration of the gut microbiota at lower taxonomic levels is still likely to have important functional consequences for the host. Whereas it is acknowledged that pyrosequencing of the 16S rRNA genes, as undertaken in this study, did not provide quantitative

population data, it did yield an overview of the effects of administration of these strains on the entire microbial population.

SCFA (acetate, propionate, butyrate, and isobutyrate) are major products of the fermentation processes of the gut microbiota on nondigestible carbohydrates in the intestine. These fatty acids have recently attracted significant interest because of their positive effect on human gastrointestinal health and diseases, including colon cancer, gastrointestinal infections, and inflammatory bowel disease (D'Argenio et al., 1996; Emenaker et al., 2001; Galvez et al., 2005; Topping & Clifton, 2001). SCFA are also believed to be a driver of energy-sparing and are portrayed as a potential mechanism involved in the increase of fat mass storage in microbiota-bearing mice (Turnbaugh et al., 2006). The differences in stool SCFA concentrations between lean and obese people have been considerable. The mean total SCFA concentration in faecal samples of obese volunteers was shown to be >20% higher than in lean volunteers, with the highest increase observed for propionate, which was 41% higher in obese volunteers (Schwiertz et al, 2010). Administration of *B. breve* DPC 6330 and *B. breve* NCIMB 702258 led to an increase in propionate in the caecum that was 37% higher in mice supplemented with *B. breve* DPC 6330 and 30% higher in mice supplemented with *B. breve* NCIMB 702258 than in unsupplemented mice. Noteworthy, neither of these *B. breve* strains produce propionate *in vitro*, which suggests that administration of these strains may result in an increase in propionate-producing bacteria in the gastrointestinal tract. Well-known propionate producers belong to the genera *Bacteroides* spp., *Prevotella* spp., and *Propionibacterium* spp. (Hosseini et al., 2011; Schwiertz et al, 2010). However, because many metabolic properties are shared between lower

microbial taxa, it is difficult to link the capacity of producing specific SCFA, such as propionate, to the phylogenetic information obtained in the current study (Zoetendal et al., 2008). Increases in propionate in the *B. breve*–fed mice could also be due to crossfeeding, in which one bacterial species metabolizes the fermentation products of another, thus producing a different end product, a common cooperation in a complex microbial community. Indeed, *Bifidobacterium* are known to produce lactate, ethanol, and succinate (Macfarlane & Macfarlane, 2003; Van der Meulen et al., 2006), all of which could be used as substrates for the production of propionate by other bacteria, such as *Bacteroides* spp., *Propionibacterium* spp., and *Clostridium propionicum* in a sequential fermentation, thus increasing the propionate concentration in the gut (Hosseini et al., 2011; Satms at al., 1998).

In conclusion, our results indicate that the effect of bifidobacteria on host fatty acid metabolism is dependent on the strain administered and that strain-strain differences are important factors that influence the modulation of the gut microbial community by ingested microorganisms. Additional studies are needed to draw conclusions about the role of specific *Bifidobacterium* species and strains in obesity and weight management.

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Chapter 5

Effects of dietary administration of GABA and GABA-producing bacteria *Lactobacillus brevis* DPC 6108 on the development of diabetes in a streptozotocin rat model

5.1 ABSTRACT

Background: Gamma-aminobutyric acid (GABA) has been shown to attenuate or prevent type 1 diabetes (Soltani et al., 2011). GABA-producing bacteria have been isolated from several types of food sources (Cho et al., 2006; Komatsuzaki et al., 2005; Siragusa et al., 2007) and from the human gut (Barrett et al., 2012).

Objective: To investigate whether dietary administration of GABA-producing *Lactobacillus brevis* DPC 6108 and pure GABA (as commercial powder) exert protective and/or regenerative effects on islet β-cells and reverse diabetes in Sprague Dawley rats in which diabetes was induced by streptozotocin (STZ) injection.

Methods: Male Sprague Dawley rats received pure GABA or *Lb. brevis* DPC 6108 mixed in the drinking water daily. In a first experiment, healthy rats were divided in 3 groups (n=10/group) receiving placebo, 2.6 mg/Kg body weight (BW) pure GABA or *Lb. brevis* DPC 6108 (~10^9 microorganisms) and differences in body weight, serum glucose and insulin, corticosterone, liver cholesterol and brain amines were accessed. Animals were also tested for anxiety and depressant-like behaviours. In a second experiment, rats (n=15/group) were randomized to five groups and 4 of these received an injection of STZ to induce type 1 diabetes while the fifth (non-diabetic control) group received injection of citrate buffer vehicle only. Diabetic and non-diabetic control groups received placebo [4% (w/v) yeast extract in dH2O], while the other three diabetic groups received one of the following dietary supplements: 2.6 mg/Kg BW GABA (low GABA), 200 mg/Kg BW GABA (high GABA) or ~10^9 *Lb. brevis* DPC 6108 bacterial cells. Serum samples were analysed for glucose, triglycerides, cholesterol, insulin, glucagon, c-peptide, peptide YY (PYY),

active glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide (GIP) and leptin, circulating GABA, and stress hormones (corticosterone and melatonin). Liver samples were analysed for cholesterol and triglycerides. Open field, forced swim test and elevated plus maze behavioural tests were performed to evaluate anxiety and anti-depressants effects.

Results: *Lb. brevis* DPC 6108 supplementation was associated with increased serum insulin levels ($p<0.05$), but did not alter other metabolic markers in healthy animals. Moreover, healthy animals receiving GABA or *Lb. brevis* DPC 6108 were less anxious in comparison with placebo control group, whereas rats receiving the GABA treatment displayed a depressant-like behaviour during the forced swim test. GABA powder and *Lb. brevis* DPC 6108 supplementation did not change brain monoamines levels in healthy rats. Diabetes induced by STZ injection decreased body weight, increased intestinal length and stimulated water and food intake and these features were not altered by dietary GABA or *Lb brevis* DPC 6108 administration. Moreover, corticosterone levels were increased in diabetic rats ($p<0.05$) when compared to non-diabetic rats and levels were not normalized by GABA or *Lb. brevis* DPC 6108 supplementation. Insulin and c-peptide levels were significantly decreased ($p<0.05$), whereas glucose ($p<0.001$) was increased in all diabetic groups when compared with the non-diabetic control group. However, a significant decrease ($p<0.01$) in glucose levels was observed in diabetic rats receiving *Lb. brevis* DPC 6108 when compared with the diabetic-control group. STZ-induced diabetic rats were more anxious compared to non-diabetic rats, but dietary intervention with *Lb. brevis* DPC 6108 or GABA did not alter this.

Conclusions: *Lb. brevis* DPC 6108 attenuated high levels of glucose cause by diabetes, but additional studies are needed to understand the mechanisms involved in this reduction. Furthermore, as GABA protective effect may be primarily due to modulation of inflammatory response, animal models of auto-immune-induced diabetes may constitute better models for studying this type of treatment than single-high-dose STZ injections in rats.

5.2 INTRODUCTION

Type 1 diabetes (T1D) is a chronic and progressive disorder in which genetically susceptible individuals may develop an autoimmune response leading to pancreatic β-cell damage and insulin insufficiency (Atkinson and Einsenbarth, 2001). T1D is associated with complications such as diabetic nephropathy, retinopathy and neuropathy (Ahmadpour, 2012; Vujicic et al., 2012), and may exert deleterious effects on structure and functions of the brain (Huang et al., 2012; Northam et al., 2009).

Insulin administration is the main therapeutic strategy for T1D and, although there have been improvements in treatment, achieving euglycaemia without risk of severe hypoglycaemia is difficult and requires strict control of glucose levels (Atkinson & Eisenbarth, 2001). Evidence indicates that the most effective interventions involve the early treatment of individuals, when the β-cell mass is not damaged and the accuracy for disease prediction is low. However, this type of preventive therapy would have to be safe and benign as some individuals diagnosed at such early stage might never develop T1D (Atkinson & Eisenbarth, 2001). Another approach involves the identification of endogenous growth factors

that could stimulate the regeneration and expansion of residual β-cells after T1D onset (Bosi 2010). Among those, incretin-based agents have been shown to promote β-cell proliferation and inhibit apoptosis, delaying the onset of T1D in rodents (Drucker 2006; Hadjiyanni et al., 2008). However, although this type of treatment is safe and effective in patients with type 2 diabetes (T2D), it does not prevent the autoimmune process seen in T1D (Bosi 2010). On the other hand, gamma-aminobutyric acid (GABA) may constitute a very effective therapy for T1D. In a study conducted by Soltani et al. (2011), injections of GABA not only promoted β-cells proliferation, but also inhibited immune responses, reversing the disease in severely diabetic mice.

GABA is the major inhibitory neurotransmitter in the central nervous system (CNS) and plays a role in the sensation of pain and anxiety. In peripheral tissues, GABA acts not only as a neurotransmitter in the enteric and parasympathetic nervous system but also as a hormone in non-neuronal tissues (Erdo, 1992). In the endocrine pancreas, GABA is produced by β-cells and once released, activates $GABA_A$ and $GABA_B$ receptors in both α- and β-cells (Bonaventura et al., 2007; Dong et al., 2006). Through a paracrine signal, GABA released from β-cells inhibits glucagon release from α-cells (Bailey et al., 2007), whereas an autocrine signal increases or decreases insulin secretion from β-cells, depending on extracellular glucose levels (Dong et al., 2006).

GABA is produced through the conversion of L-glutamate by the enzyme glutamate decarboxylase (GAD) and is widely distributed in nature, from single cell microorganisms to plants and animals (Ueno, 2000). GABA has been found in different types of bacteria and its synthesis is believed to be a response to adverse

conditions such exposure to acidic environments (Jung & Kim, 2003; Sanders et al., 1998). Indeed, potential probiotic bacteria isolated from different food sources (Cho et al., 2006; Komatsuzaki et al., 2005; Siragusa et al., 2007) and from the human gastrointestinal tract (Barrett et al., 2012) can efficiently convert glutamate to GABA *in vitro*.

A disturbed microbiota has been shown to be a potential environmental factor contributing to metabolic diseases, affecting the immune system and even influencing the perception of pain and animal behaviour (Brown et al., 2011; Cryan and Dinan, 2012; Lam et al., 2011). Interestingly, the use of dietary strategies such as probiotics and prebiotics may be effective for the alleviation of these disorders by the inhibition of pathogens growth, attenuation of immune responses (Saulnier et al., 2009), modulation of brain function and behaviour (Bravo et al., 2011) and by the production of bioactive compounds that may have a direct or indirect action on the host's metabolism (Lyte, 2011). Thus, considering the role of GABA on glucose homeostasis and the evidence showing how probiotics may affect host's health, we investigated the impact of oral administration of pure GABA and GABA-producing bacteria *Lactobacillus brevis* DPC 6108 on the prevention or alleviation of streptozotocin (STZ)-induced diabetes in rats. Furthermore, we explored the behavioural changes caused by such treatments on healthy and STZ-induced diabetic animals.

5.3 MATERIALS AND METHODS

Animals

All experimental procedures were carried out in accordance with the protocols approved by the University College Cork Ethics Committee, under a license issued from the Department of Health and Children. Male Sprague Dawley rats, 5 weeks of age, were obtained from Harlan UK and housed under barrier-maintained conditions. All animals were allowed to acclimate for one week in the housing facility before the experiments. Animals were group-housed, with 5 animals per cage at standard conditions (room temperature of 21°C, with a 12-h light–dark cycle, lights on at 07:00). All groups were fed *ad libitum* with Teklad Global rodent standard diet (#2018S; Harlan Laboratories) and allowed free access to water. Water containing either *Lactobacillus brevis* DPC 6108, GABA (Sigma) and/or placebo freeze-dried powder was the only water supply provided to the animals throughout the experiments. Bottles were replaced daily. Two experiments were conducted in this study – Experiment 1 using healthy rats and Experiment 2 using streptozotocin-induced diabetic rats.

Lactobacillus brevis DPC 6108 and placebo preparation

Lb. brevis DPC 6108 is an efficient GABA producer, with maximum conversion *in vitro* when growing on MRS broth supplemented with 30 mg/mL monosodium glutamate (MSG) (Barrett et al, 2012). Rifampicin-resistant variants of *Lb. brevis* DPC 6108 were isolated by spread-plating ~10^9 CFU (colony forming units) from an overnight culture onto MRS agar (Difco Laboratories) containing 500 µg rifampicin/mL (Sigma) and stocked at -80^{o}C. Before freeze-drying, frozen stocks

were plated in MRS agar, and then inoculated in small aliquots of fresh MRS broth supplemented with 30 mg/mL MSG and incubated overnight at 37^{o}C under anaerobic conditions. The overnight culture aliquots were then inoculated into large volumes of MRS containing MSG and allowed to grow overnight at 37^{o}C under anaerobic conditions. The overnight culture was washed twice in phosphate buffered saline (Sigma) and the pellet resuspended in 15% (w/v) trehalose (Sigma) in dH2O (Experiment 1) or 4% (w/v) yeast extract (Difco Laboratories) in dH2O (Experiment 2). One-millilitre aliquots of bacterial culture were freeze-dried by using a 24-h program (freeze temperature, -40^{o}C; condenser set point, -60^{o}C; vacuum set point, 600 mTorr). Vials containing one millilitre of placebo solution (15% (w/v) trehalose or 4% (w/v) yeast extract) were freeze-dried using the same program. All the vials containing freeze-dried powder were stored at -20^{o}C until use. Each animal that received the bacterial strain consumed ~1 x 10^{9} live microorganisms/day. This was achieved by resuspending appropriate quantities of freeze-dried powder in water, which rats consumed *ad libitum*. The groups that did not receive the bacterial strain received placebo freeze-dried powder [15% (w/v) trehalose or 4% (w/v) yeast extract in dH2O]. Freeze-dried powder underwent continuous quality control of cell counts for the duration of the experiments by plating serial dilutions on MRS agar supplemented with 100 µg/mL rifampicin and incubating plates anaerobically for 48 h at 37^{o}C.

Faecal sample microbial analysis and GABA production assay

Fresh faecal samples were taken every week for microbial analysis in order to verify if the strain survived gut transit, animals were receiving enough bacterial

cells and there was cross-contamination in the groups not receiving the strain. Microbial analysis involved enumeration of the *Lb. brevis* DPC 6108 strain after plating serial dilutions on MRS agar supplemented with 100 µg rifampicin/mL (Sigma) and incubating anaerobically for 48 h at 37^{o}C. In addition, isolated colonies were tested for GABA production as described previously (Barrett et al., 2012). Briefly, isolated colonies were grown anaerobically in MRS containing 3% (w/v) MSG at 37^{o}C for 55 h. Samples were then deproteinized by mixing equal volumes of 24% (w/v) trichloroacetic acid (TCA) and culture, allowed to stand for 10 min and centrifuged at 14000 x g for 10 min. Supernatants were removed and diluted with 0.2 mol/L sodium citrate buffer, pH 2.2 to yield 250 nmol of each amino acid residue. Samples were then diluted with the internal standard, norleucine, to give a final concentration of 125 nm/mL. Amino acids were quantified using a Jeol JLC-500/V amino acid analyzer (Jeol Ltd, Garden City, Herts, UK) fitted with a Jeol Na^{+} high-performance cation exchange column.

Experiment 1: Effects of GABA powder and Lb. brevis DPC 6108 on healthy rats

Animals were divided in 3 groups with 10 rats each, receiving either GABA (2.6 mg/Kg BW + 15% w/v trehalose), *Lb. brevis* DPC 6108 (~10^{9} cells in 15% w/v trehalose) or placebo (15% trehalose) mixed in the drinking water for 5 weeks. All groups received 15% (w/v) trehalose as this solution was used as cryoprotectant while freeze-drying *Lb. brevis* DPC 6108. The standard diet used contained 3.4% (w/w) glutamic acid in its composition and was sufficient substrate for the conversion to GABA by *Lb. brevis* DPC 6108 (Barrett et al., 2012). Body weight was assessed weekly. After 5 weeks dietary intervention, animals were sacrificed by

decapitation and blood samples were collected, allowed to clot at 4^{o}C, centrifuged for 20 min at 2 000 x g and the serum collected into clean microtubes. The brain was rapidly removed from the cranium and dissected out on an ice-cold plate into the following areas: amygdala, brain stem, cerebellum, cortex, hippocampus, striatum and hypothalamus. These brain regions were weighed and placed in 1.5-mL tubes, which contained 1 mL of chilled homogenizing high performance liquid chromatography (HPLC) buffer spiked with an internal standard (n-methyl 5-HT). Liver was removed and flash-frozen on dry ice. All samples were stored at -80^{o}C prior to analysis.

Experiment 2: Effects of GABA powder and Lb. brevis DPC 6108 on streptozotocin-induced diabetic rats

Animals were divided in 5 groups with 15 rats each**:** non-diabetic control group and diabetic control group received only placebo freeze-dried powder [4% (w/v) yeast extract], diabetic low dose GABA group received 2.6 mg/Kg BW GABA powder, diabetic high dose GABA group received 200 mg/Kg BW GABA powder, and diabetic *Lb. brevis* DPC 6108 group received ~10^{9} bacterial cells in 4% (w/v) yeast extract. GABA, *Lb. brevis* DPC 6108 and placebo freeze-dried powders were diluted in fresh drinking water every day during the 9 weeks trial. Every group received 4% (w/v) yeast extract as this solution was used as cryoprotectant while freeze-drying *Lb. brevis* DPC 6108. On experimental week 3, type 1 diabetes was induced in rats in four of the groups by intraperitoneal injection with a single dose of 60 mg/kg streptozotocin (STZ; ≥ 75% α-anomer basis; Sigma) freshly prepared in 50 mM sodium citrate buffer (pH 4.5) and injected within 10 to 15 min after dissolving,

according to a previously described procedure (Wu and Huan, 2008). Non-diabetic control group received an injection of citrate buffer. After 7 days of STZ-injection, glucose level was measured in triplicate using a Contour Next blood glucose meter (Bayer) in blood samples collected from a tail vein. Rats with glucose levels higher than 200 mg/dL were considered diabetic and STZ-induced rats that had lower glucose level were excluded. From a total of 60 rats treated with STZ, three died 1-2 days after injection, whereas ten rats did not develop diabetes and were excluded. In the remaining 47 animals, body weight and glucose levels were assessed weekly, before and after inducing diabetes. After 9 weeks of dietary intervention animals were killed by decapitation and blood samples were collected, allowed to clot at 4^{o}C, centrifuged for 20 min at 2000 x g and the serum collected into clean microtubes. Liver was removed and flash-frozen on dry ice. All samples were stored at -80^{o}C prior analyses.

Brain monoamine analysis

Neurotransmitter concentrations were determined using a modification of a previously described procedure (O'Mahony et al., 2008). Samples were sonicated in homogenising buffer which consisted of mobile phase spiked with 2ng/20ul of the internal standard N-Methyl 5-HT (Sigma). The mobile phase contained 0.1 M citric acid, 0.1 M sodium dihydrogen phosphate, 0.01 mM EDTA (Alkem/Reagecon, Cork), 5.6 mM octane-1-sulphonic acid (Sigma) and 9% (v/v) methanol (Alkem/Reagecon), and was adjusted to pH 2.8 using 4 N sodium hydroxide (Alkem/Reagecon). The homogenates were centrifuged for 20 min at 14 000 x g with the temperature maintained at 4°C and 20 µl of the supernatant injected onto

the HPLC system. The system included SIL-10A autoinjector (with sample cooler maintained at 40C), LC-10AS pump, SCL 10-Avp system controller, CTO-10A oven, LECD 6A electrochemical detector (Shimadzu) and an online Gastorr Degasser (ISS, UK). A reverse-phase column (Kinetex 2.6 u C18 100 x 4.6 mm, Phenomenex) maintained at 30^{o}C was employed in the separation at a flow rate of 0.9 ml/min, the glassy carbon working electrode combined with an Ag/AgCl reference electrode (Shimdazu, Japan) was operated a +0.8V and the chromatograms generated were analysed using Class-VP 5 software (Shimadzu). The neurotransmitters were identified by their characteristic retention times as determined by standard injections which were run at regular intervals during the sample analysis. Analyte/Internal standard peak height ratios were measured and compared with standard injections and results were expressed as ng of neurotransmitter per g fresh weight of tissue (O'Mahony et al. 2008).

Serum analyses

Commercial kits were used for measurement of GABA, metabolic markers and stress hormones in serum. GABA was determined using the GABA research ELISA kit (Invitech Ltd, UK). Glucose was determined using the QuantiChrom glucose assay (BioAssay Systems, Hayward, CA), triglycerides measured by using EnzyChrom Triglyceride Assay kit (BioAssay Systems) and cholesterol using LabAssay Cholesterol kit (Wako, Japan). Insulin, glucagon, c-peptide, peptide YY (PYY), active glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide (GIP) and leptin were measured using Rat Metabolic Hormone Magnetic Bead Panel (Merck Millipore,

Germany). Corticosterone and melatonin were measured using Rat Stress Hormone Magnetic Bead Panel (Merck Millipore).

Liver analyses

Hepatic lipids were extracted according to the method of Folch et al. (1957). After extraction, samples were dried under a stream of nitrogen and resuspended in 5% (v/v) solution of Triton X-100 in distilled water). Triglycerides concentration was determined using the commercial kit EnzyChrom Triglyceride Assay (BioAssay Systems) and cholesterol using LabAssay Cholesterol kit (Wako).

Behavioural tests

Behavioural tests were undertaken at end of Experiment 1 and along the course of Experiment 2 after STZ-injection. Open Field and Elevated Plus Maze (EPM) tests were included, as indicators of anxiety and locomotor activity. The Forced Swim Test (FST) was included to evaluate anti-depressants effects. The FST and the open field test were conducted in both Experiment 1 and 2. EPM was conducted only in Experiment 2.

Forced Swim Test (FST)

The modified forced swim test was conducted as previously described (Bissiere et al., 2006, Cryan et al., 2002, Cryan et al., 2005a and Cryan et al., 2005b). The rats were placed individually in 21 cm × 46 cm Pyrex cylinders filled to a 30 cm depth with 23–25°C water. Two swimming sessions were conducted: a 15 min pre-test followed by a 5 min test 24 h later. After 15 min on day 1, the rats were

removed, towel dried and placed back in their home cage. The water in each cylinder was changed between animals. Twenty-four hours after their first exposure, rats were placed back in the swim apparatus for 5 min and a video camera positioned above the cylinders was used to monitor and record behaviour for subsequent analysis. The total duration of predominant behaviour in each 5 s period of the 300 s test was scored on day 2. Swimming, immobility and active climbing were the main behaviours scored with swimming described as horizontal movements throughout all 4 quadrants of the cylinder, climbing was defined as vigorous fore-paw movements directed toward the walls of the apparatus and immobility defined as floating, with only enough movement necessary for the rat to keep its head above water (see Cryan et al., 2002 for pictorial representations). The experiments were analysed by an observer blind to the experimental conditions.

Open field

The open field test was performed as previously described (O'Mahony et al., 2009). Briefly, the open field consisted of a circular white arena, 90 cm in diameter, 40 cm in height, 900 lux light. Testing was conducted between 9:00 a.m. and 1:00 p.m. At the beginning of each trial, animals were placed gently into the centre of the arena and allowed to explore the arena for 10 min. The behaviour of the animals was recorded by an overhanging camera that was attached to a personal computer. Ethovision 3.1 (Noldus, The Netherlands) was used to track the movement of the animals. The total distance moved in the arena, time spent and distance moved in the inner zone of the arena were recorded. When rats are anxious they usually display freezing behaviour and stay close to the sides of the

arena, which will result in a reduction in the amount of time spent in the brightly lit inner zone and distance travelled.

Elevated Plus Maze (EPM)

Animals were acclimatized to the testing room for 30 min prior to testing. Animals were placed one at a time in a novel maze for 5 min. The maze consisted of two open arms and two closed arms (24 inches in height), elevated to a height of 28 inches. Animals were placed in the centre of the maze facing an open arm to begin. Behaviour was videotaped for the duration of the test. Frequency of entry and time spent in each of the open and closed arms entries were scored.

Statistical analysis

To assess whether differences between treatment groups were significant, statistical analysis was performed by using one factor ANOVA followed by Tukey's or Dunnett's post hoc multiple comparison tests (Graph-Pad Software, San Diego, CA, USA). Treatment effects with $p<0.05$ were considered significant. Data in the text, tables, and figures are presented as mean values plus minus standard error of the mean (SEM).

5.4 RESULTS

Lb. brevis DPC 6108 survived the transit through the rat gastrointestinal tract

Quantification of the numbers of the administered rifampicin-resistant *Lb. brevis* strain in the faeces of rats confirmed its survival during gastrointestinal transit. Stool recovery of *Lb. brevis* DPC 6108 was ~ 1.1 x 10^7 CFU/g faeces after 1

week of feeding and remained at similar numbers until the end of experiments. Colonies isolated from the plates were tested for GABA production in order to assess if gut transit affected the strain for GABA production. All such colonies tested produced similar amounts of GABA *in vitro* when compared to the wild type strain (data not shown). There was no cross-contamination between groups as *Lb. brevis* DPC 6108 was not detected in the stool of rats not receiving the strain.

EXPERIMENT 1

Lb. brevis DPC 6108 increased insulin serum levels but did not alter other metabolic markers in healthy animals

After 5 weeks supplementation with *Lb. brevis* DPC 6108, there were no differences in body weight gain between groups (data not shown) and final body weight, liver cholesterol, basal corticosterone and serum glucose. However, serum insulin levels were increased ($p<0.05$) in rats receiving *Lb. brevis* DPC 6108, when compared with rats in the GABA and control groups (Table 5.1).

Table 5.1 Final body weight and metabolic markers concentrations in healthy rats receiving GABA powder, *Lb. brevis* DPC 6108 or placebo for 5 weeks

	Control	GABA	*Lb. brevis* DPC 6108
Final body weight (g)	385.2 ± 9.7	380.5 ± 10.1	378.3 ± 6.7
Glucose (mg/dL)	99.1 ± 4.8	94.3 ± 5.0	104.1 ± 7.2
Insulin (ng/mL)	1.51 ± 0.19[a]	1.52 ± 0.19[a]	2.55 ± 0.35[b]
Liver cholesterol (mg/g)	1.88 ± 0.15	2.16 ± 0.22	1.71 ± 0.13
Corticosterone (ng/mL)	12.6 ± 3.0	12.1 ± 2.0	34.3 ± 10.6

All values are means ± SEMs. Values in the same row with different superscript letters are significantly different, $p<0.05$ (ANOVA followed by post hoc Tukey's multiple comparisons tests).

GABA and Lb brevis DPC 6108 supplementation did not change brain monoamines levels

The monoamines noradrenaline (NA), dopamine (DA), serotonin (5-HT), and the monoamine metabolites 3, 4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) were measured in the cortex, hippocampus, amygdala, striatum, hypothalamus, cerebellum and brain stem. GABA and *Lb. brevis* DPC 6108 supplementation did not affect the levels of monoamines or their metabolites in any of the brain regions analysed. In addition, there were no differences in DOPAC/Dopamine, HVA/Dopamine and 5-HIAA/5-HT turnover ratios when comparing the treatments to the control (Table 5.2).

Table 5.2 Brain concentrations (ng/g) of monoamines and their metabolites in the brain of healthy rats receiving GABA powder, *Lb. brevis* DPC 6108 or placebo for 5 weeks

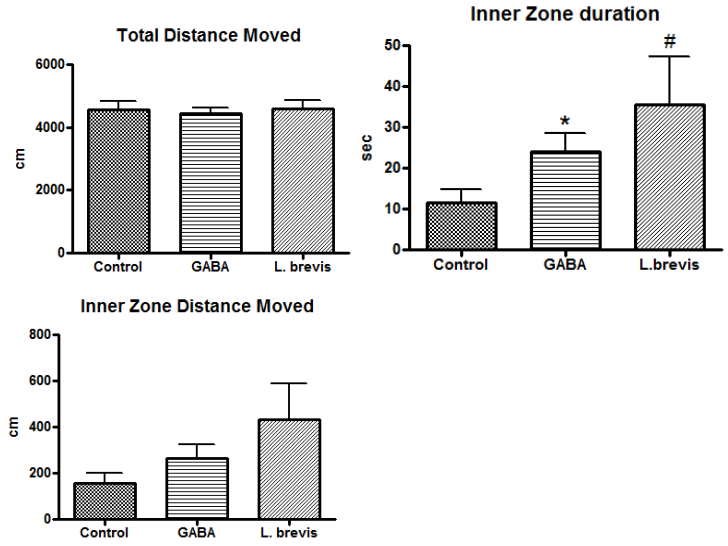
	Monoamines			Metabolites			Turnover ratio		
	Noradrenaline	Dopamine	5-HT	DOPAC	HVA	5-HIAA	DOPAC/ Dopamine	HVA/ Dopamine	5-HIAA/ 5-HT
Prefrontal cortex									
Control	1422.2 ± 66.3	194.2 ± 65.5	968.4 ± 36.4	1895.9 ± 89.5	228.6 ± 20.1	323.0 ± 13.9	12.0 ± 3.2	1.37 ± 0.35	0.34 ± 0.01
GABA	1325.7 ± 53.5	107.0 ± 13.4	927.5 ± 23.5	1887.7 ± 135.5	226.8 ± 24.8	340.9 ± 9.7	13.7 ± 2.6	1.50 ± 0.25	0.37 ± 0.01
Lb. brevis DPC6108	1079.7 ± 27.1	124.8 ± 19.6	960.0 ± 33.7	1881.6 ± 69.8	221.5 ± 13.7	328.4 ± 12.4	14.4 ± 2.4	1.67 ± 0.27	0.34 ± 0.01
Hippocampus									
Control	1633.7 ± 72.0	17.4 ± 1.7	688.8 ± 24.5	1834.1 ± 74.6	96.8 ± 6.9	417.3 ± 14.9	110.8 ± 6.4	5.88 ± 0.53	0.59 ± 0.02
GABA	1642.2 ± 54.9	17.6 ± 1.2	686.3 ± 19.9	1940.7 ± 95.0	99.5 ± 6.2	434.8 ± 14.2	113.1 ± 5.6	5.79 ± 0.41	0.65 ± 0.03
Lb. brevis DPC6108	1582.9 ± 75.9	21.5 ± 3.1	700.7 ± 29.8	1831.5 ± 72.5	92.4 ± 8.8	395.8 ± 14.0	100.9 ± 17.6	5.17 ± 0.99	0.57 ± 0.02
Amygdala									
Control	1466.8 ± 59.2	345.1 ± 36.0	1258.5 ± 46.5	1864.6 ± 79.6	93.8 ± 8.2	364.7 ± 13.9	6.10 ± 0.57	0.27 ± 0.02	0.29 ± 0.01
GABA	1369.1 ± 59.2	379.7 ± 48.5	1118.1 ± 43.3	2004.7 ± 140.2	91.5 ± 8.0	382.4 ± 25.7	5.39 ± 0.73	0.23 ± 0.02	0.32 ± 0.02
Lb. brevis DPC6108	1401.3 ± 56.3	323.2 ± 31.7	1201.3 ± 52.4	1903.7 ± 84.3	86.5 ± 3.3	343.6 ± 15.5	5.72 ± 0.67	0.27 ± 0.03	0.29 ± 0.01
Striatum									
Control	975.7 ± 104.7	14012 ± 1316	996.6 ± 59.0	3371.9 ± 283.0	1139.0 ± 130.8	679.9 ± 29.8	0.25 ± 0.02	0.08 ± 0.00	0.69 ± 0.03
GABA	931.9 ± 154.7	15134 ± 1250	1094.1 ± 104.2	3613.2 ± 400.2	1232.3 ± 175.6	735.1 ± 58.0	0.23 ± 0.01	0.07 ± 0.00	0.69 ± 0.03
Lb. brevis DPC6108	1002.3 ± 100.3	11915 ± 1008	1098.2 ±50.9	3313.1 ± 256.5	961.2 ± 90.2	714.0 ± 29.4	0.26 ± 0.02	0.07 ± 0.00	0.67 ± 0.02
Hypothalamus									
Control	7029.6 ± 962.9	732.5 ± 79.2	1816.5 ± 215.4	2161.1 ± 271.9	ND	742.9 ± 92.0	2.96 ± 0.22	ND	0.40 ± 0.01
GABA	6478.2 ± 512.7	719.2 ± 67.3	1679.7 ± 69.8	2069.8 ± 199.2	ND	786.9 ± 70.8	3.06 ± 0.12	ND	0.43 ± 0.02
Lb. brevis DPC6108	6581.4 ± 532.1	797.4 ± 122.2	1718.3 ± 131.2	1881.4 ± 246.2	ND	792.9 ± 87.6	2.72 ± 0.44	ND	0.42 ± 0.02
Cerebellum									
Control	925.0 ± 24.9	7.46 ± 0.50	135.1 ± 5.1	2555.8 ± 86.6	258.1 ± 11.1	88.0 ± 2.5	343.0 ± 36.8	30.6 ± 5.3	0.64 ± 0.02
GABA	884.2 ± 21.2	7.42 ± 0.99	129.8 ± 7.0	2704.9 ± 127.9	227.7 ± 22.3	89.9 ± 3.6	392.3 ± 40.4	31.5 ± 3.2	0.71 ± 0.04
Lb. brevis DPC6108	950.5 ± 13.8	8.11 ± 1.19	127.8 ± 5.7	2606.3 ± 79.2	247.5 ± 18.0	84.9 ± 4.5	354.9 ± 72.0	32.4 ± 6.7	0.67 ± 0.03
Brain stem									
Control	1542.1 ± 93.5	68.1 ± 3.2	1023.4 ± 29.7	1675.3 ± 81.4	118.5 ± 6.8	465.4 ± 29.6	25.25 ± 0.62	1.82 ± 0.07	0.49 ± 0.02
GABA	1693.5 ± 58.7	71.9 ± 2.5	1032.2 ± 32.8	1839.1 ± 72.3	129.8 ± 4.9	530.1 ± 11.3	25.81 ± 1.25	1.82 ± 0.08	0.49 ± 0.01
Lb. brevis DPC6108	1714.1 ± 51.3	72.7 ± 2.2	1033.6 ± 32.4	1798.6 ± 58.3	130.9 ± 5.8	505.7 ± 13.0	25.51 ± 0.75	1.81 ± 0.09	0.48 ± 0.01

Data are presented as Means ± SEMs; Control (n=10), GABA (n=10), *Lb. brevis* DPC6108 (n=9). ND= not detected

GABA and Lb. brevis DPC 6108 interventions have an anxiolytic effect in healthy animals

In the Open Field test, no differences in total distance moved by the animals were found, but significantly ($p<0.05$) higher time was spent by animals in the GABA group in the inner zone, and data approaching significance were recorded for the *L. brevis* DPC 6108 group ($p=0.0511$), compared with unsupplemented control group, suggesting an anxiolytic-like effect in the former two groups, compared with the latter group. The pre-swim results demonstrated no differences between any of the groups in immobility, swimming or climbing. However, the FST on day 2 revealed significant increase in immobility in the GABA treated group compared with control animals indicating depressant-like activity (Fig. 5.1).

Open Field



Forced Swim Test

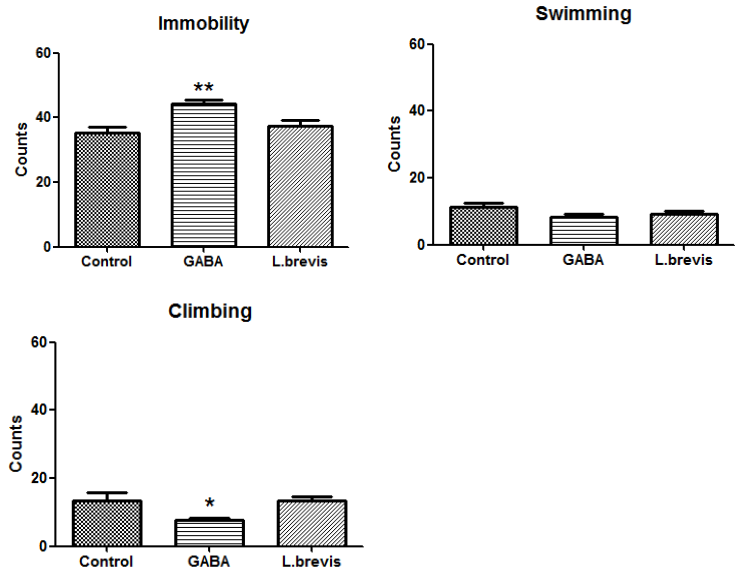


Figure 5.1 Effects of GABA powder (2.6 mg/Kg BW) and *Lb. brevis* DPC 6108 on behaviour of healthy animals (Experiment 1). Mean ± SEM (n=10) *p<0.05, **p<0.01, #p=0.051; One-way ANOVA followed by Dunnett's Multiple Comparison Test.

EXPERIMENT 2

GABA and Lb brevis DPC 6108 supplementation did not alter changes in body weight, small intestine length, water and food intake induced by diabetes

After STZ injection, animals that developed diabetes lost body weight continuously until the end of the experiment. Final body weight was significantly reduced in all diabetic animals including the diabetic control group and treatment groups, when compared with non-diabetic controls ($p<0.001$). Dietary supplementation with GABA and *Lb. brevis* DPC 6108 did not improve weight gain in diabetic animals. The abdomens of diabetic animals were distended, filled with a swollen intestine, and the small intestinal length significantly increased when compared to intestines of non-diabetic rats ($p<0.001$). Moreover, mean daily food and water intakes were significantly higher in diabetic rats when compared with rats non-diabetic control group ($p<0.001$) with no differences between treatments (Table 5.3). Diabetic rats exhibited polyuria and excessive faecal output (data not shown).

Table 5.3 Final body weight, small intestine length, food and water intake after STZ injection

	Non-diabetic Control	Diabetic Control	Low GABA	High GABA	*L. brevis* DPC 6108
Final body weight (g)	372.6 ± 7.5[a]	247.0 ± 5.6[b]	246.3 ± 8.4[b]	247.2 ± 5.8[b]	238.7 ± 8.0[b]
Small intestine length (cm)	98.6 ± 2.8[a]	129.4 ± 4.0[b]	133.8 ± 4.6[b]	135.7 ± 3.7[b]	132.2 ± 4.7[b]
Average food intake (g/100g BW)	6.0 ± 0.1[a]	14.3 ± 0.4[b]	12.8 ± 0.6[b]	13.6 ± 0.4[b]	12.7 ± 0.5[b]
Average water intake (mL/100g BW)	12.1 ± 1.1[a]	72.2 ± 2.5[b]	68.7 ± 1.9[b]	66.3 ± 2.0[b]	62.6 ± 3.9[b]

All values are means ± SEMs. Values in the same row with different superscript letters are significantly different, $p<0.05$ (ANOVA followed by post hoc Tukey's multiple comparisons tests).

Supplementation with high doses of GABA increased GABA levels in serum

Serum analysis results revealed a significant increase in GABA levels in the diabetic rats receiving a high dose of GABA (200 mg/Kg BW) relative to animals treated with lower dose of GABA, *Lb. brevis* DPC 6108 and both diabetic and non-diabetic controls ($p<0.05$; Table 5.4).

Lb. brevis DPC 6108 reduced serum glucose levels in diabetic rats but did not alter other metabolic markers

Serum glucose levels were significantly increased in all diabetic groups when compared with the non-diabetic control group ($p<0.001$). However, lower glucose levels were observed in rats receiving *Lb. brevis* DPC 6108 supplementation relative to rats in the diabetic control group ($p<0.01$). Insulin and c-peptide levels were significantly decreased, whereas glucagon was increased in all diabetic groups when compared with the non-diabetic control group ($p<0.05$; Table 5.4). GLP-1 and GIP serum levels were not different between treatments. PYY was 4- to 5-fold higher in diabetic rats when compared with non-diabetic rats ($p<0.05$), with no differences between treatments. As expected, due to the lower adipose tissue mass in diabetic rats (data not shown), leptin levels were significantly decreased in all diabetic groups compared with the non-diabetic control group ($p<0.001$; Table 5.4).

Lb. brevis DPC 6108 and high dose GABA increased serum cholesterol in diabetic rats

Serum cholesterol was significantly higher in rats supplemented with *Lb. brevis* DPC 6108 and the high GABA dose when compared with rats in the diabetic

and non-diabetic control groups ($p<0.05$), whereas no differences in liver cholesterol were observed between groups. Moreover, serum and liver triglycerides were significantly higher in diabetic groups compared with non-diabetic control ($p<0.05$), but there was no differences between treatments (Table 5.4).

Table 5.4 Metabolic markers concentrations in non-diabetic rats and diabetic rats receiving GABA powder, *Lb. brevis* DPC 6108 or placebo for 9 weeks

	Non-diabetic Control	Diabetic Control	Low GABA	High GABA	*L. brevis* DPC 6108
Serum GABA (ng/mL)	144.1 ± 9.8[a]	145.0 ± 2.9[a]	157.7 ± 9.4[a]	195.1 ± 7.7[b]	137.4 ± 9.4[a]
Glucose (mg/dL)	125.8 ± 5.9[a]	579.5 ± 34.3[b]	513.9 ± 28.7[b,c]	508.6 ± 23.8[b,c]	444.5 ± 18.6[c]
Insulin (ng/mL)	1.45 ± 0.10[a]	0.12 ± 0.02[b]	0.13 ± 0.03[b]	0.19 ± 0.05[b]	0.11 ± 0.03[b]
Glucagon (pg/mL)	9.0 ± 3.2[a]	24.1 ± 1.8[b]	24.0 ± 3.7[b]	26.3 ± 3.8[b]	31.2 ± 3.8[b]
C-peptide (ng/mL)	1.23 ± 0.13[a]	0.20 ± 0.02[b]	0.21 ± 0.02[b]	0.23 ± 0.03[b]	0.19 ± 0.02[b]
PYY (pg/mL)	38.5 ± 4.3[a]	167.5 ± 30.3[b]	150.8 ± 10.0[b]	186.7 ± 22.9[b]	185.5 ± 33.7[b]
GLP-1 (pg/mL)	80.5 ± 17.2	182.3 ± 41.4	113.5 ± 21.0	170.5 ± 20.8	151.9 ± 17.3
GIP (pg/mL)	275.5 ± 37.1	230.5 ± 29.9	196.2 ±25.1	227.0 ± 42.9	237.7 ± 40.2
Leptin (ng/mL)	1.38 ± 0.20[a]	0.07 ± 0.01[b]	0.06 ± 0.01[b]	0.08 ± 0.01[b]	0.06 ± 0.01[b]
Serum cholesterol (mg/dL)	58.2 ± 3.0[a]	63.7 ± 3.4[a]	68.1 ± 6.0[a]	103.4 ± 13.4[b]	90.0 ± 7.4[a]
Liver cholesterol (mg/g)	1.81 ± 0.06	1.92 ± 0.06	1.81 ± 0.08	1.79 ± 0.05	1.94 ± 0.08
Serum triglycerides (mg/dL)	155.8 ± 9.1[a]	712.8 ± 87.1[b]	634.8 ± 106.3[b]	1034.5 ± 127.2[b]	1013.5 ± 114.9[b]
Liver triglycerides (mg/g)	1.83 ± 0.08[a]	5.38 ± 0.31[b]	5.10 ± 0.21[b]	5.30 ± 0.14[b]	5.46 ± 0.28[b]

All values are means ± SEMs. Values in the same row with different superscript letters are significantly different, $p<0.05$ (ANOVA followed by post hoc Tukey's multiple comparisons tests).

Lb. brevis DPC 6108 and GABA supplementation did not attenuate increase in corticosterone levels induced by diabetes

Increased basal levels of serum corticosterone were found in diabetic rats when compared with non-diabetic rats ($p<0.05$), with no differences between treatments. Melatonin was increased in diabetic rats with a significant difference in the diabetic control group when compared with the non-diabetic control group ($p<0.05$; Table 5.5).

Table 5.5 Stress hormones concentrations in non-diabetic rats and diabetic rats receiving GABA powder, *Lb. brevis* DPC 6108 or placebo for 9 weeks

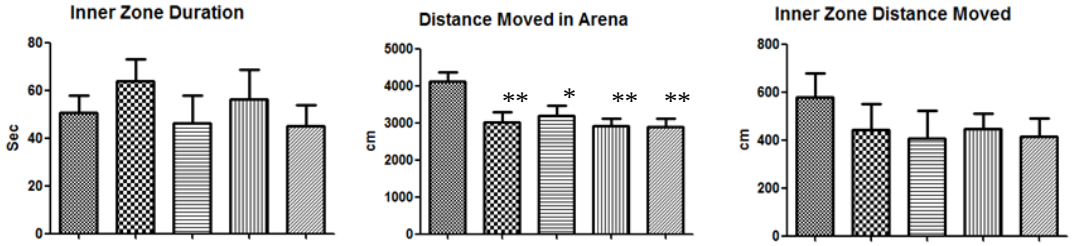
	Non-diabetic Control	Diabetic Control	Low GABA	High GABA	*L. brevis* DPC 6108
Corticosterone (ng/mL)	64.5 ± 11.8[a]	183.0 ± 23.4[b]	193.2 ± 20.7[b]	207.8 ± 29.1[b]	244.2 ± 14.6[b]
Melatonin (pg/mL)	138.7 ± 25.6[a]	407.7 ± 104.1[b]	338.9 ± 38.9[a]	373.8 ± 39.2[a]	345.0 ± 68.4[a]

All values are means ± SEMs. Values in the same row with different superscript letters are significantly different, $p<0.05$ (ANOVA followed by post hoc Tukey's multiple comparisons tests).

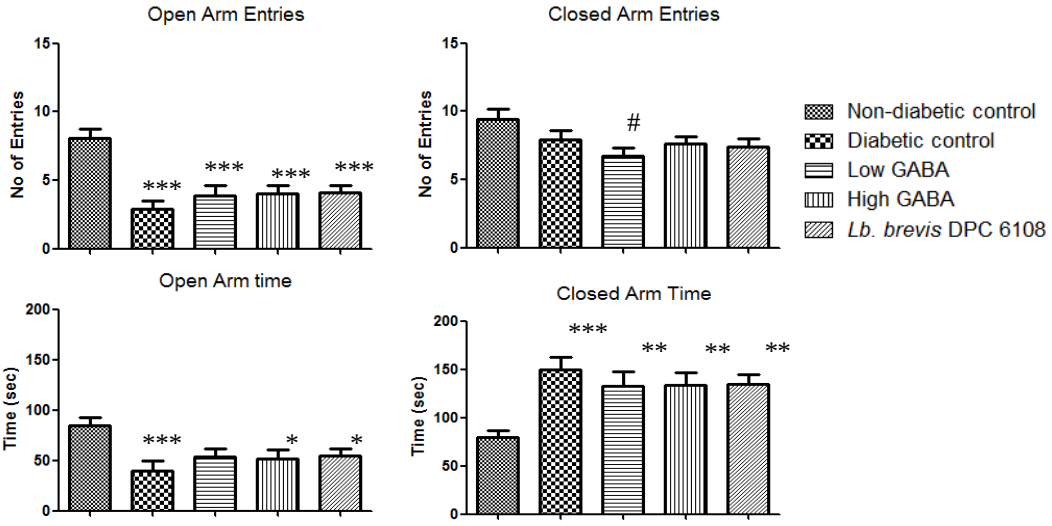
Diabetic rats displayed an anxiety-like behaviour that was not ameliorate by GABA or Lb brevis DPC 6108 interventions

The Open Field test revealed no significant differences between groups in either the amount of time spent in the inner-zone or the distance moved in the inner zone. A significant reduction in the total distance moved in the arena was seen for diabetic-control, diabetic-high GABA and diabetic-*L. brevis* DPC 6108 treated groups when compared with the non-diabetic control group ($p<0.05$). In the FST, diabetic *L. brevis* DPC 6108-treated group spent more time immobile when compared with the non-diabetic control group ($p<0.05$). Results seen in the EPM test showed significantly reduced entries into the open arms of all diabetic groups when compared with non-diabetic controls ($p<0.001$). Diabetic control, diabetic-high GABA and diabetic *L. brevis* DPC 6108 treated groups spent less time in the open arms when compared with the non-diabetic control ($p<0.05$). Furthermore, the diabetic low GABA-treated group had reduced closed arm entries when compared to non-diabetic control ($p=0.059$) and all diabetic groups spent significantly more time in the closed arms, when compared with the non-diabetic control group ($p<0.05$) (Fig. 5.2).

Open Field



Elevated Plus Maze



Forced Swim Test

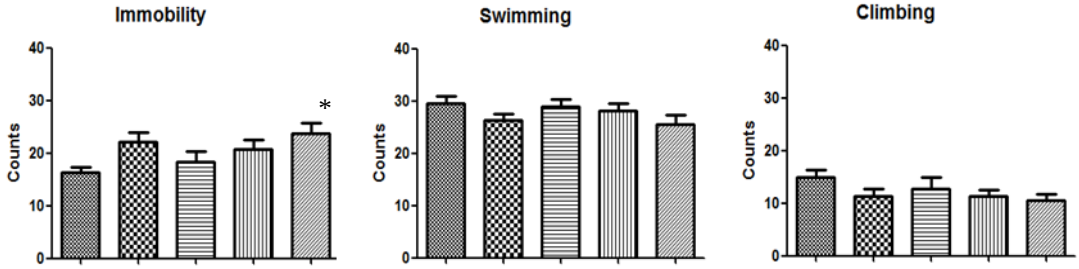


Figure 5.2 Effects of GABA powder and *Lb. brevis* DPC 6108 on behaviour of diabetic animals (Experiment 2). Mean ± SEM *p<0.05, **p<0.01, ***p<0.001, #p=0.059; One-way ANOVA followed by Dunnett's Multiple Comparison Test.

5.5 DISCUSSION

In the present study, we demonstrated that *Lb. brevis* DPC 6108 dietary intervention for 5 wk significantly increase (69%, $p<0.05$) serum insulin in healthy rats. Leading on from this data, we conducted a second experiment to investigate whether dietary GABA and *Lb. brevis* DPC 6108 would exert protective and/or regenerative effects on islet β-cells and reverse diabetes in Sprague Dawley rats in which diabetes was induced by streptozotocin-injection. Oral administration of GABA and *Lb. brevis* DPC 6108 for 9 weeks had no effect on the prevention or reversion of diabetes induced by streptozotocin on male Sprague-Dawley rats, and animals developed an overt basal hypoinsulinemia and extreme hyperglycemia, suggesting a drastic decrease in β-cells numbers. Our data support the results obtained by Adeghate and Ponery (2002), where pancreas isolated from STZ-diabetic rats presented a significant decrease in cell mass with concomitant insulin depletion that were not restored by GABA treatment. However, they contradict studies showing that oral GABA administration attenuated hyperglycemia and oxidative stress in STZ-diabetic rats (Nakagawa et al., 2005), whereas GABA i.p. injections prevented and reversed high glucose levels caused by multiple low doses of STZ in mice (Soltani et al., 2011). GABA therapy was also reported to be beneficial in other models of diabetes, such as the non-obese diabetic (NOD) mouse model of T1D and high-fat diet (HFD)-fed mouse model of T2D (Soltani et al., 2011; Tian, Dang, Kaufman, et al., 2011; Tian, Dang, Yong, et al., 2011). In all these studies, the positive GABA effect was related to the modulation of the immune response present in diabetes, confirming the anti-inflammatory effects of GABAergic agents seen in other models of autoimmune diseases, such as

rheumatoid arthritis (Tian, Yong, Dang et al., 2011) and multiple sclerosis (Bhat et al., 2010).

The results obtained in this study further support the hypothesis that the beneficial effect of GABA may be primarily due to modulation of immune cell function. In animal models such as the NOD mouse, HFD-induced T2D mouse and the multiple low-dose STZ-induced diabetes (MDSD) mouse, pancreatic islets are partially damaged and the inflammatory process causes the further loss of β-cells. On the other hand, the procedure commonly applied in rats and used in this study induces T1D with one single dose of STZ, destroying the β-cells rapidly and completely, with absence of an immune response (Wu and Huan, 2008). In this case, GABA administration may have no effect, as there is no immune response to be attenuated. However, beside its anti-inflammatory effect, evidence suggest that GABA may stimulate β-cell replication and inhibit apoptosis *in vitro* (Soltani et al., 2011) and *in vivo* (Tian et al., 2013), if a sufficient residual islet mass is present to permit the recovery. In this study, the extremely high levels of glucose and low levels of insulin and c-peptide in diabetic animals indicate an extensive loss of β-cell mass that could not be reversed by dietary GABA. While STZ is known to be a highly selective β-cells cytotoxic agent, not affecting the other pancreatic islet cells, a large variation is observed between studies related to the extension of β-cell mass destruction and, consequently circulating levels of glucose and insulin. Several factors may interfere with STZ toxicity, such as animal age, sex, species and strain (Abeeleh et al., 2009; Blondel et al., 1989; Deeds et al., 2011; Wu and Huan, 2008). Furthermore, the preparation of STZ solution recommended by most protocols and used in this study seems to be inappropriate, leading to considerable

interexperimental variation. Following the protocol, STZ solution should be prepared fresh each time and injected within 5 min of being dissolved. However, STZ occurs in two anomeric forms, the ratio of α to β anomer varies considerably between lots, and freshly prepared solutions are unstable and may contain higher concentrations of the more toxic α anomer. Thus, diabetes induction using solutions prepared few hours before the injections instead of freshly prepared may be a better alternative, allowing anomeric equilibrium and yielding more uniform and reproducible results (Garza-Rodea et al., 2010).

Previous studies investigating the benefits of probiotic administration in STZ-diabetic animals showed contrasting results on glucose homeostasis-related outcomes. Davari et al. (2013) and Lin et al. (2013) reported a significant decrease in glucose levels in animals receiving probiotic in relation to diabetic controls, as was seen in this study. Tabuchi et al. (2003) and Yadav et al. (2008) observed an improvement in glucose tolerance, whereas Zarfeshani et al. (2011) found no significant differences in blood glucose levels in the probiotic supplemented group. Increased serum insulin levels in probiotic-fed groups were demonstrated only in the studies by Davari et al. (2013) and Tabuchi et al. (2003). Although we observed a significant increase in serum insulin levels in healthy rats receiving *Lb. brevis* DPC 6108 (Experiment 1; 69%, $p<0.05$), the same was not seen in diabetic rats in Experiment 2, most likely because the β-cell mass was considerably destroyed and could not be stimulated. However, although administration of *Lb. brevis* DPC 6108 did not affect serum glucose levels in healthy animals, it caused a significant decrease in glucose levels in diabetic rats when compared with diabetic controls (23%; $p<0.05$). The balance between glucose uptake, regulated mainly by the CNS,

and glucose production from the liver is complex and involves several mechanisms. Leptin has been shown to induce reduction of food intake in diabetic animals, lowering plasma glucose levels (Sindelar et al., 1999), and acting in the CNS inhibiting hepatic glucose production and increasing glucose utilization by other tissues (German et al., 2011). However, food intake and leptin levels were not altered in the diabetic group receiving the bacteria when compared to the other diabetic groups in the present study. Moreover, the levels of other metabolic markers involved in glucose homeostasis such as insulin, glucagon, corticosterone, GLP-1 and GIP were similar among all diabetic groups and could not account for this reduction in glucose levels in the *Lb brevis* DPC 6108-fed group. Bacteria produce several types of bioactive metabolites which affect the host's health and, thus, there is the possibility that probiotic strains administered in different studies may produce compounds that may interfere with glucose homeostasis. For example, α-glucosidase inhibitors, saccharides used as drugs to modulate glucose absorption in the treatment of T2D have been found to be produced by a wide range of marine bacteria (Pandey et al., 2013). Other mechanisms that could explain the difference in glucose level may be related to increased glucose excretion in the urine, reduced hepatic glucose production or increased glucose uptake by tissues.

GABA released by β-cells has been shown to play a role in regulating glucagon secretion from α-cells (Bailey et al., 2007; Bansal et al., 2011). In this study, the extensive damage to the β-cell mass caused by STZ most likely reduced the amount of endogenous GABA (Adeghate and Ponery, 2002), stimulating glucagon release in all diabetic groups. Even though higher level of circulating GABA was detected in the diabetic group receiving high dose of GABA, this

supplementation did not contribute to glucagon suppression. The increased glucagon secretion together with high level of corticosterone and low level of circulating insulin seen in all diabetic rats, most likely caused insufficient suppression of hormone-sensitive lipase (HSL) activity in the adipose tissue leading to hyperglycemia and hypertriglyceridemia. Increased food intake is another probable factor that contributed to hypertriglyceridemia. Moreover, high levels of corticosterone and glucose may have stimulated hepatic triglyceride production leading to the fatty liver observed in diabetic rats (Dallman & Bhatnagar 2011; Tamura & Shimomura, 2005).

The marked hyperphagia observed in diabetic animals has been associated with insulin and leptin deficiencies and high concentration of corticosterone (Dallman & Bhatnagar 2011; Sindelar et al., 1999; Sipols et al 1995). Hyperphagia leads to small intestine hypertrophy (Saudek & Young, 1981) as seen in all diabetic rats in this study. With increased intestinal mass, the density of gastrointestinal cells also increases, altering gut motility and secretion/absorption (El-Salhy & Sitohy, 2001). We observed an increased faecal output and diarrhoea, and significantly higher PYY levels in serum in all diabetic groups. In a study by El-Salhy (2001), PYY secretion was shown to delay intestinal transit and gastric emptying and to be produced in order to reduce diarrhoea. Small intestinal hypertrophy is also associated with increased intestinal cholesterol synthesis and absorption in diabetes (Feingold, 1989; Gleeson et al., 2000). Interestingly, in this study, serum total cholesterol was increased only in diabetic rats receiving high dose of GABA or *Lb. brevis* DPC 6108 when compared with the non-diabetic control. As food intake and intestinal length were similar among all diabetic groups as well as hepatic

cholesterol levels, the mechanism by which *Lb. brevis* DPC 6108 and high dose of GABA mediated the changes in serum cholesterol observed in the current study is unclear and remains to be elucidated.

In addition to high corticosterone levels, we observed an increase in melatonin level in diabetic rats, although the increase was significant only in the diabetic control group when compared with the non-diabetic control group. This is in agreement with results by Peschke et al. (2008) that showed increased levels of melatonin in STZ-induced diabetic rats and, further suggested that this change was due to the great reduction in insulin and increase in catecholamines (Peschke et al., 2012). The group hypothesised that the increased melatonin levels in type 1 diabetic rats could be a mechanism to protect β-cell mass damage, as melatonin has been shown to attenuate oxidative stress caused by STZ treatment (Aksoy et al., 2003; Yavuz et al., 2003).

In the present study, we also investigated behavioural effects of GABA and *Lb. brevis* DPC 6108 administration on healthy and diabetic rats. In the first experiment, healthy animals receiving GABA powder or *Lb. brevis* DPC 6108 were less anxious in comparison with placebo control group, whereas rats receiving the GABA treatment displayed a depressant-like behaviour during the FST. The behavioural changes seen as a result of GABA administration in Experiment 1 in healthy rats were not observed in diabetic rats in Experiment 2. STZ-induced diabetic rats displayed an anxiety-like behaviour in comparison to non-diabetic rats in the open field and the EPM tests, and no differences were observed in rats receiving either GABA or *Lb brevis* DPC 6108. Furthermore, even though diabetic rats presented lower weight and appeared to be weaker than non-diabetic controls,

no differences were observed between groups in the FST, suggesting that the behavioural changes were related to anxiety rather than motor or energy deficits. Previous studies have reported the same increase in anxiety in STZ-induced diabetic rats and have correlated this behaviour change with altered catecholaminergic and serotonergic systems in different brain regions (Ramanathan et al. 1998; Thorré et al., 1997). The exacerbated hypothalamo–pituitary–adrenal (HPA) axis activation due to excess of corticosterone was another mechanism strongly associated to impaired cognitive performance and increased anxiety in rodents (Magariños et al., 2000; Revsin et al., 2009; Stranahan et al., 2008).

Diabetes has been associated to decreased neurogenesis, increased oxidative stress and demyelination of brain cells with effects on neural excitability and brain function (Alvarez et al., 2009; Davari et al., 2013; Malone et al., 2006; Suzuki et al., 2011). There are several reports about diabetes-induced neurological complications and behavioural/cognitive changes in animal models and humans (Reagan, 2012; Roriz-Filho et al., 2009). GABA and probiotic therapies have been studied as alternatives to attenuate diabetes metabolic and neurological symptoms by a range of different mechanisms. Indeed, GABA administration has been shown to affect the brain monoamine system improving the symptoms of depression after stress stimulation (Chuang et al., 2011), while GABAergic agents prevented and delayed experimental autoimmune encephalomyelitis (EAE) onset in mice by decreasing the severity of symptoms through modulation of the immune system (Bhat et al., 2010). In a study by Bravo et al. (2011), the strain *L. rhamnosus* (JB-1) was shown to consistently modulate mRNA expression of GABA receptors, exerting anxiolytic and anti-depressant effects in a manner dependent on the vagus nerve,

whereas Davari et al. (2013) demonstrated that feeding a probiotic mixture elevates antioxidant enzymes and protects the brain from cell damage, preventing memory impairments and electrophysiological deficits caused by STZ-induced diabetes. In this study, GABA or *Lb. brevis* DPC 6108 administration did not affect monoamines and their metabolites in the brain of healthy animals, and the elevated basal corticosterone levels observed in diabetic rats were not ameliorated by the treatments; therefore, further analyses are required to draw conclusions about the mechanisms involved in the anxiolytic and depressant effects seen in healthy animals.

In conclusion, our results indicate that, although the use of GABA and probiotics have been shown to be effective in the prevention and attenuation of diabetes symptoms, a minimum number of healthy β-cells may be necessary for the efficacy of the treatment. Furthermore, as the main mechanism of action of GABA seems to be associated with anti-inflammatory activity, future studies using animal models of diabetes involving an autoimmune response will likely give better insights into the potential effects of GABA and *Lb. brevis* DPC 6108 on diabetes onset. Finally, additional studies are needed to understand the mechanisms involved in the glucose lowering effect caused by feeding *Lb. brevis* DPC 6108 to diabetic rats and the roles of GABA and probiotics on animal behaviour.

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Chapter 6

Evaluation of dietary bovine milk oligosaccharides versus a commercial prebiotic on microbiota composition and functionality in mice

Tatiana M. Marques and Devon Kavanaugh are co-authors of this chapter. TMM performed animal feeding, culling, and dissection, along with short chain fatty acid analysis by gas chromatography. DK performed serum cytokine and caecal secretory IgA analysis, faecal pellet preparation for 454 pyrosequencing, and contributed to the analysis of sequencing results.

6.1 ABSTRACT

Background: Human milk oligosaccharides exhibit an array of beneficial biological functions, including selective promotion of commensal bacteria and anti-adhesive activity against various pathogens. Milk oligosaccharides from animal species are currently of potential commercial interest given that they may produce similar biological outcomes when ingested.

Objective: To investigate the effect of dietary bovine milk oligosaccharides, 6'sialyllactose and the commercial prebiotic, Beneo Orafti P95 oligofructose, on murine gut microbiota composition and functionality.

Method: Animals received bovine milk oligosaccharides, 6'sialyllactose, P95 oligofructose (1 mg/g body weight) or saline by oral gavage for 21 days. Caecal short chain fatty acids were determined by gas chromatography and caecal secretory IgA and serum cytokine levels using commercial kits. Faecal microbiota composition was assessed by 16S rRNA pyrosequencing, while *Bifidobacterium* was quantified by qPCR.

Results: Caecal secretory IgA was reduced following treatment with P95 (62%; $p<0.01$) and BMO (61%; $p<0.05$). P95 and BMO supplementation did not influence SCFA content, whereas 6'SL supplementation resulted in reduced levels of butyrate by 41% ($p<0.01$) when compared with unsupplemented control. Moreover, P95 treatment reduced IL-12 (18.1%; $p<0.01$) and IL-6 (21.1%; $p<0.05$) and BMO supplementation reduced levels of IL-12 (11.9%; $p<0.05$) compared with unsupplemented control. At phylum level P95 supplementation significantly reduced *Cyanobacteria* ($p=0.003$), while administration of 6'SL decreased relative proportions of *Actinobacteria* ($p=0.017$). Bovine milk oligosaccharide

supplementation was associated with increased proportion of *Candidate division TM7* (p=0.002) and decreased proportions of *Actinobacteria* (p=0.0255) and *Cyanobacteria* (p=0.003) compared to the unsupplemented control. Significant changes were also observed at family and genus level when comparing animals receiving oligosaccharides supplementation versus unsupplemented animals. Overall, P95, BMO and 6'SL supplementations were associated with depletion or reduction of less favourable bacteria such as *Moraxellaceae*, *Vibrionaceae* and *Porphyromonaceae*. Indigenous *Bifidobacterium* was not detected in any of the groups tested.

Conclusions: This study highlights the potential of bovine milk oligosaccharides and commercial whey streams containing them, as untapped nutritional resources of prebiotics for gut microbiota modulation.

6.2 INTRODUCTION

Oligosaccharides are the third largest solid component of human milk following lactose and lipids, with concentrations of 22-24 g/L in colostrum and 12-13 g/L in mature milk (Urashima et al., 2013). Human milk oligosaccharides (HMO) have been shown to have specific biological functions including prebiotic and anti-adhesive activity, anti-inflammatory properties, modification of cell surface glycans, and modulation of growth-related characteristics of intestinal cells (for reviews see Bode, 2006; Hickey, 2009; Kunz & Rudloff, 2006; Newburg et al., 2005). However, few *in vivo* studies exist demonstrating the beneficial effects of HMO ingestion. Among these, Fuhrer *et al.* (2010) demonstrated that milk sialyllactose influences colitis in mice through selective intestinal bacterial colonization. Ingestion of milk

containing 3'sialyllactose (unaltered or 3'sialyllactose alone) resulted in reduced resistance to dextran sulphate sodium (DSS)-induced colitis, which was associated with the presence of clostridial cluster IV bacteria. Moreover, Newburg et al. (2004) showed that human milk containing higher content of 2-linked fucosyloligosaccharides is associated with lower risk of diarrhea in breastfed infants, whereas Ruiz-Palacios et al. (2003) demonstrated that fucosylated oligosaccharides present in human milk inhibit mice gut colonization with the pathogenic bacteria *Campylobacter jejuni.*

The several benefits attributed to breast milk are probably related to the structural complexity of HMO (Barile & Rastall, 2013). However, there are very few commercial products on the market which capitalize on these functions. This is mainly due to the fact that large quantities of HMO are required for clinical trials and these are not commercially available. In contrast, commercial oligosaccharides, including short-chain galactooligosaccharides and long-chain fructooligosaccharides (scGOS/lcFOS) are supplemented in certain products such as infant formula, which are currently marketed based on prebiotic health claims (Fanaro et al., 2005). However, the structure and composition of commercial oligosaccharides differ from that of HMO. Many efforts are, therefore, being made to replicate the effects of HMO by searching for alternative compounds which may produce similar biological outcomes particularly for infant formula applications.

Little information exists regarding the biological activity associated with alternative compounds, such as bovine milk oligosaccharides (BMO), for infant formula applications. However, a number of *in vitro* studies have already shown that bovine oligosaccharides possess anti-adhesive activity against certain

pathogens (Hakkarainen et al., 2005; Matrosovich et al., 1993; Wang et al., 2001). Moreover, studies demonstrated that sialylated oligosaccharides, such as 3'-sialyllactose and 6'-sialyllactose, which are present in both human and bovine milk, are potent inhibitors of pathogen adhesion in the gut (Gopal & Gill, 2000; Lane et al., 2010).

Given the potentially wide availability of BMO from dairy streams and the accumulating positive data from *in vitro* studies on their health-promoting effects, their inclusion in functional foods, such as infant formula may be a promising dietary strategy. Therefore, the objective of the current study was to assess physiological parameters (safety and tolerance) and modulatory effects of dietary supplementation with BMO or 6'sialyllactose (6'SL), and their effects on intestinal microbiota of healthy mice when compared with the commercial prebiotic fibre, Beneo Orafti P95 oligofructose (P95) and an unsupplemented control.

6.3 MATERIALS AND METHODS

Oligosaccharides

P95 (composed of 95% FOS [Degree of polymerization 3 to 10]) was kindly provided by Beneo Orafti (Tienen, Belgium), and 6'SL was purchased from Carbosynth (Berkshire, UK). BMO powder was kindly provided by Food for Health Ireland (FHI, Moorepark, Ireland).

Animals and treatments

All animal experiments were approved by the University College Cork Animal Ethics Committee, and experimental procedures were conducted under the

appropriate license from the Irish Government. BALB/c mice were purchased from Harlan (Becester, Oxfordshire, UK) and housed within the Biological Services Unit, University College Cork (UCC), under barrier-maintained conditions. Mice were delivered at 6 weeks of age and allowed to acclimatise for one week prior the beginning of the study. Animals were divided into four groups (n=10) and housed under standard conditions with access to standard chow and water *ad libitum*. P95, BMO or 6'SL were resuspended in saline solution and administered daily (1mg/g body weight) for 21 days by oral gavage. Unsupplemented group received the same volume of saline by oral gavage. Body weight was assessed weekly. Faecal pellets were collected at day-0 and day-21 of the trial and immediately placed on ice, and frozen at -80°C until bacterial DNA was extracted (see below). At the conclusion of the study, animals were sacrificed by decapitation, blood serum collected, and the liver, small intestine, and caecum harvested, washed with PBS, blotted dry on filter paper and weighed. Caecal content was divided for analysis of secretory IgA and short chain fatty acid (SCFA) composition.

Caecal secretory IgA analysis

Caecal secretory IgA concentration was determined using the mouse IgA ELISA quantification kit (Bethyl Laboratories, Cambridge, UK). Essentially, anti-mouse IgA antibody is adsorbed on the surface of microtitre wells and blocked using BSA. Following addition and binding of caecal secretory IgA, wells were washed and bound secretory IgA was detected using a biotinylated detection antibody, followed by streptavidin-linked horse radish peroxidase. Secretory IgA levels were colorimetrically detected using the TMB (3,3',5,5'-

tetramethylbenzidine) substrate and read on a BioTek plate reader at 450nm. Concentrations were determined based on a standard curve using the mouse IgA standard provided.

SCFA analysis

SCFA analysis was carried out according to previously published methods (Chapter 2 and Wall et al., 2012).

Serum cytokine analysis

Measurement of cytokines IL-1β, IL-6, IL-10, IL-12, IFN-γ, mKC, and TNF-α in serum was performed using the Meso Scale Discovery 7-plex mouse pro-inflammatory kit (Meso Scale Discovery, Gaithersburg, MD, USA) following the manufacturer's instructions.

Microbial composition analysis

DNA extractions and pyrosequencing were as described in Murphy et al. (2013). Total metagenomic DNA was extracted from individual faecal samples using the QIAmp DNA Stool Mini Kit (Qiagen, Crawley, West Sussex, UK). Samples were initially bead-beaten and then processed according to the manufacturer's instructions. For compositional analysis, isolated microbial DNA was subjected to pyrosequencing of 16S rRNA tags (V4 region; 239 nt long) amplified using universal 16S primers. The forward primer, F1, (5'-AYTGGGYDTAAAGNG) and a mixture of four reverse primers, R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC), and R4 (5'-

TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp) were used. To allow emulsion-based clonal amplification for the 454 pyrosequencing system, proprietary 19-mer sequences at the 5'end were incorporated into the primers. Unique molecular identifier (MID) tags were incorporated between the adaptamer and the target-specific primer sequence, allowing for identification of individual sequences from pooled amplicons. The resulting amplicons were cleaned using the AMPure purification system (Beckman Coulter, Takeley, UK) and sequenced within the Teagasc 454 Sequencing Platform. Raw sequences were quality trimmed using the Qiime Suite of programmes (Caporaso et al., 2010); any reads not meeting the quality criteria of a minimum quality score of 25 and sequence length shorter than 150 bps for 16S amplicon reads were discarded. Trimmed FASTA sequences were then BLASTed (Altschul et al., 1997) against a previously published 16S-specific database (Urich et al., 2008) by using default parameters. The resulting BLAST output was parsed by using MEGAN (Huson et al., 2007). MEGAN assigns reads to NCBI taxonomies by using the Lowest Common Ancestor algorithm. A bit-score of 86, as previously used for 16S ribosomal sequence data (Urich et al., 2008), was used within MEGAN for filtering the results before tree construction and summarisation. Phylum, family and genus counts for each subject were extracted from MEGAN. Sequences were clustered into operational taxonomical units (OTUs), chimera checked and aligned using the default pipeline within Qiime (Kuczynski et al., 2011); subsequently alpha and beta diversities were generated. A phylogenetic tree was calculated using the FastTree software (doi:10.1093/molbev/msp077). Resulting Principal Coordinate analysis was visualised using KING.

Detection of Bifidobacterium

To quantify *Bifidobacterium* in murine faecal pellets, a plasmid standard was first created. A PCR fragment from within the 16 rRNA gene of *B. longum* subsp. *infantis* ATCC 15697 was generated using species-specific primers. The primers used were as follows: forward 5'- CTCCTGGAAACGGGTGG- 3' and reverse 5'- GGTGTTCTTCCCGATATCTAC- 3', according to Matsuki et al. (2002). Purified amplicons were cloned into the pCR®2.1-TOPO vector using the TOPO-TA cloning system (Invitrogen, Life Technologies, Carlsbad, California) in accordance with manufacturer's instructions. The complete vector was transformed into chemically competent TOP-10 *Escherichia coli* cells (Invitrogen, Life Technologies, Carlsbad, California) and harvested on LB media containing 50 µg/ml ampicillin. The cloned amplicon was confirmed by restriction analysis and DNA sequencing. Quantitative real-time PCR (qPCR) standards were prepared following the linearization of plasmid DNA with *Kpn*I restriction enzyme and quantification using a Nanodrop ND-1000 (Thermo Fisher Scientific Inc, USA). A standard curve was then generated via a series of dilutions from 10^9 to 10^2 copies/µl DNA. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics GmbH, Mannheim, Germany) was used for quantification according to the manufacturer's instructions. Each PCR reaction contained 10 µl Sybr green master mix, 1 µl of both forward and reverse primer (10 pmol), 1 µl of DNA and was made up to a final volume of 20 µl with nuclease free distilled water. The PCR conditions were as follows: denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 5 sec and elongation 72°C for 25 sec. To quantify by qPCR, it was necessary to correct copy number of 16S rRNA gene to cfu/ml. Based on the amplification of

DNA, which was isolated from *Bifidobacterium* cultures of known cfu/ml against a standard curve generated from the Lightcycler 480 (copy number/µl), a formula was used to correct copy number values for cfu/ml as outlined in Quigley et al. (2013).

[(C/µl)*(TV)]/TCN x T cfu/ml = cfu/ml(S)

Where; C/µl = Copy number/µl, TV = Template volume, TCN = Total copy number of the standard used, T cfu/ml = Total cfu/ml of standard used and cfu/ml(S) = cfu/ml of test sample.

Statistical Analysis

Data are presented as mean values ± standard errors of the mean (SEM). Statistical analysis was performed by ANOVA and the Student t-test (Graph-Pad Software, San Diego, CA, USA). For microbiota analysis, SPSS was utilised to establish non-parametric significance using the Mann-Whitney and Kruskal-Wallis tests. $P<0.05$ was considered as statistically significant.

6.4 RESULTS

Oligosaccharides effects on body and organs weight, caecal secretory IgA, SCFA composition and serum cytokine levels

BALB/c mice body weight did not differ significantly throughout the study as a result of dietary intervention with BMO, P95 or 6'SL compared to unsupplemented control. Following 3 weeks of dietary supplementation with oligosaccharides no differences on liver, caecum, or small intestine weight were observed (Table 6.1).

Caecal secretory IgA was assessed at day 21 and levels were reduced following treatment with P95 (62%; $p<0.01$) and BMO (61%; $p<0.05$), whereas 6'SL supplementation showed a similar trend but did not reach statistical significance (Fig. 6.1).

To examine the effects of oligosaccharide supplementation on caecal SCFA, levels of butyrate, propionate, and acetate were determined (Fig. 6.2). P95 and BMO supplementation did not influence SCFA content when compared with unsupplemented control. 6'SL supplementation resulted in reduced levels of butyrate by 41% ($p<0.01$) in comparison with the control, while levels of propionate and acetate were unaltered in caecal contents.

Serum was isolated from collected blood and analysed for cytokine levels (Table 6.2), and results indicated that dietary supplementation with both BMO and P95 were not associated with changes in the levels of IL-1β, IL-10, IFN-γ, or TNF-α. P95 treatment was associated with a reduction in IL-12 (18.1%; $p<0.01$) and IL-6 (21.1%; $p<0.05$) versus the unsupplemented control. BMO supplementation resulted in reduced levels of IL-12 (11.9%; $p<0.05$) versus the unsupplemented control, and increased mouse keratinocyte-derived chemokine (mKC) levels in relation to 6'SL (42.7%; $p<0.01$).

Table 6.1 Murine body and organs weights by treatment group

	Control	**P95**	**BMO**	**6'SL**
Final body weight (g)	19.3 ± 1.0	19.4 ± 1.2	19.1 ± 1.1	19.5 ±0.9
Liver weight (g)	0.77 ± 0.16	0.82 ± 0.15	0.88 ± 0.16	0.89 ± 0.09
Caecal tissue weight (g)	0.06 ± 0.02	0.06 ± 0.02	0.07 ± 0.02	0.06 ± 0.01
Small intestine tissue weight (g)	0.40 ± 0.09	0.39 ± 0.06	0.37 ± 0.09	0.32 ± 0.07

All values are means ± SEMs.

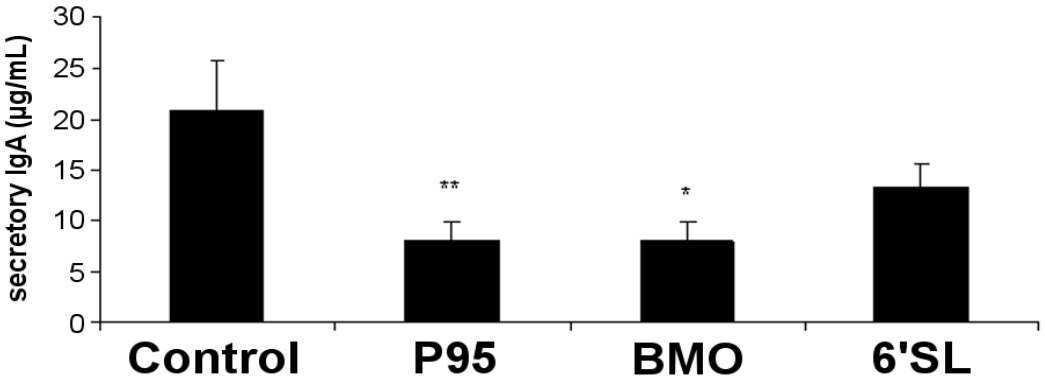


Figure 6.1 Caecal secretory IgA measured following 21 days of oligosaccharide supplementation. *p<0.05; **p<0.01 (ANOVA followed by post hoc Tukey's multiple comparisons tests).

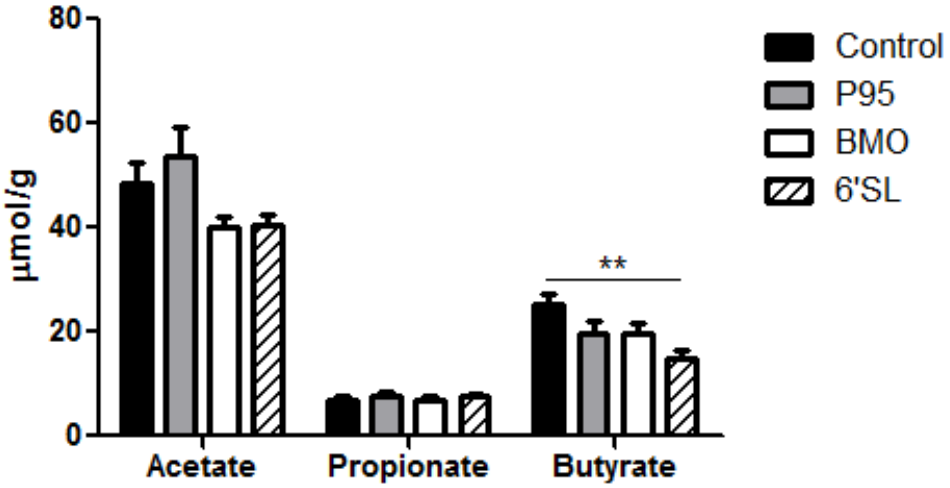


Figure 6.2 Caecal short-chain fatty acids measured following 21 days of oligosaccharide supplementation. **p<0.01. (ANOVA followed by post hoc Tukey's multiple comparisons tests).

Table 6.2 Serum cytokines (pg/ml) detected following 21-day oligosaccharide supplementation

	Control	P95	BMO	6'SL
IFN-γ	4.4 ± 0.4	3.9 ± 0.2	3.9 ± 0.2	4.3 ± 0.3
IL-10	55.3 ± 3.0	54.7 ± 2.6	55.3 ± 1.6	56.5 ± 1.9
IL-12	110.6 ± 3.3	90.6 ± 2.3**	97.4 ± 2.7*	99.6 ± 4.8
IL-1β	15.6 ± 0.8	14.7 ± 1.9	15.6 ± 0.9	13.3 ± 1.1
IL-6	57.7 ± 3.4	45.5 ± 2.3*	54.2 ± 2.7	55.7 ± 2.8
mKC	103.2 ± 7.3	92.7 ± 6.8	121.3 ±10.0^	85.0 ± 4.5^
TNF-α	16.2 ± 1.2	12.9 ± 0.3	14.7 ± 0.7	14.1 ± 0.8

All values are means ± SEMs. Statistical significance was determined using one-way ANOVA followed by post hoc Tukey's multiple comparisons tests; *p <0.05; **p<0.01; ***p<0.001 in relation to control; ^ denotes significance between two treatment groups.

Effects of oligosaccharide supplementation on murine intestinal microbiota

At the conclusion of the 21-day trial, the relative proportions of gut bacteria among the treatment groups were assessed by DNA sequencing (Roche-454 titanium, Roche Diagnostics Ltd, West Sussex, UK) of the V4-region of 16S rRNA amplicons derived from total faecal DNA and bioinformatic analysis. Sequencing resulted in a total of 421,986 reads with an average of 5540.481 reads per animal. Reads were quality trimmed and clustered into operational taxonomical units (OTUs) based on 98% sequence identity. Subsequently alpha diversity was estimated using 5 different metrics (Shannon, Simpson, Chao1, Phylogenetic diversity and observed species). There were some temporal differences in diversity, with a reduction in diversity over time (phylogenetic diversity p=0.019; observed species p=0.032; data not shown). However, no significant differences were observed in alpha diversity between the treatment groups at day-21 only. Rarefaction curves for each group were at or approaching parallel, indicating that sufficient depth of sequencing was undertaken. Principal coordinate analysis, based on unweighted Unifrac distances illustrated that the control and P95 samples cluster closer to each other than to the 6'SL and BMO samples (Fig. 6.3).

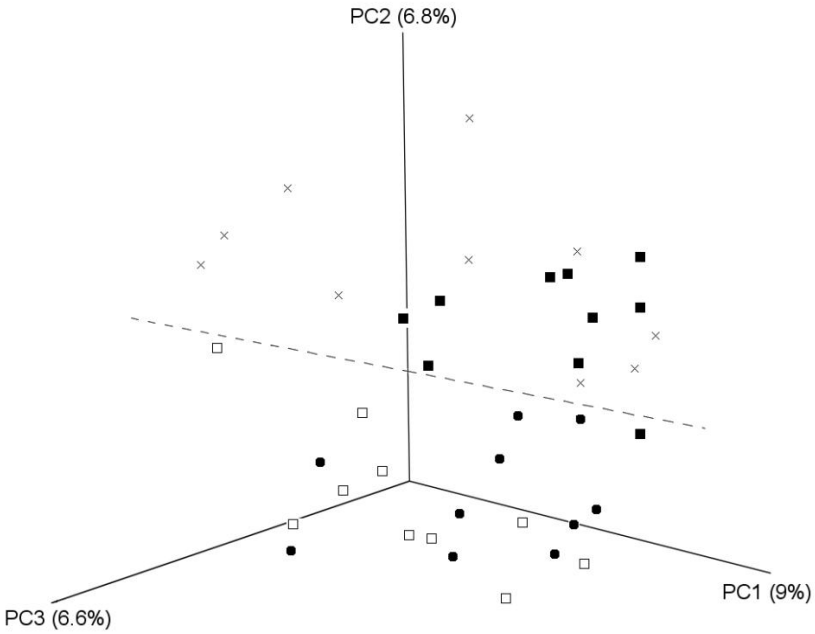


Figure 6.3 Unweighted Unifrac analysis of oligosaccharide treatment clustering on microbial composition. Oligosaccharide treatments are denoted as follows: Control – black circle; P95 – white square; BMO – black square; and 6'SL – x symbol.

Consistent with previous studies, *Firmicutes* and *Bacteroidetes* were found to be the predominant gut microbiota in the BALB/c caecal content at the phylum level (Pedron et al., 2012) (Fig. 6.4). *Proteobacteria, Deferribacteres, Actinobacteria, Viridiplantae*, and Candidate division TM7 contribute to the remaining proportion (Fig. 6.4). A comparison of intestinal microbiota composition between unsupplemented control and oligosaccharide-treated mice revealed multiple alterations. At the phylum level, P95 supplementation was associated with a significant reduction in *Cyanobacteria* ($p=0.003$). BMO supplementation was associated with an increase in the relative proportion of Candidate division TM7 ($p=0.002$) and decreases in *Actinobacteria* ($p=0.0255$) and *Cyanobacteria* ($p=0.003$) compared to the unsupplemented control, while administration of 6'SL was associated with a decrease in the relative proportion of *Actinobacteria* ($p=0.017$) (Fig. 6.4).

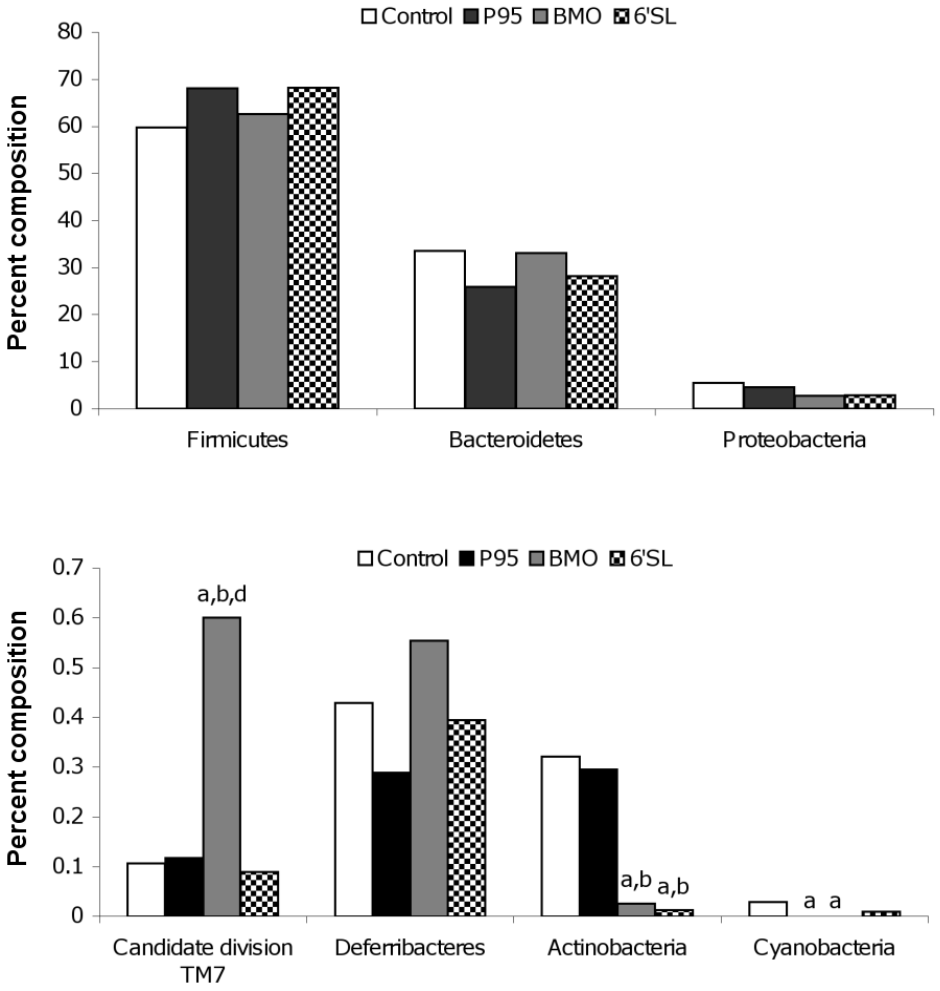


Figure 6.4 Microbial composition at the phylum level. Top panel refers to microbial compositions >2%, bottom panel refers to <1%.[a] denotes significance in comparison to control; [b] denotes significance in comparison to P95; [c] denotes significance in comparison to BMO; and [d] denotes significance in comparison to 6'SL. Significance is determined as $p<0.05$.

At the family level, supplementation with P95 was associated with increased *Enterobacteriaceae* ($p<0.0001$), *Enterococcaceae* ($p=0.016$), *Alcaligenaceae* ($p=0.018$), a decrease in *Porphyromonadacaea* ($p=0.04$) in caecal content, and the non-detection of *Moraxellaceae* and *Vibrionaceae* in pair-wise comparisons against the unsupplemented control (Fig. 6.5). Supplementation with BMO resulted in a significant reduction in numbers of 3 families; *Veillonellaceae* ($p=0.003$), *Succinivibrionaceae* ($p=0.001$), *Peptostreptococcaceae* ($p=0.038$), an increase in *Alcaligenaceae* ($p=0.006$), and non-detection of *Moraxellaceae* and *Vibrionaceae*. 6'SL supplementation was associated with a significant reduction in numbers of *Moraxellaceae* ($p=0.003$) and *Porphyromonadaceae* ($p=0.016$) and non-detection of *Prevotellaceae* ($p=0.035$), *Vibrionaceae* ($p=0.003$), *Veillonellaceae* ($p=0.003$), and *Succinivibrionaceae* ($p=0.001$). Moreover, *Lactobacillaceae* numbers were significantly reduced in the 6'SL-treated mice, compared with the unsupplemented control and P95 supplemented animals ($p=0.001$ and 0.005 respectively).

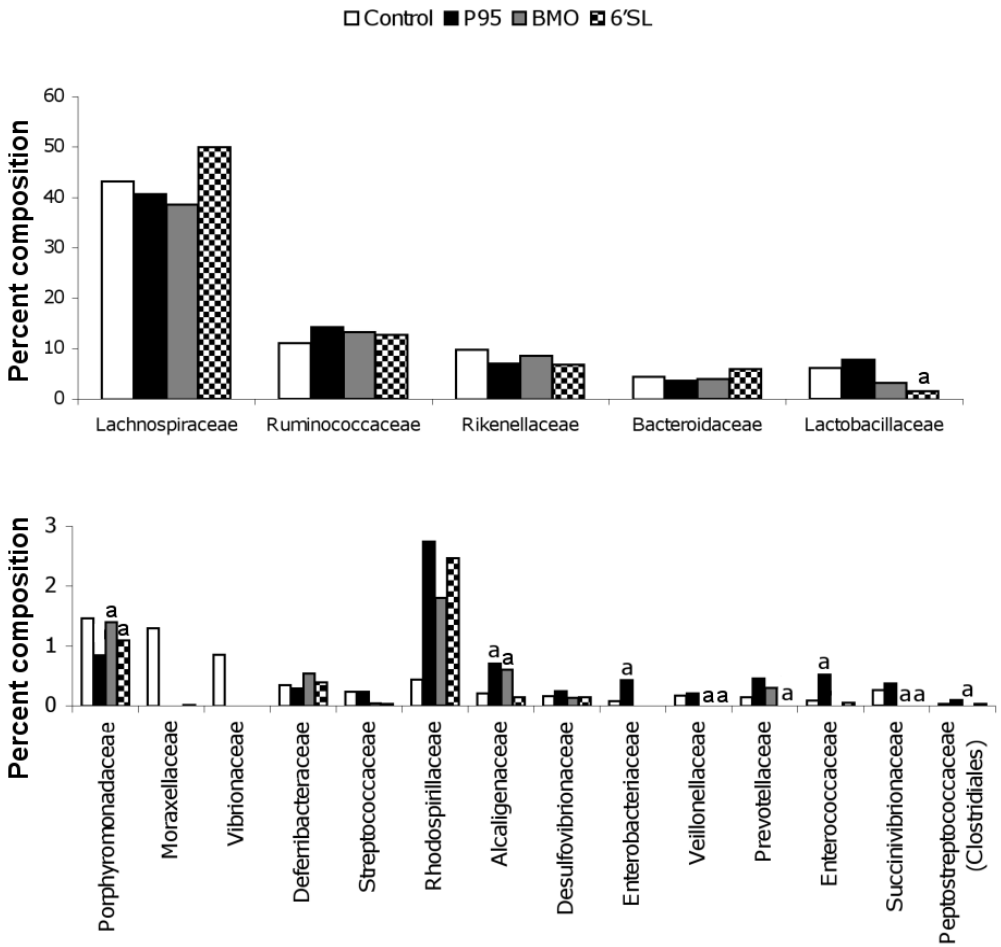


Figure 6.5 Microbial composition at the family level. [a] denotes significance in comparison to control; [b] denotes significance in comparison to P95; [c] denotes significance in comparison to BMO; and [d] denotes significance in comparison to 6'SL. Significance is determined as $p<0.05$.

In pair-wise comparisons versus the control, P95 was associated with significantly increased numbers of *Enterobacteriaceae* spp. ($p<0.0001$), *Pasteurellales* ($p<0.0001$), *Sutterella* ($p=0.018$), *Parabacteroides* ($p=0.004$), *Ruminococcaceae* Incertae Sedis ($p<0.0001$), *Enterococcus* ($p=0.030$), and *Erysipelotrichales* Incertae Sedis ($p=0.001$), and decreases in *Lachnospiraceae* Incertae Sedis ($p=0.017$), uncultured *Lachnospiraceae* ($p=0.003$), *Marvinbryantia – Bryantella* ($p<0.0001$), and *Acetitomaculum* ($p=0.008$) (Fig. 6.6). BMO was associated with a significant increase in *Sutterella* ($p=0.006$) and decreases in *Anaerobiospirillum* ($p=0.001$), uncultured *Lachnospiraceae* ($p=0.016$), *Acetitomaculum* ($p=0.008$), *Anaerovibrio* ($p=0.018$), and *Ruminococcaceae* Incertae Sedis ($p=0.028$). 6'SL was associated with an increase in *Parabacteroides* ($p=0.008$) and *Ruminococcaceae* Incertae Sedis ($p=0.002$), and the largest number of genus decreases; *Anaerobiospirillum* ($p=0.001$), *Lachnospiraceae* Incertae Sedis ($p=0.049$), uncultured *Lachnospiraceae* ($p=0.009$), *Marvinbryantia – Bryantella* ($p<0.0001$), *Acetitomaculum* ($p=0.008$), *Anaerovibrio* ($p=0.018$), and *Lactobacillus* ($p=0.001$). Notably, members of *Ruminococcaceae* Incertae Sedis were significantly increased in the P95 and 6'SL supplemented groups versus the unsupplemented control ($p<0.0001$ and $p=0.002$, respectively), while decreased in the BMO-supplemented mice ($p=0.028$). Interestingly, members of *Lactobacillus* were significantly reduced in 6'SL supplemented group, compared with P95 and unsupplemented controls ($p=0.006$ and 0.001 respectively). Moreover, qPCR analysis of faecal pellets revealed absence of *Bifidobacterium* in samples taken at day 0 and day 21, with no differences among groups.

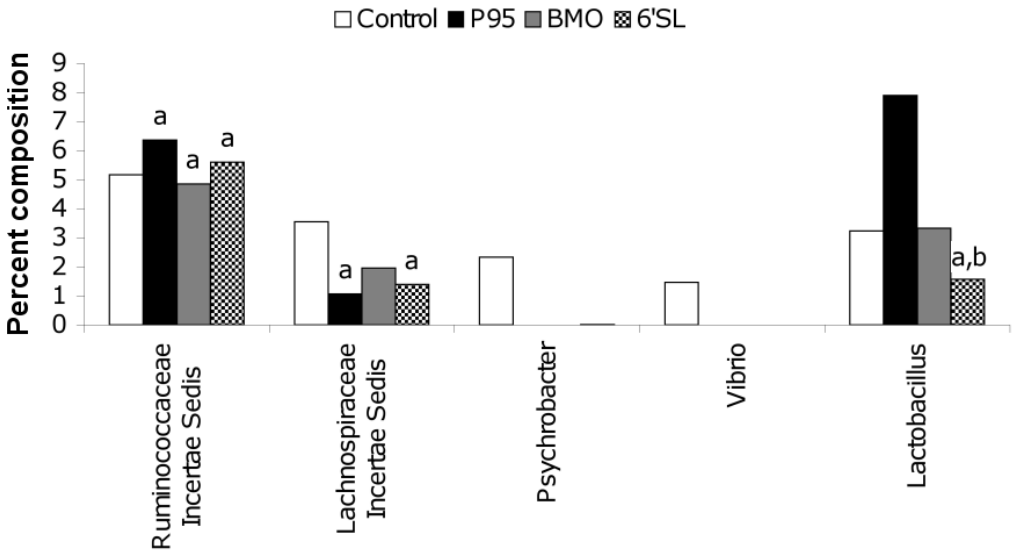


Figure 6.6 Microbial composition at the genus level. [a] denotes significance in comparison to control; [b] denotes significance in comparison to P95; [c] denotes significance in comparison to BMO; and [d] denotes significance in comparison to 6'SL. Significance is determined as $p<0.05$.

6.5 DISCUSSION

Dairy whey streams present an abundant resource from which to mine biologically active bovine oligosaccharides, offering an attractive alternative to HMO as functional food ingredients (Zivkovic & Barile, 2011). In the current study, we report the influence of carbohydrate supplementation (1 mg/g BW) of P95, an enriched BMO powder, or 6'SL on the composition of the murine microbiota.

Following the 3-week administration period, individual carbohydrate interventions resulted in clustering of bacterial species detected, with similar effects on bacterial composition among the control and P95, and BMO and 6'SL. In the current study, P95 demonstrated the ability to modulate the faecal bacterial populations of mice, promoting the growth of specific families, while reducing others. P95 significantly increased the relative proportions of *Enterococcaceae* and *Enterobacteriaceae*, which have been demonstrated to be associated with the intestinal microbiota of preterm infants (Arboleya et al., 2012; Hoy et al., 2000; Magne et al., 2006). The current study findings are in contrast to a prior study in which supplementation with oligosaccharides resulted in reduced concentrations of *Enterococcaceae* in the caecum (Pan et al., 2009), although this may be attributable to differences naturally present between the caecal and faecal microbiota (Marteau et al., 2001). Of interest, P95 supplementation was associated with non-detectable numbers of the *Moraxellaceae* and *Vibrionaceae* families of bacteria, both of which include notable human pathogens, *Moraxella catarrhalis* and *Vibrio cholerae*, respectively.

Overall, BMO and 6'SL supplementations were generally associated with reductions in less favourable bacteria. At the phylum level, BMO reduced the

proportion of Candidate division TM7, a currently uncultivable bacteria which has previously been isolated from periodontitis (Paster et al., 2001) and is thought to rely on group interactions within biofilms for survival (Wade, 2002). BMO was also associated with a decrease in *Actinobacteria*, a diverse phylum to which the family *Bifidobacteriaceae* belong (Ventura et al., 2007). BMO treatment was also associated with the absence of *Moraxellaceae* and *Vibrionaceae*. In contrast to P95, BMO reduced *Enterococcaceae*, as well as *Enterobacteriaceae*, which are both predominant in the flora of preterm human infants (Arboleya et al., 2012). Of the three treatments, BMO was the sole carbohydrate source to significantly reduce the *Peptostreptococcaceae* family.

In contrast to P95 supplementation, 6'SL significantly reduced the proportion of *Actinobacteria* at the phylum level. At the family level, 6'SL reduced *Moraxellaceae*, *Lactobacillaceae*, and *Porphyromonaceae*, while 4 families of bacteria were no longer detected. The inability to detect *Prevotellaceae* was unique to treatment with 6'SL. *Prevotellaceae* are commonly linked to periodontal disease and abscesses along with bacteremia and upper respiratory tract infections (Tanaka et al., 2008). Although investigation into the mechanisms of action was not carried out, the fact that 6'SL did not promote the growth of any bacterial families may suggest this molecule is acting in an anti-infective manner or as a decoy molecule, as reported for acidic oligosaccharides in previous studies (Coppa et al., 2006; Hester et al., 2013; Thomas & Brooks, 2004).

The fact that indigenous *Bifidobacterium* were undetectable through both real-time PCR analysis and 454 pyrosequencing in baseline and 3-week faecal samples limited our ability to comprehensively assess the prebiotic nature of the

supplemented oligosaccharides, as prebiotic supplementation is often associated with increases in *Bifidobacterium* and *Lactobacillus* (Gibson et al., 1995; Kaplan & Hutkins, 2000; Roberfroid, 2000). *Lactobacillus* typically comprise 0.2-1% of the total microbiota in infant faecal samples (Mueller et al., 2006), and display adaptations to specific ecological niches. Isolates from milk exhibit an adaptation for growth on lactose, while those isolated from the intestinal niche often encode pseudogenes associated with sugar internalization and metabolism (Makarova et al., 2006; Ventura et al., 2007). While FOS have been reported to act as fermentable substrates for *Lactobacillus* (Gänzle & Follador, 2012), FOS supplementation has been demonstrated to reduce levels of *Lactobacillus* in conventionally raised C57Bl/6J mice (Pachikian et al., 2011), while other studies have reported limited or no promotion of growth (Campbell et al., 1997; Petersen et al., 2010). Furthermore, digestion of sialyllactose and mixtures of complex HMO lie largely beyond the scope of their enzymatic activities (Idota et al., 1994; Schwab & Gänzle, 2011), possibly accounting for the lack of effect of the supplemented oligosaccharides on the *Lactobacillus* numbers.

While the murine model provides an accessible and comparatively cheap *in vivo* intestinal model for the study of prebiotic substances, there are several inherent and important differences to that of the human environment. The commensal flora of mice is adapted to the murine intestinal environment and daily diet (utilizing different nutrients, binding sites, and adhesins), and may respond differently to the provision of human/bovine oligosaccharides when compared to the human microbiota. For example, *Lactobacillus* likely employ different adhesion mechanisms depending on host tissue, given that a widespread mucus-adhesion

protein of *Lactobacillus* will bind to human, rabbit, and guinea pig tissues, yet does not adhere to the mucus of murine intestinal biopsies (Coïc et al., 2012), indicating significant differences in the murine mucosal composition. Despite numerous studies demonstrating the presence and dominance of *Bifidobacterium* in human neonates, the microbiota in mice depends on the particular strain used (e.g. C57BL/6 or BALB/c) and the environment in the housing facilities (Buddington et al., 2000). This may allow for variable inter-study intestinal microbiota composition, and in some cases, an absence or low levels of *Bifidobacterium* (Hasegawa et al., 2010).

The predominant bacterial fermentation end-products in the large bowel are butyrate, acetate, and propionate, which result from the breakdown of oligosaccharides, polysaccharides, proteins, peptides, and glycoproteins (Macfarlane & Macfarlane, 2002). The production of SCFA relies on the number and type of bacteria present, as well as their associated ability to metabolize relevant precursors. *Clostridium*, *Eubacterium* and *Ruminococcus* genera are associated with the production of butyrate, while acetate is attributed to lactic acid producing bacteria of the *Lactobacillus* and *Bifidobacterium* genera (Gourbeyre et al., 2011). In the current study, levels of SCFA were generally unaffected, with the exception of 6'SL treatment, which resulted in decreased levels of butyrate. Faecal butyrate concentrations in breast-fed infants have been found to be lower than their formula-fed counterparts (Knol et al., 2005), potentially highlighting a similarity between breast-feeding and supplementation with 6'SL. This reduction in butyrate may be the result of elimination of a family or combination of families of bacteria which are involved in the production of butyrate. 6'SL and P95 treatment

were associated with increased proportions of *Ruminococcus*, with only the 6'SL treatment reducing butyrate concentrations. In addition, 6'SL significantly reduced the proportion of *Lactobacillus,* but did not affect caecal acetate concentrations, leading to the inference that the *Lactobacillus* populations did not significantly contribute to the observed levels of acetate in this particular study. It should be noted that in the current study caecal flow and volume were not assessed. An increased volume or rate of caecal transit could explain the lack of impact or reduced concentrations of SCFA detected (Cheng et al., 1987; Peuranen et al., 2004).

Blood serum cytokine levels were assessed to determine whether oligosaccharide supplementation would modulate the murine systemic immune response. Supplementation with oligosaccharides did not result in significant changes in the serum concentrations of the pro-inflammatory cytokines – IL-1β, IFN-γ, and TNF-α, or the anti-inflammatory cytokine, IL-10. The production of these cytokines have been demonstrated to be enhanced in response to probiotic bacteria (reviewed in Gill, 2003), therefore the lack of significant change in their levels may be explained by the fact that *Bifidobacterium* were found to be absent during the present study and *Lactobacillus* were significantly reduced or otherwise unaffected following oligosaccharide supplementation. The stimulation of intestinal epithelial cells by pathogens is associated with the production of TNF-α and IL-8 (Kagnoff & Eckmann, 1997), both of which were unaffected by oligosaccharide supplementation. Therefore, despite the fact that it appears that probiotic-associated cytokines were unaffected, there was also a lack of production of

pathogen-associated cytokines, possibly indicating that the oligosaccharide supplements did not result in an increased pathogenic burden.

P95 supplementation led to significantly decreased levels of IL-12 and IL-6, while BMO decreased IL-12 levels. IL-12 is a pro-inflammatory cytokine (Shida et al., 2002), while IL-6 may act as a pro- or anti-inflammatory cytokine, which is necessary to fight infection of *Streptococcus pneumoniae* in a murine model (van der Poll et al., 1997), thereby demonstrating both positive and negative properties associated with the two interventions. mKC is the murine analogue of human IL-8, which is associated with inflammation and is released in response to pathogenic stimulation of intestinal epithelial cells *in vitro* (Gill, 2003). 6'SL significantly decreased levels of mKC in relation to the BMO group, demonstrating the potential to modulate inflammation. As the exact mechanism of action of the oligosaccharides in this particular study is unknown, further studies would prove beneficial.

The primary role of secretory IgA is in the prevention of pathogenic attachment to host tissues (Kagnoff, 1993). Furthermore, the induction of IgA antibodies and $CD4^+$ regulatory T cells mediate oral tolerance to intestinal contents (Mowat, 2003). In the current study, both P95 and BMO resulted in significant decreases in caecal secretory IgA, while 6'SL did not differ significantly from the unsupplemented control. In contrast to our current findings, previous studies typically report increased levels following the administration of prebiotics (Seifert & Watzl, 2007). Ingestion of RaftiloseR Synergy1 (a commercial blend of inulin and oligofructose) resulted in increased levels of caecal secretory IgA in a rodent model (Roller et al., 2004). Hosono et al. (2003) found a transient increase in faecal IgA

levels at week two, returning to control levels at weeks three and four, while Nakamura et al. (2004) found elevated levels throughout the small and large intestine, though feeding the mice nearly ten times as much oligofructose (50g/kg diet) as used in the current study. Although 6'SL supplementation did not induce a significant change, BMO and P95 reduced caecal levels of secretory IgA. Pathogens and probiotic bacteria are both able to stimulate the production of IgA, though typically differing on whether they are T-cell dependent or independent, respectively (Cerutti & Rescigno, 2008). The fact that P95 and BMO supplementation both significantly reduced the levels of caecal secretory IgA leads to several different hypotheses. Though many scenarios are possible, the most probable situation in agreement with the sequencing data is that the BMO and P95 treatments are reducing either a particular bacterial group responsible for the production of IgA, or the overall bacterial numbers in the caecum, resulting in a decreased concentration of secretory IgA. Given the current findings, it appears likely that 6'SL supplementation does not produce a significant effect on intestinal and oral tolerance due to its negligible effects on levels of secretory IgA. Another hypothesis is that the amount of oligosaccharide used in this study was not enough to stimulate the production of IgA.

The present study has demonstrated the gut-modulatory properties of oligosaccharides in a murine model. While BMO and 6'SL treatments have resulted in a significant reduction in proportions of less favourable bacterial families, next steps will include determining their effects upon probiotic bacterial strains, with specific emphasis on *Bifidobacterium* which were absent in the current study. Future studies would benefit from the use of mice colonized with a human-derived

microbiota, a synbiotic treatment (provision of *Bifidobacterium* and oligosaccharides) as implemented in previous studies (Bielecka et al., 2002; Furrie et al., 2005), or higher doses of oligosaccharides/longer period of supplementation to allow for a better interpretation of the interplay between supplemented oligosaccharides, their effects on pathogens, probiotic commensals, and overall host health.

6.6 CONCLUSIONS

The results from this study demonstrate the ability of BMO and 6'SL supplementation to significantly reduce or eliminate families of bacteria which potentially harbour pathogenic organisms. Additionally, supplementation with 6'SL reduced caecal butyrate, which is primarily produced by *Clostridium*, *Eubacterium* and *Ruminococcus*, resembling the SCFA pattern commonly found in faeces of breast-fed infants. Finally, caecal secretory IgA was not significantly impacted by 6'SL, demonstrating that supplementation with this specific oligosaccharide, which is naturally found in human and bovine milk, likely would not impact oral tolerance of the host. Despite the differences and limitations of the murine model, this study has demonstrated that ingestion of BMO is a safe and effective approach to modulate populations of the intestinal microbiota.

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Chapter 7

General Discussion

The adult intestinal microbiota comprises a microbial ecosystem of approximately 100 trillion organisms (Qin et al., 2010), the majority of these microorganisms being bacteria (Sommer & Backhed, 2013) with a collective genome containing at least one hundred times as many genes as the human genome (Barberan & Mine, 2013). This bacterial community has huge metabolic activity (Bocci, 1992; O'Hara & Shanahan, 2006), producing bioactive compounds that may influence microbial community structure (McHardy et al, 2013) and may directly or indirectly affect the regulation of multiple host metabolic pathways (Roshchina, 2010), and have a role in mood disorders and chronic diseases (Forsythe et al., 2010). Moreover, gut bacteria are responsible for the metabolism and energy harvest from nondigested nutrients entering the colon, generating a wide range of products, mainly SCFA (Macfarlane & Macfarlane, 2003).

SCFA are among the most important gut microbial products, affecting a range of host processes from gut motility (Fukumoto et al., 2003; Grider & Piland, 2007) to modulation of inflammation (Maslowski et al., 2009), fat metabolism (Ge *et al*., 2008; Hong et al., 2005) and appetite (Karaki et al., 2008; Samuel et al., 2008). In Chapter 2, we optimized a gas chromatograpic method previously described by Tangerman & Nagengast (1996) for analysing SCFA in human and animal intestinal samples. A high polarity capillary column specific for aqueous samples, with a programmed oven temperature and a narrow glass liner to simulate on-column injection was used to create a rapid and efficient assay for SCFA analysis with better resolved chromatograms. The optimized method proved to be efficient for the detection of faecal/caecal acetate, propionate, isobutyrate and butyrate with good recovery and reproducibility rates, provided that sample

storage at -20^{o}C did not exceed 2 weeks. The method was then applied for quantifying SCFA production in animals as a response to dietary interventions with *t*10, *c*12 conjugated linoleic acid (CLA) (Chapter 3), probiotics (Chapter 4) and prebiotics (Chapter 6).

The gut microbiota play an important role in host health, being involved in nutritional, immunological and physiological functions. Recent evidence from animal and human studies indicates that changes in gut microbiota composition may be involved in inflammatory bowel disease (IBD) (Maynard et al., 2012), necrotizing enterocolitis (NEC) (AlFaleh et al., 2012) and extra-intestinal disorders such as obesity (Backhed et al., 2004; Ley et al., 2006; Turnbaugh et al., 2006), insulin resistance (Cani et al., 2008) and non-alcoholic fatty liver disease (NAFLD) (Dumas et al., 2006). In Chapter 3, we investigated the effects of dietary *t*10, *c*12 CLA supplementation on intestinal microbiota composition and production of short chain fatty acids in mice. *T*10, *c*12 CLA has been shown to affect body composition by reducing body fat and increasing lean body mass in mice. However, in most studies using mice, this anti-obesity effect has been accompanied by such adverse effects as hepatic steatosis and hyperinsulinemia (Clément et al., 2002; Liu et al., 2012). Recent studies indentified the gut microbiota as an environmental factor with an important role in host fat metabolism and the development of hepatic steatosis (Le Roy et al., 2012). Indeed, we found that dietary *t*10, *c*12 CLA supplementation was associated with altered gut microbiota composition with greater proportions of *Bacteroidetes* ($p=0.027$), when compared with unsupplemented controls. *Bacteroidetes* are known to produce LPS and induce inflammation, whereas *Porphyromonadaceae,* also observed at higher proportions

(p=0.002), have previously been linked with negative effects on lipid metabolism and induction of hepatic steatosis (Henao-Mejia et al., 2012). Moreover, SCFA levels were altered, with higher levels of caecal acetate, propionate and isobutyrate in mice receiving *t*10, *c*12 CLA supplementation. These data indicate that the mechanism of dietary *t*10, *c*12 CLA on lipid metabolism in mice may be at least partially mediated by alterations in gut microbiota composition and functionality. Future studies comparing the effects of dietary *t*10, *c*12 CLA with dietary *c*9, *t*11 CLA and other trans-fatty acids on human microbiota composition would give important information about the mechanisms of action of these fatty acids on metabolic pathways, especially considering that the severe collateral effects seen in rodents while feeding *t*10, *c*12 CLA have not yet been confirmed in humans.

The maintenance of the homeostasis in the gut ecosystem is essential for health and diet has been shown to be the main environmental factor contributing to human gut microbiota modulation (De Filippo et al., 2010; Wu *et al.*, 2011). Dietary strategies such as probiotics and prebiotics may be used to impact positively on microbiota composition and consequently, host health. In Chapter 4 we compared the effect of dietary supplementation with CLA-producing *Bifidobacterium breve* strains on fat distribution and composition and on the composition of the gut microbiota in mice. Both *B. breve* DPC 6330 and *B. breve* NCIMB 702258 survived gastrointestinal transit and were detected in faecal samples (~1.1 x 10^6 CFU/g faeces and 8.2 x 10^6 CFU/g faeces, respectively). After 8 weeks supplementation, visceral fat mass and brain stearic acid, arachidonic acid (AA), and docosahexaenoic acid (DHA) were higher in mice supplemented with *B. breve* NCIMB 702258 than in mice receiving no supplementation ($p<0.05$), but these

changes were not observed in mice supplemented with *B. breve* DPC 6330. Caecal propionate was significantly higher in mice fed *B. breve* DPC 6330 and *B. breve* NCIMB 702258 than in unsupplemented mice ($p<0.05$). However, administration of these strains altered the composition of the gut microbiota differently at the phylum, family, and genus levels. PCoA analysis showed that mice fed *B. breve* DPC 6330 had a more divergent clustering pattern and were separated from the unsupplemented mice and from mice fed *B. breve* NCIMB 702258, suggesting a greater effect of *B. breve* DPC 6330 on murine microbiota composition. The study showed that the response of fatty acid metabolism to administration of bifidobacteria is strain-dependent and furthermore highlighted the importance of intra strain differences in modifying the gut microbiota composition of the host. Potential future studies could involve the investigation of the contrasting effects of *B. breve* NCIMB 702258 and *B. breve* DPC 6330 administration on human microbiota composition. Moreover, since ARA and DHA are neuroprotective agents, involved in the improvement of cognitive function including memory and learning (Henriksen et al., 2008; Yurko-Mauro et al., 2010), and administration of *B. breve* NCIMB 702258, as outlined in this chapter, led to increased concentrations of both fatty acids in brain, it would be interesting to evaluate its effects in humans with psychiatric conditions.

In Chapter 5, we investigated metabolic and behavioural effects of dietary administration of GABA-producing *Lb. brevis* DPC 6108 and pure GABA (as commercial powder). GABA is the major inhibitory neurotransmitter in the central nervous system (CNS) and plays a role in the sensation of pain and anxiety (Erdo, 1992). In the endocrine pancreas, GABA is produced by β-cells and modulates

insulin and glucagon secretions (Bailey et al., 2007; Bonaventura et al., 2007; Dong et al., 2006). Using healthy animals, we observed a significant increase ($p<0.05$) in serum insulin in rats receiving *Lb. brevis* DPC 6108 for 5 weeks, compared with placebo controls. Following on from the observation that ingested GABA-producing *Lb. brevis* DPC 6108 led to increased serum insulin, we investigated the potential of this probiotic for prevention of the onset of diabetes in streptozotocin (STZ) induced type-1-diabetic (T1D) rats, in comparison to dietary GABA. The dietary interventions were not effective and diabetic animals developed an overt basal hypoinsulinemia and extreme hyperglycemia compared with animals receiving placebo. However, similar to previous observations in studies investigating the benefits of probiotic administration in STZ-diabetic animals (Davari et al., 2013; Lin et al. , 2013), probiotic *Lb brevis* DPC 6108 caused a significant decrease in glucose levels in diabetic rats when compared with diabetic controls (23%; $p<0.05$). Moreover, although healthy animals receiving GABA powder or *Lb. brevis* DPC 6108 were less anxious in comparison with the placebo control group, they did not ameliorate the anxiety effect caused by diabetes. However, the beneficial effect of GABA therapy has been shown in other models of diabetes, such as the non-obese diabetic (NOD) mouse model of T1D and high-fat diet (HFD)-fed mouse model of T2D (Soltani et al., 2011; Tian, Dang, Kaufman, et al., 2011; Tian, Dang, Yong, et al., 2011). Considering that in all these studies, the protective effect of GABA was related to the modulation of the immune response present in diabetes, we then hypothesised that induction of diabetes with a single-dose of streptozotocin may not be appropriate for studying GABA treatment, as in this method β-cells are rapidly and completely destroyed, with absence of an immune response. Future

studies are required in order to determine the mechanisms by which orally administered *Lb. brevis* DPC 6108 mediated the changes in host levels of insulin and glucose. Moreover, administration of *Lb. brevis* DPC 6108 to animal models of diabetes involving an autoimmune response would give better insights on the potential effects of this strain on the prevention or reversion of diabetes.

Breast milk is an important dietary source of nutrients for the healthy growth and development of infants and promotes optimum microbiota development. It contains a complex and diverse mixture of oligosaccharides (Barile & Rastall, 2013; Zivkovic et al., 2011) that may exert anti-adhesive effects, inhibiting the binding of pathogenic bacteria and toxins and may selectively support the establishment of a healthy intestinal microbiota (Zivkovic et al., 2011). As human milk oligosaccharides (HMO) are not commercially available, many efforts are being made to replicate their beneficial effects using alternative compounds. Therefore, in Chapter 6 we investigated the effects of dietary bovine milk oligosaccharides (BMO), 6'sialyllactose (6'SL) and the commercial prebiotic, Beneo Orafti P95 oligofructose (P95), on murine gut microbiota composition and functionality. Following 3 weeks oligosaccharide supplementation, significant changes in gut microbiota composition were observed. Overall, BMO and 6'SL supplementations were associated with reductions of less favourable bacteria. Dietary supplementation with BMO was associated with the absence of *Moraxellaceae* and *Vibrionaceae,* while administration of 6'SL was associated with reduced proportions of *Moraxellaceae* ($p=0.003$), and *Porphyromonaceae* ($p=0.016$) among the gut microbiota. As indigenous *Bifidobacterium* were undetectable in baseline and 3-week faecal samples, the ability to comprehensively assess the oligosaccharides

prebiotic nature was limited. Moreover, SCFA levels were unaltered in animals receiving P95 and BMO with only 6'SL administration being associated with decreased levels of butyrate ($p<0.01$). The results from this study demonstrated the potential of BMO and 6'SL supplementation for significantly reducing or eliminating families of bacteria which potentially harbour pathogenic organisms, and indicate that BMO and 6'SL have potential as alternatives to commercial prebiotics such as P95. However, future studies using synbiotic treatment (oligosaccharides and *Bifidobacterium*), higher doses or longer periods of dietary supplementation would be necessary to assess prebiotic properties of these oligosaccharides. Since this study has demonstrated that ingestion of BMO and 6'SL is safe, human studies comparing their effects with breast milk or infant milk formula on microbiota composition could give important information for their future use as functional food components.

While we are still at the early stages of understanding the complex communication systems between gut microbiota and host metabolism, it is now apparent that certain bacteria species and strains have the ability to produce molecules with a range of bioactive functions. The data presented in this thesis have demonstrated the impact of some diverse dietary strategies on gut microbiota composition and thus host metabolism. Further *in vivo* studies are required to elucidate the role of metabolite-producing probiotics on host metabolism in health and disease. Such studies should address what effect these metabolite-producing bacteria and their components have on the host, including effects on nervous system function and behaviour.

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