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Modulation of the Gut Microbiota and Implications for Host Health

A thesis presented to the National University of Ireland for the Degree of Doctor of Philosophy

By

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March 2014

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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 herein was performed independently by the author, with the following exceptions:

Chapter 2

Dr. Orla O’ Sullivan conducted part of the bioinformatic analysis of pyrosequencing data.

Chapter 3

Dr. Rebecca Wall assisted with the design of the experiments and tissue analyses.

Chapter 4

Elaine Patterson and Dr. Lis London are joint first authors of this chapter. Elaine Patterson contributed towards animal feeding, culling and
dissection. Real-time PCR analyses and fatty acid analyses for gas chromatography was conducted by Elaine Patterson.

Dr. Kanishka Nilaweera conducted TSE Phenomaster cage metabolic analyses.

Chapter 5
Dr. Tatiana M. Marques injected animals with streptozotocin and collected faecal pellets. Elaine Patterson contributed towards glucose testing, culling and dissection. DNA isolation and preparation for pyrosequencing and statistical analyses were conducted by Elaine Patterson.

Dr. Orla O’ Sullivan conducted part of the bioinformatic analysis of pyrosequencing data.

Dr. Tatiana M. Marques performed gas chromatography analyses.

Chapter 6
Mr. Patrick Fitzgerald and Ms. Colette Manley contributed towards animal feeding and faecal pellet collection. Mr. Patrick Fitzgerald contributed towards behaviour testing and analyses.

Elaine Patterson

Abbreviations
AA  Arachidonic acid
AAD  Antibiotic associated diarrhoea
AD  Alzheimer’s disease
ALA  α-linolenic acid
ANOVA  Analysis of variance
AMPK  AMP-activated protein kinase
ASD  Autism spectrum disorder
BBDP  Bio-breeding diabetes prone
BBDR  Bio-breeding diabetes resistant
BHI  Brain heart infusion
CALA  Conjugated α-linolenic acid
CD  Crohn’s disease
CD-36  Cluster of differentiation-36
cDNA  Complementary DNA
CHD  Coronary heart disease
CIA  Collagen induced arthritis
CLA  Conjugated linoleic acid
CNS  Central nervous system
COPD  Chronic obstructive pulmonary disease
COX  Cyclooxygenase
CPT-1b  Carnitine palmitoyltransferase-1b
CRP  C-reactive protein
CVD  Cardiovascular disease
CysLT  Cysteinyl leukotriene receptors
DGAT  Diacylglycerol acyltransferase
<table>
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<tr>
<td>DGLA</td>
<td>Dihomo-gamma-linolenic acid</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DIO</td>
<td>Diet-induced obesity</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ED</td>
<td>Endothelial dysfunction</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELOVL</td>
<td>Elongation of very long-chain fatty acids</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>EPS</td>
<td>Exopolysaccharide</td>
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<tr>
<td>FABP</td>
<td>Fatty acid binding protein</td>
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<td>FADS</td>
<td>Fatty acid desaturase</td>
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<td>FAME</td>
<td>Fatty acid methyl ester</td>
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<td>Fatty acid transport protein</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GLA</td>
<td>Gamma linolenic acid</td>
</tr>
<tr>
<td>Gla</td>
<td>Gamma-carboxyglutamyl</td>
</tr>
<tr>
<td>GLC</td>
<td>Gas liquid chromatography</td>
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<tr>
<td>GLP</td>
<td>Glucagon-like peptide</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GMO</td>
<td>Genetically modified organism</td>
</tr>
<tr>
<td>GOS</td>
<td>Galactooligosaccharide</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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GRAS  Generally recognised as safe
HDL  High density lipoprotein
HETE  Hydroxyeicosatetraenoic acid
HF  High-fat
HLA  Human leukocyte antigen
HNE 4-hydroxy-20-nonenal
IBD  Inflammatory bowel disease
IBS  Inflammatory bowel syndrome
IL  Interleukin
LA  Linoleic acid
LC  Long chain
LDL  Low density lipoprotein
LF  Low-fat
LOX  Lipooxygenase
LPL  Lipoprotein lipase
LPS  Lipopolysaccharide
LT  Leukotriene
LXR  Liver-X-receptor
MLN  Mesenteric lymph node
MRS  de Man, Rogosa and Sharpe
MSG  Monosodium glutamate
MUFA  Monounsaturated fatty acid
n-3  Omega-3 polyunsaturated fatty acid
n-6  Omega-6 polyunsaturated fatty acid
NAFLD  Nonalcoholic fatty liver disease
NASH  Nonalcoholic steatohepatitis
**NEFA** Nonesterified fatty acid

**NF-κB** Nuclear factor-κB

**NOD** Non-obese diabetic

**OTU** Operational taxonomy unit

**PG** Prostaglandin

**PNS** Peripheral nervous system

**PPAR** Peroxisome proliferator activated receptor

**PUFA** Polyunsaturated fatty acid

**RER** Respiratory exchange ratio

**RNA** Ribonucleic acid

**ROS** Reactive oxygen species

**RXR** Retinoid-X-receptor

**SCD** Sterol-CoA desaturase

**SCFA** Short chain fatty acid

**SFA** Saturated fatty acid

**SREBP** Sterol regulatory binding protein

**STZ** Streptozotocin

**T1D** Type-1-diabetes

**T2D** Type-2-diabetes

**TAG** Triglyceride

**TLR** Toll-like receptor

**TNF** Tumor-necrosis factor

**TX** Thromboxane

**UC** Ulcerative colitis

**UCC** University College Cork

**VLDL** Very low density lipoprotein
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through the activities of COX and LOX (Russo, 2009). The eicosanoids derived from AA are synthesised in larger quantities than ever before due to increases in $n$-6 PUFA dietary intake (Olivier et al., 2010).

Eicosanoids are biologically active lipids, and include prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), and hydroxyeicosatetraenoic acids (HETEs) which have all been implicated in various pathological processes such as inflammation and cancer (Table 1.1.2) (Wang and DuBois, 2010). When they are present in high quantities, they influence various metabolic activities besides inflammation such as platelet aggregation, haemorrhage, vasoconstriction and vasodilation (Benatti et al., 2004). In general, AA-derived eicosanoids are proinflammatory but they have important homeostatic functions in regulating both the promotion and resolution of inflammation in the immune response (Ricciotti and FitzGerald, 2011). In contrast, it is known that $n$-3 PUFA and their LC-derivatives mostly promote anti-inflammatory activities (Tai and Ding, 2010). In a recent study involving 250 clinically stable chronic obstructive pulmonary disease (COPD) patients, higher intakes of $n$-3 PUFA were associated with lower proinflammatory cytokine concentrations (e.g., tumour necrosis factor alpha (TNF$\alpha$)), while higher $n$-6 PUFA intake was associated with higher pro-inflammatory interleukin-6 (IL-6) and C-reactive protein (CRP) concentrations in the disease state (de Batlle et al., 2011). While COPD is a complex chronic inflammatory condition, it is interesting to see the association between dietary intake of $n$-6 versus $n$-3 PUFA on serum inflammatory markers associated with the disease (de Batlle et al., 2011). Despite ample evidence that increased dietary consumption of $n$-6 PUFA induces a proinflammatory response in the host, it must also be reported that recent studies have also shown the opposite (Poudel-Tandukar et al., 2009; Yoneyama et al.,
Chapter 1

Literature reviews
Chapter 1.1

Health implications of high dietary omega-6 polyunsaturated fatty acids

1.1.1 ABSTRACT

Omega-6 (n-6) polyunsaturated fatty acids (PUFA) (e.g., arachidonic acid (AA)) and omega-3 (n-3) PUFA (e.g., eicosapentaenoic acid (EPA)) are precursors to potent lipid mediator signalling molecules, termed “eicosanoids”, which have important roles in the regulation of inflammation. In general, eicosanoids derived from n-6 PUFA are proinflammatory while eicosanoids derived from n-3 PUFA are anti-inflammatory. Dietary changes over the past few decades in the intake of n-6 and n-3 PUFA, show striking increases in the n-6: n-3 ratio (~15:1), which are associated with greater metabolism of the n-6 PUFA, compared with n-3 PUFA. Coinciding with this increase in the ratio of n-6: n-3 PUFA, are increases in chronic inflammatory diseases such as nonalcoholic fatty liver disease (NAFLD), cardiovascular disease (CVD), obesity, inflammatory bowel disease (IBD), rheumatoid arthritis and Alzheimer’s disease (AD). By increasing the ratio of n-3: n-6 PUFA in the Western diet, reductions may be achieved in the incidence of these chronic inflammatory diseases.

1.1.2 INTRODUCTION

Fatty acids are hydrocarbon chains with a carboxyl group at one end and a methyl group at the other. The biological reactivity of fatty acids is defined by the length of the carbon chain and by both the number and position of any double bonds present. While saturated fatty acids do not contain double bonds within the acyl chain, unsaturated fatty acids contain at least one double bond. When two or more double bonds are present, unsaturated fatty acids are referred to as PUFA (Calder, 2008a). There are two families of PUFA and they are classified as omega-3 (n-3) and omega-6.
Based on the location of the last double bond relative to the terminal methyl end of the molecule (Wall et al., 2010). The human body can produce all but two of the fatty acids it requires. Linoleic acid (LA, 18:2n-6) (precursor to the n-6 series of fatty acids) and α-linolenic acid (ALA, 18:3n-3) (precursor to the n-3 series of fatty acids) are the simplest members of each family of PUFA and are termed essential fatty acids as the body cannot synthesise them.PUFA regulate a wide variety of biological functions, depending on the location of the last double bond, which range from blood pressure and blood clotting, to the correct development and functioning of the brain and nervous system (Wall et al., 2010). In addition, lipid mediators generated from long-chain (LC-) PUFA (AA in the n-6 series and EPA and docosahexaenoic acid (DHA) in the n-3 series) have important roles in immune regulation and inflammation (Calder, 2009). The main dietary sources of LA include plant oils such as sunflower, safflower and corn oils (Table 1.1.1), but they are also present in cereals, animal fat and wholegrain bread. Rich dietary sources of ALA include green leafy vegetables, flaxseed, and rapeseed oils (Wall et al., 2010) (Table 1.1.1).

Over the last few decades, extreme qualitative nutritional changes have taken place with the increased levels of fatty acid consumption (Olivier et al., 2010). Today, industrialised societies are characterised by an increase in saturated fat, n-6 PUFA, and trans fatty acid intake, as well as an overall decrease in n-3 PUFA intake (Simopoulos, 2011). Fatty acids now represent 28-42% of total energy consumed by European populations (Linseisen et al., 2009; Olivier et al., 2010), whereas in ancestral nutrition, fatty acid consumption was only approximately 20-30% of total energy (Eaton et al., 2010; Olivier et al., 2010; Simopoulos, 2001). As a result of the increased consumption of LA-rich vegetable oils associated with the Western diet, n-6 PUFA consumption has
become progressively much higher than that of n-3 PUFA (Anderson and Ma, 2009). Optimal dietary intakes of the n-6: n-3 ratio should be around 1-4:1, whereby in ancestral nutrition, this ratio was balanced. However, according to the nutritional changes described above in the Western diet, this ratio has now increased to be within the range of 10:1 to 20:1 (Olivier et al., 2010). In parallel, there are coinciding increases in the incidence of diseases involving inflammatory processes such as CVD, obesity, IBD, rheumatoid arthritis and cancer. Neurodegenerative and psychiatric illnesses such as AD and depression are other examples (Corsinovi et al., 2011). A study carried out by Hassan and Hanachi, involving 984 Iranian women, suggested that a good dietary pattern rich in fruits, legumes, vegetables, cereals and fish, rich in n-3 PUFA, can decrease the likelihood of developing the Metabolic Syndrome (Hassan and Hanachi, 2009). Another study carried out in France, involving 912 men, concluded that a low consumption of fish rich in n-3 PUFA is associated with a higher probability of developing the Metabolic Syndrome (Ruidavets et al., 2007). Thus, high intake of n-6 PUFA, along with low intakes of n-3 PUFA, shifts the physiological state to one that is proinflammatory and prothrombotic with increases in vasospasm, vasoconstriction and blood viscosity and the development of diseases associated with these conditions.

PUFA play an important role in the composition of all cell membranes where they maintain homeostasis for correct membrane protein function and influence membrane fluidity, thus regulating cell signalling processes, cellular function and gene expression (Das, 2006b). Other functions of PUFA require their metabolism to more highly unsaturated members of their family. For example, LA is converted to AA (20:4n-6) via γ-linolenic acid (GLA, 18:3n-6) and dihomo-γ-linolenic acid (DGLA, 20:3n-6). By the same set of enzymes, ALA can be converted to EPA (20:5n-3) and DHA (22:6n-3). The
primary site for PUFA metabolism is the liver, however, it can also take place in various other tissues (Hughes CL, 2002). It is these longer chain metabolites of LA and ALA that are of major clinical importance within different organs such as the brain, kidney and liver (El-Badry et al., 2007; Lauretani et al., 2008; Uauy and Dangour, 2006). Cyclooxygenases (COX) and lipoxygenases (LOX) can convert AA to the 2-series of prostaglandins, the 2-series of thromboxanes and the 4-series of leukotrienes. These are very important, active and short-lived hormones termed “eicosanoids” which are involved in various pathological processes involving inflammatory conditions such as atherosclerosis, obesity and IBD (Das, 2006b).

Since PUFA give rise to a variety of biologically active compounds which all have important roles in pathological and physiological processes, a proper understanding is needed regarding the contribution these active compounds have on the coinciding increases in inflammatory diseases seen with the disruption of the balance in the ratio of n-6: n-3 associated with the Western diet.

1.1.3 METABOLISM OF n-6 FATTY ACIDS AND BIOSYNTHESIS OF EICOSANOIDS

LA can be metabolised to other more unsaturated, long chain members of the n-6 family by the insertion of additional double bonds during consecutive elongation and desaturation reactions (Figure 1.1.1). The initial rate limiting desaturation of LA to GLA is catalysed by the enzyme Δ-6-desaturase (FADS2) (Stoffel et al., 2008). Elongation then takes place to convert GLA to DGLA, by elongation of very long chain fatty acids (ELOVL)-5, and finally a cycle of elongation and desaturation by Δ-5-desaturase (FADS1) generates AA (Moon et al., 2009).
The importance of the FADS2 gene in LC-PUFA synthesis has recently been demonstrated in mice (Stoffel et al., 2008; Stroud et al., 2009). The first study demonstrated that loss of the FADS2 gene abolishes synthesis of LC-PUFA, with further downstream effects on the COX and LOX pathways, eventually leading to hypogonadism and sterility of male and female mice (Stoffel et al., 2008). Further demonstrated by this FADS2 null model was the pivotal role PUFA-substituted phospholipids play in establishing cell polarity, shown here for tight junctions of Sertoli cells of the testis and the gap junction network between ovarian follicle cells (Stoffel et al., 2008). Impairment of male reproduction and both dermal and intestinal ulceration have been shown in FADS2 null mice (Stroud et al., 2009).

ELOVL5 is one of seven mammalian fatty acid condensing enzymes involved in microsomal fatty acid elongation (Moon et al., 2009), required for the elongation of GLA to DGLA. Studies using liver microsomal protein from ELOVL5 null mice found greater tissue accumulation of GLA and a decrease in the levels of downstream metabolism products such as AA for n-6 metabolism and DHA for n-3 metabolism. The metabolic consequence of this reduction of AA and DHA, was the activation (or de-repression) of sterol regulatory element-binding protein (SREBP)-1c. Activation of this transcription factor (as will be discussed in further detail later) in ELOVL5 null mice resulted in the activation of further genes involved in fatty acid and triglyceride synthesis, which culminated in the development of hepatic steatosis (Moon et al., 2009).

There are many other factors involved in the regulation of FADS1 and FADS2 enzyme activity. For example, decreased activity in both FADS1 and FADS2 have been demonstrated in the liver of obese NAFLD patients (Araya et al., 2010). Glucagon,
adrenaline, glucocorticoids and thyroxin depress FADS1 and FADS2 activity (Brenner, 2003). Low FADS2 enzyme activity was reported in diabetic rats whereby insulin acts as a FADS2 stimulator (Brenner, 1981). Since LA and ALA are metabolised by the same set of enzymes, a natural competition exists between these two fatty acids, whereby FADS1 and FADS2 will exhibit affinity to metabolise n-3 over n-6 PUFA, provided that they exist in a ratio of 1:1-4. However, the higher consumption of LA, as now seen in the Western diet, shows an increase in the preference of these enzymes to metabolise n-6 PUFA, leading to AA synthesis, despite the fact that these enzymes show higher affinity for n-3 PUFA (Das, 2006a). Supplementation of the diet with EPA and DHA has been shown to correct this imbalance by partially replacing AA from the cell membranes of platelets, erythrocytes, neutrophils, monocytes and hepatocytes where AA is usually found in high proportions (Simopoulos, 2002).

The intermediates of PUFA metabolism can either be incorporated into phospholipids or undergo further elongation/desaturation. In the n-6 pathway, AA, synthesised from the desaturation of DGLA by FADS1 can be further elongated by ELOVL2 to docosatetraenoic acid (22:4n-6) or to its respective set of eicosanoids via COX and LOX enzymes. The importance of ELOVL2 derived PUFA in mammals have recently been demonstrated in ELOVL2-ablated mice, thus demonstrating the importance of this elongase enzyme (Zadravec et al., 2011). This study showed the role ELOVL2 plays in the elongation of 20:0 and 22:0 PUFA in order to produce 24:4n-6 up to 30:5n-6 PUFA in testis, where they are required for normal spermatogenesis and fertility (Zadravec et al., 2011). Binding of growth factors and hormones to membrane receptors leads to activation of phospholipase A₂ which releases AA from the cell membrane where the free acid can become a substrate for eicosanoid biosynthesis.
A recent review has suggested that n-6 PUFA have some anti-inflammatory actions such as those of n-3 PUFA (Fritsche, 2008). For example, mean serum CRP concentrations tended to decrease with increased n-6 PUFA consumption in both Japanese men (Poudel-Tandukar et al., 2009) and women (Yoneyama et al., 2007). Nevertheless, evidence of these associations is limited.

Metabolism of AA by the COX enzymes (COX-1 a constitutive enzyme, or COX-2 an inducible enzyme) leads to the synthesis of the 2-series of prostaglandins: PGE₂, PGI₂, PGD₂ and PGF₂α, (largely produced by monocytes and macrophages) and thromboxanes A₂ and B₂. Collectively, the prostaglandins and thromboxanes are referred to as prostanoids. The synthesis of AA-derived eicosanoids is however dependent on the concentration of DGLA, as DGLA competes with AA for COX and LOX. When DGLA is in excess, it inhibits the synthesis of AA-derived eicosanoids due to its higher affinity for the COX and LOX enzymes (Levin et al., 2002). The activity of 5-LOX metabolises AA to hydroxyl and hydroperoxy derivatives: 5-HETE and 5-hydroperoxyeicosatetraenoic acid (5-HPETE). These derivatives in turn produce the 4-series of leukotrienes: LTA₄, LTB₄, LTC₄, LTD₄ and LTE₄. Monocytes, macrophages and neutrophils produce LTB₄, while mast cells, eosinophils and basophils produce LTC₄, LTD₄ and LTE₄ (Bagga et al., 2003).

Prostaglandin overproduction has various proinflammatory effects. For example, PGI₂ and PGE₂ exert their acute inflammatory response in arthritis (Kojima et al., 2005; Pulichino et al., 2006). PGE₂ can also increase its own synthesis through induction of COX-2 leading to the production of the pro-inflammatory cytokine IL-6 in macrophages (Bagga et al., 2003; Tilley et al., 2001). TXB₂ is a potent vasoconstrictor and platelet activator. LTB₄ has many proinflammatory functions, acting as an important activator...
of neutrophils, a chemotactic agent for leukocytes, induces release of lysosomal enzymes, accelerates reactive oxygen species (ROS) production and increases vascular permeability (Schmitz and Ecker, 2008). LTB₄ also leads to the production of inflammatory cytokines like TNFα, IL-1β and IL-6 by macrophages (Peters-Golden and Henderson Jr, 2007). However, the overall pathophysiological outcome will depend on the cells present, the nature of the stimulus, the timing of eicosanoid generation, the concentrations of different eicosanoids generated and the sensitivity of target cells and tissues to the eicosanoids generated (Calder, 2008a).

In contrast, EPA can also act as a substrate for COX and LOX enzymes and gives rise to an entirely different set of eicosanoids (Table 1.1.2). These are the 3-series prostaglandins and thromboxanes and the 5-series leukotrienes, which are considered to be less inflammatory or even anti-inflammatory in comparison to the eicosanoid family derived from AA (Robinson and Stone, 2006).

1.1.4 HOW n-6 PUFA-DERIVED EICOSANOIDS INFLUENCE INFLAMMATORY RESPONSES

The mode by which prostaglandins and leukotrienes exert their biological homeostatic and inflammatory actions depends on binding to their respective G-protein coupled receptors (GPCRs). Specific GPCRs have been identified for all the prostanoids, where there are at least nine known prostanoid receptor forms in mouse and man (Hata and Breyer, 2004; Narumiya and FitzGerald, 2001). Although most of the prostaglandin GPCRs are localised at the plasma membrane of platelets, vascular smooth muscle cells and mast cells, some are situated at the nuclear envelope (Ide et al., 2003). Four of these receptor subtypes bind PGE₂ (EP₁-EP₄), two bind PGD₂ (DP₁
and DP₂) and more specific receptors bind PGF₂α, PGI₂ and TXA₂ (FP, IP and TP, respectively) (Harizi et al., 2008). PGE₂ and PGI₂ are the predominant proinflammatory prostanoids and through their activation of EP₂ and IP, respectively, they can increase vascular permeability and leukocyte infiltration. In individuals with asthma, a bronchial allergen leads to enhanced PGD₂ production. Thus, during asthmatic attacks in humans, PGD₂ is released in large amounts by mast cells (Arima and Fukuda, 2011). PGD₂ can also promote inflammation via DP₂ through activation of eosinophils (Hata and Breyer, 2004; Pettipher et al., 2007).

Four distinct GPCRs for leukotrienes have been characterised. LTB₄ interacts with BTL₁ and BTL₂ through which important roles in host defence of cells and inflammation are mediated (Lundeen et al., 2006). LTB₄ induces leukocyte infiltration and as already mentioned above, leads to the release of proinflammatory cytokines. In patients with IBD, the colonic mucosa contains 3- to 7- fold higher counts of cells expressing the 5-LOX pathway, thus increasing the tissue synthesis of LTB₄ (Jupp et al., 2007). LTC₄ and LTD₄ can contract smooth muscle by interacting with two subtypes of cysteiny1 leukotriene receptors, CysLT₁ and CysLT₂ (Funk, 2001).

The proinflammatory effects of the AA-derived prostanoids and leukotrienes have been described (Harizi et al., 2008). A mechanism has been proposed whereby a co-ordinated program for resolution initiates in the first few hours after the inflammatory response. A switch occurs whereby the AA-derived prostanoids and leukotrienes, which have set the inflammatory response to begin, undergo further metabolism to become another generation of eicosanoids derived from AA, termed lipoxins, and hence terminate inflammation at the local contained sites (Serhan and Savill, 2005). Since these lipoxins are involved in the resolution of the acute inflammation that
occurs as a result of the overproduction of the proinflammatory eicosanoids derived from AA, they are said to have ‘pro-resolving’ and anti-inflammatory functions. These events coincide with the biosynthesis of resolvins and protectins from n-3 fatty acids, which act to shorten the period of neutrophil infiltration (Serhan and Savill, 2005). However, while the initial response of the AA-derived eicosanoids to promote inflammation is beneficial in one respect, for example, in the control of blood flow and vessel dilation, with the increase in the ratio of n-6: n-3 PUFA, there is an overall increase in the production of proinflammatory cytokines and an unnecessary over reactive inflammatory response leading to the pathogenesis of inflammatory diseases. In addition, the decrease in consumption of n-3 PUFA which leads to an overall decrease in resolvin and protectin production is detrimental to the inflammatory response as these products, which have the ability to dominate the resolution phase of inflammation, can no longer exert this potential, thus the inflammatory response cannot be terminated effectively.

1.1.5 n-6 PUFA REGULATION OF INFLAMMATORY GENE EXPRESSION

Nuclear receptors are a family of ligand-activated transcription factors that either directly or indirectly control various genes of lipid metabolism and inflammatory signalling (Schmitz and Ecker, 2008). Upon ligand binding, nuclear receptors can undergo conformational changes which dissociate corepressors and facilitate recruitment of coactivator proteins to enable transcription activation (Chawla et al., 2001; Schmitz and Ecker, 2008). LC-PUFA and their eicosanoid derivatives can act as ligands for these transcription factors and hence, elicit changes in gene expression by governing the activity of nuclear transcription factors. The regulation of gene
expression by dietary fats is believed to be one of the greatest factors impacting on the development of certain diseases related to the Metabolic Syndrome, such as hepatic steatosis and NAFLD.

The peroxisome proliferator-activated receptor (PPAR) family is composed of three proteins; PPARα, PPARβ/δ and PPARγ and although they each have different tissue distributions, their biological functions overlap (Bensinger and Tontonoz, 2008). The PPARs have emerged as important regulators of metabolic and inflammatory signalling, in both metabolic disease and immunity (Bensinger and Tontonoz, 2008). The role PPARα plays in the regulation of genes involved in lipid metabolism was first identified in the early 1990s, on the basis of being a target of the hypolipidaemic fibrate drugs and other compounds that induce peroxisome proliferation in rodents (Bensinger and Tontonoz, 2008; Issemann and Green, 1990). PUFA, especially those of the n-3 family and their eicosanoid derivatives are ligands for the PPARs. The n-3 PUFA EPA and DHA have been shown as more potent as in vivo activators of PPARα than n-6 PUFA (Couet et al., 1997; Hu et al., 1999; Mori et al., 1999; Power and Newsholme, 1997). Once PPARs become activated, they form heterodimers with the retinoid X receptor (RXR) and these dimers then bind to PPAR responsive elements (PPREs) in target genes to alter coactivator/corepressor dynamics and induce transcription (Chakravarthy et al., 2009). PPARα has recently been shown to exert hypolipidaemic effects through activation of skeletal muscle, cardiac and hepatic genes encoding proteins which are involved in lipid oxidation (Chakravarthy et al., 2009; Fujita et al., 2008; Yoon et al., 2006). Thus, the PPARs, particularly PPARα, play an important role in insulin sensitisation, atherosclerosis and metabolic diseases. In the regulation of inflammatory signalling, PPARs inhibit nuclear factor-κB (NF-κB) expression.
NF-κB, another transcription factor regulated by PUFA, is found in almost all animal cell types, has a crucial role in inflammatory signalling pathways and plays a key role in regulating the immune response to infection. It controls several cytokines (e.g., IL-1, IL-2, IL-6, IL-12 and TNFα), chemokines (e.g., IL-8, monocyte chemoattractant protein-1), adhesion molecules and inducible effector enzymes (e.g., inducible nitric oxide synthase and COX-2) (Wall et al., 2010). NF-κB becomes activated as a result of a signalling cascade triggered by extracellular inflammatory stimuli (such as free radicals, bacterial or viral antigens), which involves phosphorylation of an inhibitory subunit of NF-κB (IκB), which in turn allows the translocation of the remaining NF-κB dimer to the nucleus, with the result of an increase in expression of inflammatory genes (Perkins, 2007). Since n-3 LC-PUFA exhibit anti-inflammatory action, they inhibit NF-κB activity. As an example, both EPA and DHA have been shown to block the activity of NF-κB through decreased degradation of IκB, in human monocytes and human THP-1 monocyte derived macrophages (Weldon et al., 2007; Zhao et al., 2004). However, this effect is not observed to the same extent with n-6 LC-PUFA, due to potency in the inhibition of NF-κB (De Caterina et al., 1999). Interestingly, 5-LOX, the enzyme which converts AA to the 4-series leukotrienes and 5-HETE translocates into the nucleus in association with NF-κB (Lepley and Fitzpatrick, 1998; Soberman and Christmas, 2003).

SREBP-1c is a transcription factor required for the insulin-mediated induction of hepatic fatty acid and triglyceride synthesis. Responsive targets in mammalian cells include genes of fatty acid metabolism, such as fatty acid synthase (FAS) and its expression is most commonly found in high levels in macrophages, liver, white adipose tissue, adrenal glands, and the brain of both mice and humans (Ecker et al., 2007). PUFA have the ability to modulate SREBP-1c activity and expression. For example, n-3
LC-PUFA have been shown to suppress SREBP-1c gene expression and so inhibit transcription of lipogenic and hepatic genes involved in lipid biosynthesis (Teran-Garcia et al., 2007; Xu et al., 2002). Studies have shown that a decrease in hepatic SREBP-1c leads to a decrease in hepatic FAS, thus reducing lipid accumulation within the liver (Howell III et al., 2009; Xu et al., 1999; Xu et al., 2001). However, n-3 PUFA are more potent inhibitors of SREBP-1c, than n-6 PUFA (Schmitz and Ecker, 2008). More recently, the liver X receptors (LXR-α and –β) have been shown to play a major role in lipogenesis through regulation of transcription of the gene encoding SREBP-1c (Howell III et al., 2009). This study concluded that the downregulation of SREBP-1c transcription by n-3 PUFA results from attenuated transactivation of the ligand-activated nuclear receptor LXR-α (Howell III et al., 2009). A more recent study in mice fed an n-3 PUFA depleted diet showed increased activation of SREBP-1c and related pathways which was consistent with increased LXR activity, thus highlighting the importance of n-3 PUFA depletion related to lipid accumulation in the liver (Pachikian et al., 2011). However, another study demonstrated that fish oil feeding in rats suppressed hepatic SREBP-1c target genes with change in expression of genes directly regulated by LXR (Pawar et al., 2003). Inhibition of LXR may also be an indirect effect of PUFA stimulation of PPAR transcription factors (Schmitz and Ecker, 2008). Cross talk between PPARα and LXR via SREBP-1c has been reported, whereby overexpression of PPARα inhibited LXR induced SREBP-1c promoter activity, through a reduction of LXR binding to its activator, RXR (Yoshikawa et al., 2003). Both n-6 and n-3 PUFA are often interchangeable in regulating gene expression. However, it is well known that n-3 PUFA are more potent ligands to these nuclear receptors than n-6 PUFA (Schmitz and Ecker, 2008). Through n-3 PUFA mediated activation of PPARα and inhibition of SREBP-
1c, lipid biosynthesis can be reduced and lipid degradation can be increased (Russo, 2009; Schmitz and Ecker, 2008).

By targeting the transcription of various nuclear receptors involved in regulating lipogenic gene expression through dietary fatty acids, prevention of certain diseases related to the Metabolic Syndrome, such as hepatic steatosis and NAFLD can be reduced. The contribution n-6 PUFA make to the development of liver disease due to the increased consumption of LA-rich foods and the decreased consumption of ALA rich foods will be discussed in further detail. Already discussed are the positive contributions of n-3 PUFA in the prevention of lipid biosynthesis in various organs, such as the liver, for example, through the activation of PPARα and inhibition of NF-κB and SREBP-1c. However, since these n-3 PUFA are more potent ligands for these nuclear receptors and Western diets overall consumption of n-6: n-3 has increased dramatically over the last 50 years in particular, what now becomes the fate of these nuclear receptors and how have our dietary changes impacted upon our health status through regulation of inflammatory gene expression? Determination of the molecular and cellular mechanisms regulated by PUFA, may identify novel sites for pharmacological intervention.

1.1.6 n-6 PUFA CONTRIBUTION TO CHRONIC INFLAMMATORY CONDITIONS

Clinical studies indicate that inflammation is at the base of many diseases including NAFLD, CVD, atherosclerosis, IBD, and neurodegenerative diseases such as AD (Figure 1.1.2). The contributions of n-6 PUFA to inflammatory conditions are reviewed, with a particular focus on NAFLD.
1.1.6.1 **NAFLD**

NAFLD is often described as the hepatic component of the Metabolic Syndrome and is rapidly becoming a serious public health problem (Byrne et al., 2009). The range of liver damage associated with NAFLD begins with steatosis and can often persist to further steatohepatitis (NASH), advanced fibrosis and cirrhosis (Byrne, 2010). NAFLD itself is an independent risk factor for CVD. Occurrences of NAFLD are much higher in subgroups of the population with obesity, Metabolic Syndrome and type 2 diabetes, whereby prevalence in developing the disease for those with type 2 diabetes may be as high as 70% (Byrne, 2010; Erickson, 2009). Both nutritional factors and alterations in lipid metabolism of the liver are the primary metabolic abnormalities which lead to hepatic steatosis (Videla et al., 2006).

The role of \( n-3 \) LC-PUFA as a potential therapeutic target in the pathogenesis of NAFLD has recently been demonstrated (Larter et al., 2008). Within the liver, \( n-3 \) LC-PUFA presence is associated with an increased ability to direct fatty acids away from triacylglycerol storage and to enhance their oxidation. However, \( n-3 \) LC-PUFA levels are decreased in the hepatic tissue of patients with NAFLD (Araya et al., 2004; Spadaro et al., 2008). Depletion of \( n-3 \) LC-PUFA within the liver of NAFLD patients is a major problem as liver fatty acids now become directed away from oxidation and secretion and instead towards triacylglycerol storage. In addition, a higher \( n-6 : n-3 \) LC-PUFA ratio within the liver of NAFLD patients may contribute to the development of fatty liver due to a derangement in the capacity to regulate liver lipid metabolism (Araya et al., 2004). A recent comparative review also demonstrated various mechanisms through which consumption of fish oil has been beneficial in the alleviation of NAFLD such as (i) decreased plasma nonesterified fatty acids (NEFA) concentrations; (ii) decreased \( de \)
novo lipogenesis, very low-density lipoprotein (VLDL) export and plasma triglyceride concentrations; (iii) decreased adipocyte size and visceral fat content (Zivkovic et al., 2007). The mechanisms which lead to the development of fatty liver, such as impaired fatty acid oxidation and increased de novo fatty acid synthesis are regulated by hepatic gene transcription.

$n$-3 LC-PUFA regulate lipid metabolism in the liver by acting as ligand activators of the transcription factor PPARα. Activation of PPARα results in the upregulation of genes which are involved in fatty acid and lipid metabolism and which stimulate fatty acid oxidation (El-Badry et al., 2007; Jump, 2008). In two separate studies employing murine models of NASH, administration of a PPARα agonist prevented steatohepatitis and reversed the establishment of the disease (Ip et al., 2004; Ip et al., 2003).

VLDL is a type of lipoprotein synthesised by the liver from triglycerides, cholesterol and apolipoproteins. Within the bloodstream, VLDL transports cholesterol from the liver, thus enabling fats to move within the bloodstream and it is here that VLDL itself can also act as a precursor to low-density lipoprotein (LDL), often referred to as “bad cholesterol”. PPARα activation increases the secretion of apolipoprotein B-100 (apo B-100), which is the main structural protein of VLDL and upregulates the expression of liver fatty acid binding protein (LFABP) which is essential for the secretion of apo B-100 (Carlsson et al., 2001; Linden et al., 2002). Since $n$-3 LC-PUFA upregulate PPARα, hepatic fatty acid oxidation has the potential to occur within the liver and since more apo B-100 is secreted out of the liver, less VLDL is synthesised, with the result of less of this harmful cholesterol entering the bloodstream, where the downstream further effects on the development of atherosclerosis are attenuated (Savage and Semple, 2010). However, with the reduced availability of $n$-3 LC-PUFA from dietary intake, and
the increases in n-6 PUFA consumption, PPARα does not become activated to its full potential. This results in PUFA favouring fatty acid and triglyceride synthesis over fatty acid oxidation. As demonstrated by PPARα$^{-/-}$ mice, rates in their ability to oxidise fatty acids are decreased during periods of food deprivation, thus, they develop characteristics of adult-onset diabetes including fatty livers, elevated blood triglyceride concentrations and hyperglycemia (Kersten et al., 1999).

$n$-3 LC-PUFA are also involved in the negative regulation of the transcription factor SREBP-1c within the liver, thus acting as inhibitors in the expression of lipogenic genes such as FAS (Xu et al., 2002). The effect $n$-3 LC-PUFA have on SREBP-1c is to reduce endogenous lipid production and accumulation of triglycerides in the liver (Masterton et al., 2010) and this is achieved by reducing the amount of mature SREBP-1c available for de novo lipogenesis within the nucleus (Yahagi et al., 1999). Therefore, depletion of $n$-3 LC-PUFA and an increase in the ratio of $n$-6: $n$-3 LC-PUFA in the liver of NAFLD patients results in fatty acid and triacylglycerol synthesis over oxidation, again leading to fatty liver. A recent study by Pachikian et al., using mice fed a depleted $n$-3 PUFA diet showed increases in hepatic activation of SREBP-1c leading to increased lipogenesis, contributing to hepatic steatosis (Pachikian et al., 2011). This is consistent with a previous study in rats fed an $n$-3 PUFA depleted diet whereby hepatic accumulation of triglycerides and esterified cholesterol led to both macro- and microvesicular steatosis caused by changes in the fatty acid pattern that resulted from $n$-3 PUFA depletion (Malaisse et al., 2009).

Another mechanism involved in the depletion of $n$-3 LC-PUFA from the liver of obese NAFLD patients and which further exacerbates the disease progression is the decreased hepatic FADS1 and FADS2 activity in these patients (Araya et al., 2010).
Impairment of these enzymes affects the desaturation and elongation pathways of LA and ALA, which are required for the synthesis of their LC-PUFA derivatives within the liver (Videla et al., 2004). Decreased activity of both FADS1 and FADS2 have been demonstrated in the liver of obese NAFLD patients (Araya et al., 2010). This may be attributed to the lower intake of ALA, the imbalance in the \( n-6: n-3 \) LC-PUFA ratio and higher consumption of trans isomers (18:1, \( n-9 \) \( trans \)) inhibiting FADS2 (Araya et al., 2004). The depletion of \( n-3 \) LC-PUFA within the liver of these patients resulting from the decrease in FADS1 and FADS2 activity may lead to further development of steatosis by altering the activity of PPAR\( \alpha \) and SREBP-1c (Araya et al., 2010). This will determine a metabolic imbalance favouring lipogenesis over fatty acid oxidation since \( n-3 \) LC-PUFA depletion induces SREBP-1c expression and upregulation of lipogenic genes (Videla et al., 2006). In general, it is also understood that the adipose tissue acts as a suitable biomarker for dietary fatty acid intake. Considering that in NAFLD, there is an enhancement in \( n-6 \) PUFA adipose tissue content and a significant decrease in \( n-3 \) adipose tissue content suggests that while there is an adequate amount of \( n-6 \) PUFA for metabolism within the liver, \( n-3 \) PUFA cannot be metabolised to the same extent due to inadequate dietary intake. Also, decreased dietary intake of \( n-3 \) PUFA constitutes a limiting factor for the production of \( n-3 \) LC-PUFA in liver lipids of NAFLD patients, resulting from the competition between the two metabolic pathways (Figure 1.1.1), particularly at the desaturation steps (Araya et al., 2004). Thus, a dietary imbalance comprising inadequate intake of \( n-3 \) PUFA and an excess intake of \( n-6 \) PUFA leads to defective desaturation of PUFA (Araya et al., 2004).

Oxidative stress, caused by the accumulation of liver triglycerides and insulin resistance are major contributors in the pathogenesis of NAFLD (Narasimhan et al.,
Both oxidative stress and mitochondrial dysfunction are often associated with the increased production of ROS and proinflammatory cytokines related to NAFLD (Byrne, 2010). Recent human studies have described a strong association between the severity of NASH and the degree of oxidative stress (Chalasani et al., 2004; Narasimhan et al., 2010; Yesilova et al., 2005). The increased pro-oxidant activity associated with oxidative stress, leads to elevation in hepatic lipid peroxidation status. Lipid peroxidation can also cause immunological dysfunction, which could lead to the development of hepatic fibrogenesis (Byrne, 2010). This could potentially lead to an increase in the release of 4-hydroxy-20-nonenal (HNE), which can bind hepatocyte proteins forming new antigens and therefore provoking a harmful immunological response (Byrne, 2010). For example, a correlation between hepatic expression of HNE and the degree of severity of necroinflammation and fibrosis has been reported (Seki et al., 2002). Oxidative stress associated with NAFLD has also been shown to increase production of proinflammatory cytokines. This hepatotoxicity associated with the production of inflammatory cytokines, induced through oxidative stress may indirectly activate transcription factors such as NF-κB. The accumulation of NEFA within hepatocytes of NAFLD patients is another source of NF-κB activation (Feldstein et al., 2004).

Oxidative stress and changes in dietary intake trends may contribute to low hepatic LC-PUFA (Allard et al., 2008). The increase in lipid peroxidation associated with NAFLD, as discussed, may contribute to the decrease in LC-PUFA, as they are particularly susceptible to lipid peroxidation (Allard et al., 2008; Sevanian and Hochstein, 1985). Thus, oxidative stress-dependent lipid peroxidation may represent an alternative mechanism to liver n-3 LC-PUFA depletion in NAFLD (Videla et al., 2004), since lipids
containing PUFA are more susceptible to peroxidation and the availability of n-6 LC-PUFA in the liver of NAFLD patients results in enhanced peroxidation of these LA derived LC-PUFA into their eicosanoid derivatives (Videla et al., 2004). For example, LTB₄, an AA-derived eicosanoid, is involved in acceleration of ROS production. The increased production of proinflammatory cytokines and eicosanoids, produced from n-6 PUFA metabolism, cause enhanced liver Kupffer cell production of inflammatory cytokines causing activation of NF-κB, further exacerbating systemic and hepatic insulin resistance with worsening inflammation and fibrosis (Byrne, 2010). Insulin resistance as seen in NAFLD may be related to the depletion in n-3 LC-PUFA because they are expected to modify membrane-mediated processes such as insulin signalling (Lombardo and Chicco, 2006).

In summary, the depletion in n-3 LC-PUFA, the decrease in the ratio of product/precursors of LC-PUFA, the increase in n-6 PUFA and the increase in n-6 LC-PUFA derived eicosanoid production within the liver, all contribute to the development of NAFLD and related pathophysiologies such as insulin resistance. The relationship between the n-6: n-3 PUFA ratio within the liver and severity of steatosis has also been demonstrated (Vuppalanchi et al., 2007). In this study, patients with NAFLD showed significant correlation between the n-6: n-3 PUFA ratio and the quantity of hepatic triglycerides, as a marker of the severity of hepatic steatosis (Vuppalanchi et al., 2007). Defective desaturation of PUFA due to dietary imbalance comprises inadequate intake of n-3 PUFA and a higher intake of n-6 PUFA further enhances the contribution of desaturase inhibition in NAFLD.

1.1.6.2 CVD AND ATHEROSCLEROSIS
Atherosclerosis is now considered a “systemic disease” characterised by low grade arterial inflammatory lesions that can mature along with disease progression (Montecucco and Mach, 2009). It is the underlying cause of coronary heart disease (CHD), and abnormalities in the metabolism of essential fatty acids are characteristic of the associated risk factors (Das, 2007). Under normal physiological conditions, healthy endothelial cells synthesise and release adequate amounts of NO, PGI$_2$ and PGE$_1$, maintaining a downstream balance between pro- and anti-inflammatory molecules. However, in atherosclerosis, this balance becomes disrupted, leaning towards an increase in the production of proinflammatory cytokines such as IL-1, IL-2, IL-6 and TNF$\alpha$, resulting in the further progression of the disease (Das, 2007). These proinflammatory cytokines can induce oxidative stress by enhancing the production of ROS by monocytes, macrophages and leukocytes. Since PUFA and their eicosanoid derivatives modulate inflammation, they play a significant role in this disease (Das, 2006a). Decreases in ALA-derived LC-PUFA such as EPA and DHA seen in recent endothelial cell PUFA deficiency, increases the production of proinflammatory cytokines and free radicals which results in the development of insulin resistance (Das, 2006a). As an example, early studies in Greenland Eskimos, a population consuming a high fat diet, but rich in $n$-3 PUFA, showed that ingestion of EPA and DHA led to decreases in the mortality rate from CVD (Bang HO, 1976). Similarly, Japanese populations eat more fish than North Americans and present a lower rate of acute myocardial infarction and atherosclerosis (Holub, 2002; Menotti et al., 1999). Later studies have further demonstrated strong associations between $n$-3 PUFA and decreased risks of CVD (He et al., 2004; Lemaitre et al., 2003; Mozaffarian et al., 2005).
The role of n-6 PUFA in CVD is much more complex than the role of n-3 PUFA. PGE₂, PGF₂α, TXA₂ and LTs produced from AA metabolism are proinflammatory (Das, 2007). TXA₂ acts as a potent vasoconstrictor and powerful activator of platelet aggregation (Sellers and Stallone, 2008). Studies have shown that TXA₂ promotes the initiation and progression of atherosclerosis by regulating platelet activation and leukocyte-endothelial cell interactions (Kobayashi et al., 2004). LTB₄ acts as a potent chemotactic agent, by inducing the generation of ROS, activating neutrophils and inducing the aggregation and adhesion of leukocytes to the vascular endothelium (Das, 2007). The leukotrienes LTC₄, LTD₄ and LTE₄ induce vasoconstriction and bronchospasm (Das, 2007). Since AA is derived from LA, it is a substrate for the synthesis of a variety of proinflammatory molecules and therefore, a reduction of LA intake will reduce tissue AA content, which in turn will reduce any inflammatory potential and therefore lower the risk for CVD (Harris et al., 2009). There are many other lines of evidence that link LA with atherosclerosis. Endothelial dysfunction (ED), is a characteristic of early-state atherosclerosis common in patients with insulin resistance and diabetes (Maingrette and Renier, 2005). One recent review reported that diets enriched with LA increase the LA content of LDL and its susceptibility to oxidation, whereby oxidative modification increases the atherogenicity of LDL cholesterol (Simopoulos, 2008). Studies have also shown that in patients with type 2 diabetes susceptible to developing ED, there are substantial increases in LA concentrations in all LDL subfractions (Prescott et al., 1999). Cellular oxidative stress associated with LA oxidation of LDL and LA mediated ED, is a critical signal transduction pathway involved in NF-κB activation, whereby NF-κB is critical for the expression of inflammatory genes associated with ED (Maingrette and Renier, 2005). The susceptibility of LDL to oxidation by LA and its associated
metabolites is linked to the severity of coronary atherosclerosis development (Regnstrom et al., 1992; Simopoulos, 2008). Despite the evidence to suggest that n-6 PUFA consumption increases the risk of developing CVD, recent evidence has suggested that both LA and ALA have the ability to prevent CVD (Poudel-Tandukar et al., 2009). In this study, LA significantly reduced levels of CRP, an inflammatory marker, upregulated in CVD in Japanese men (Poudel-Tandukar et al., 2009). However, other evidence to suggest that n-6 PUFA have an anti-inflammatory effect when consumed in such high quantities, such as that seen in the Western diet, is limited.

Since it has been proposed that diets high in LA reduce ALA metabolism (Liou et al., 2007) and since ALA metabolites such as EPA and DHA have been shown to reduce mortality rates from CVD (Bang HO, 1976; Holub, 2002; Menotti et al., 1999), the balance of n-6 and n-3 PUFA is important in the prevention of atherosclerosis and CVD.

1.1.6.3 IBD

IBD is classified as a group of chronic systemically natured diseases of unclear pathology which cause inflammation of the digestive tract, including Crohn’s disease (CD) and ulcerative colitis (UC) (Lucendo and De Rezende, 2009). While environmental factors indeed play a significant role in the etiology of the disease, more recent attention has been placed on various dietary and nutritional factors, specifically the lipid components of the diet as triggers of IBD (Cashman and Shanahan, 2003; Lucendo and De Rezende, 2009). It is difficult to suggest that dietary influences or supplementation can reduce the incidence of IBD or impact beneficially (through anti-inflammatory effects) upon disease progression since, like many chronic diseases, IBD is multifactorial. Despite this, lower prevalence of IBD has been observed with
consumption of diets rich in n-3 LC-PUFA derived from fish oils, such as that seen of the Greenland Eskimos (Bang et al., 1980; Kromann and Green, 1980). It has also been reported that patients of IBD who supplement their diets with n-3 PUFA, show anti-inflammatory actions, with decreased production of LTB₄ by neutrophils and colonic mucosa, resulting from incorporation of the n-3 PUFA into the gut mucosal tissue (Hawthorne et al., 1992; Shimizu et al., 2003). A recent study using IL-10 knockout mice (mice that spontaneously develop colitis) demonstrated significantly reduced colonic inflammation when fed n-3 PUFA-rich fish oil compared with mice that were fed n-6 PUFA-rich corn oil (Chapkin et al., 2007). In Japan, increased reports in the incidence of IBD correlate with the increased dietary intake of n-6 PUFA (Sakamoto et al., 2005; Shoda et al., 1996). Importantly, while n-3 PUFA decrease production of LTB₄ by neutrophils and colonic mucosa (Hawthorne et al., 1992; Shimizu et al., 2003), metabolism of AA increases the production of LTB₄ within the inflamed intestinal mucosa of IBD (Sharon and Stenson, 1984). A more recent report demonstrated abnormal prevalence of the enzymes that co-ordinate to generate LTB₄ from membrane-derived AA in active IBD biopsies (Jupp et al., 2007). The recruitment of neutrophils and other leukocytes to the IBD gut mucosa seen with colonic injury may be a direct result of the increased ability to generate LTB₄ from AA (Jupp et al., 2007). It is clear from the literature that n-3 PUFA have a positive effect on reducing the risk of IBD (Belluzzi et al., 1996; Ferrucci et al., 2006; Sijben and Calder, 2007). The situation is less clear for n-6 PUFA, although the proinflammatory eicosanoids derived from AA have been shown to play a crucial role in the pathogenesis of all these related inflammatory disorders. Since n-3 PUFA have been shown to alleviate the progression
of IBD, while n-6 PUFA have been implicated in the origin of IBD, the importance of a balance in the ratio of n-6: n-3 in today’s dietary regime is highlighted.

1.1.6.4 RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a long-term disease that leads to inflammation of the joints and surrounding tissues, causing pain, swelling and impaired function. It is characterised by infiltration of T-lymphocytes, macrophages, and plasma cells into the synovium, with the initiation of a chronic inflammatory state that involves the overproduction of proinflammatory cytokines (Calder, 2008b). Studies have shown that AA-derived eicosanoids, PGE<sub>2</sub> and PGI<sub>2</sub>, play a role in the pathogenesis of rheumatoid arthritis (Honda et al., 2006; Pulichino et al., 2006). PGI receptor-deficient (IP<sup>-/-</sup>) mice subjected to collagen induced arthritis (CIA), showed a significant reduction in arthritic scores and reduction in IL-1β and IL-6 levels in arthritic paws (Honda et al., 2006). Inhibition of both PGE receptors (EP2 and EP4), suppressed inflammatory events and arthritis in CIA. These results suggest that both PGE<sub>2</sub> and PGI<sub>2</sub> participate in rheumatoid arthritis. Supplementation with n-3 PUFA has been demonstrated to modulate the activity of inflammatory factors that cause cartilage destruction during arthritis (Calder, 2008b; Curtis et al., 2000). Moreover, by decreasing n-6 PUFA intake (particularly AA) down to less than 90 mg/day through an anti-inflammatory lactovegetarian (vs. normal Western) diet was shown to improve the clinical symptoms associated with rheumatoid arthritis (Adam et al., 2003).

1.1.6.5 ALZHEIMER’S DISEASE (AD)
AD is the most common form of dementia in the elderly, clinically characterised by memory dysfunction, loss of lexical access, spatial and temporal disorientation and impaired judgement (Corsinovi et al., 2011). The pathogenesis of AD is extremely complex, with genetic factors, education and lifestyle all playing crucial roles in disease onset. However, a poor understanding of the pathogenesis of AD means that there are no curative treatments yet available. Recently, the role of diet has gained much interest in both the pathogenesis and prevention of this disease. The role of $n$-6 PUFA and oxidised eicosanoid derivatives of $n$-6 PUFA have recently been reviewed as contributing to β-amloid deposition, a hallmark of AD onset and progression (Björkhem et al., 2009; Whelan, 2008). AA is distributed in several different cell types in both the grey and white matter in the brain (Corsinovi et al., 2011). The role AA plays in oxidative stress and lipid peroxidation has already been discussed in relation to NAFLD, however, oxidative stress and production of ROS has also been suggested to play a role in AD, thus suggesting a role of AA and lipid oxidation products (eicosanoids) in the onset and progression of the disease (Lee et al., 2010; Rothman and Mattson, 2010). Furthermore, the enhanced consumption of $n$-6 PUFA leads to an excessive production of the proinflammatory cytokines derived from AA through COX and LOX enzymatic activity which lead to brain damage (Farooqui et al., 2007; Tassoni et al., 2008). As an example, a study using transgenic mice with memory impairment and β-amyloid deposition, fed a diet poor in $n$-3 PUFA but rich in $n$-6 PUFA was found to have a significant decrease in the postsynaptic receptor complex in the brain which regulates memory and learning and a net potentiation of programmed cell death (Calon et al., 2005). In contrast, $n$-3 PUFA may play a role in the prevention of AD. Studies have shown that DHA provides support to learning and memory events in
animal models of AD and protection against the disease (Hashimoto et al., 2002; Lim et al., 2005; Schaefer et al., 2006). Another recent epidemiological study indicated a relationship between higher fish consumption and improved cognitive function in later life (Dangour et al., 2009). Both DHA and EPA have been shown to competitively counteract the production of proinflammatory eicosanoids derived from n-6 PUFA in the brain of AD patients (Freeman et al., 2006). The neuroprotective role of EPA has been demonstrated since EPA competes with AA for incorporation into cell membrane phospholipids and for oxidation by the COX enzyme, thus exerting anti-inflammatory actions. The resulting production of anti-inflammatory PGE\(_3\) might result in decreased levels of proinflammatory PGE\(_2\) (Freemantle et al., 2006). The balance between the n-6: n-3 PUFA ratio may therefore play a crucial role in the onset of AD. A recent study showed that a lower n-6: n-3 PUFA ratio was associated with a lower incidence of dementia, especially in depressed patients (Samieri et al., 2008). Furthermore, we have previously demonstrated in patients with depression, increases in plasma AA and IL-6 associated with inflammation (Dinan et al., 2009). Therefore, a dietary pattern consisting of lower n-6 PUFA and higher n-3 PUFA or a more balanced n-6: n-3 PUFA ratio may be therapeutic in the pathogenesis of AD.

1.1.7 CONCLUSION

Increases in the ratio of n-6: n-3 PUFA, characteristic of the Western diet, could potentiate inflammatory processes and consequently predispose to or exacerbate many inflammatory diseases. The change in ratio and increase in n-6 PUFA consumption changes the production of important mediators and regulators of
inflammation and immune responses towards a proinflammatory profile. Chronic conditions such as CVD, diabetes, obesity, rheumatoid arthritis, and IBD are all associated with increased production of LTB₄, TXA₂, IL-1β, IL-6 and TNFα, whereby the production of these factors increases with increased dietary intake of n-6 PUFA and decreased dietary intake of n-3 PUFA. In conclusion, the unbalanced dietary consumption of n-6: n-3 PUFA is detrimental to human health, and so, the impact of dietary supplementation with n-3 PUFA upon the alleviation of inflammatory diseases, more specifically, NAFLD, needs to be more thoroughly investigated.

1.1.8 ACKNOWLEDGEMENTS

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1.1.9 REFERENCES


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TABLE 1.1.1 PUFA content of dietary oils

<table>
<thead>
<tr>
<th>Fat type</th>
<th>LA</th>
<th>ALA</th>
<th>AA</th>
<th>EPA + DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lard</td>
<td>8600</td>
<td>1000</td>
<td>1070</td>
<td></td>
</tr>
<tr>
<td>Butter fat</td>
<td>2300</td>
<td>1400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coconut oil</td>
<td>1400</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef tallow</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Unsaturated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1)Monounsaturated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peanut oil</td>
<td>23900</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pecans</td>
<td>20600</td>
<td>1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Almonds</td>
<td>9860</td>
<td>260</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olive oil</td>
<td>8000</td>
<td>950</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avocado</td>
<td>1970</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2)Polyunsaturated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>-Omega-6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Safflower oil</td>
<td>74000</td>
<td>470</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>60200</td>
<td>500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean oil</td>
<td>53400</td>
<td>7600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn oil</td>
<td>50000</td>
<td>900</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotton seed oil</td>
<td>47800</td>
<td>1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walnut</td>
<td>34100</td>
<td>6800</td>
<td>590</td>
<td></td>
</tr>
<tr>
<td>Brazil nut</td>
<td>24900</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>-Omega-3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linseed oil</td>
<td>13400</td>
<td>5530</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canola oil</td>
<td>19100</td>
<td>8600</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmon</td>
<td>440</td>
<td>550</td>
<td>300</td>
<td>1200</td>
</tr>
<tr>
<td>Tuna</td>
<td>260</td>
<td>270</td>
<td>280</td>
<td>400</td>
</tr>
<tr>
<td>Herring</td>
<td>150</td>
<td>62</td>
<td>37</td>
<td>1700</td>
</tr>
<tr>
<td>Trout</td>
<td>74</td>
<td>30</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>Cod</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>300</td>
</tr>
</tbody>
</table>

Data are expressed as mg/100g edible portion. Data are elaborated from (Benatti et al., 2004; Das, 2006b; Hughes CL, 2002). Content of fatty acids may vary slightly according to species, sources and analytical factors.
**TABLE 1.1.2** Proinflammatory effects of n-6 PUFA-derived eicosanoids and anti-inflammatory effects of n-3 PUFA-derived eicosanoids

**Proinflammatory effects of n-6 PUFA-derived eicosanoids**

<table>
<thead>
<tr>
<th>AA (n-6) derived eicosanoids</th>
<th>Physiological effects</th>
<th>Organs or cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGD₂</td>
<td>Bronchoconstriction</td>
<td>Bronchi</td>
</tr>
<tr>
<td></td>
<td>Proinflammatory</td>
<td>Activation of eosinophils</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Proarrhythmic</td>
<td>Vessels</td>
</tr>
<tr>
<td></td>
<td>Induces fever</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Causes pain</td>
<td>Nociceptor sensory neurons</td>
</tr>
<tr>
<td></td>
<td>Increases production of IL-6</td>
<td></td>
</tr>
<tr>
<td>PGF₂</td>
<td>Bronchoconstriction</td>
<td>Bronchi</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Proarrhythmic</td>
<td>Vessels</td>
</tr>
<tr>
<td></td>
<td>Causes pain</td>
<td>Nociceptor sensory neurons</td>
</tr>
</tbody>
</table>

**Thromboxanes**

<table>
<thead>
<tr>
<th></th>
<th>Physiological effects</th>
<th>Organs or cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>TXA₂</td>
<td>Proaggregation</td>
<td>Platelets</td>
</tr>
<tr>
<td></td>
<td>Vasoconstriction</td>
<td>Vessels</td>
</tr>
<tr>
<td></td>
<td>Bronchoconstriction</td>
<td>Bronchi</td>
</tr>
<tr>
<td>TXB₂</td>
<td>Proaggregation</td>
<td>Platelets</td>
</tr>
<tr>
<td></td>
<td>Vasoconstriction</td>
<td>Vessels</td>
</tr>
<tr>
<td></td>
<td>Bronchoconstriction</td>
<td>Bronchi</td>
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**Leukotrienes**

<table>
<thead>
<tr>
<th></th>
<th>Physiological effects</th>
<th>Organs or cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTB₄</td>
<td>Proinflammatory</td>
<td>Leukocytes</td>
</tr>
<tr>
<td></td>
<td>Chemotaxis</td>
<td>Leukocytes</td>
</tr>
<tr>
<td></td>
<td>Release of reactive oxygen species</td>
<td>Granulocytes</td>
</tr>
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</table>

**Anti-inflammatory effects of n-3 PUFA derived eicosanoids**

<table>
<thead>
<tr>
<th>EPA and DHA (n-3) derived eicosanoids</th>
<th>Physiological effects</th>
<th>Organs or cells</th>
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</thead>
<tbody>
<tr>
<td>PGI₃</td>
<td>Antiarrhythmic</td>
<td>Vessels</td>
</tr>
<tr>
<td>PGE₃</td>
<td>Antiarrhythmic</td>
<td>Vessels</td>
</tr>
<tr>
<td>PGF₃, PGD₃</td>
<td>Antiarrhythmic</td>
<td>Vessels</td>
</tr>
<tr>
<td>TXA₃</td>
<td>Antiaggregation</td>
<td>Platelets</td>
</tr>
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<td>TXB₃</td>
<td>Antiaggregation</td>
<td>Platelets</td>
</tr>
<tr>
<td>LT₄, LTC₄, LTD₄, LTE₄</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTB₅</td>
<td>Anti-inflammatory</td>
<td>Leukocytes</td>
</tr>
<tr>
<td>RVE₁</td>
<td>Antiaggregation</td>
<td>Platelets</td>
</tr>
<tr>
<td></td>
<td>Anti-inflammatory</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>RVD</td>
<td>Anti-inflammatory</td>
<td></td>
</tr>
<tr>
<td>NPD₁</td>
<td>Anti-inflammatory</td>
<td>Retina (photoreceptor cells) and brain</td>
</tr>
<tr>
<td></td>
<td>Antiapoptotic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreases oxidative stress</td>
<td></td>
</tr>
</tbody>
</table>

Data elaborated from (Bazan, 2009; Fredman and Serhan, 2011; Harizi et al., 2008; Schmitz and Ecker, 2008).
The metabolism of PUFA is a complex process involving several enzymes of desaturation, elongation, and β-oxidation. Shown here is the pathway of both n-6 and n-3 PUFA metabolism to more unsaturated, long-chain members of each family. Also shown are their respective eicosanoid derivatives. Data elaborated from (Schmitz and Ecker, 2008).
**FIGURE 1.1.2** Effects of unbalanced $n$-$6$ : $n$-$3$ PUFA intake on various diseases of inflammation

Dietary imbalance in the consumption of $n$-$6$ and $n$-$3$ PUFA, representative of the Western diet. Greater consumption of $n$-$6$ PUFA leads to an increase in their metabolism to their LC-PUFA derivatives (AA). Decreases in $n$-$3$ PUFA consumption leads to a decrease in their metabolism to their LC-PUFA derivatives (EPA/DHA). The increase in AA in cell membrane phospholipids leads to an increase in COX and LOX enzyme production of AA derived eicosanoids and a decrease in EPA/DHA derived eicosanoids, leading to an increase in inflammation and proinflammatory cytokine production. This in turn leads to a decrease in PPARα gene expression, while there is
an increase in both SREBP-1c and NF-κB gene expression. This change in gene expression can also cause an increase in lipogenesis, as well as increasing inflammation. The result is an increase in various diseases of inflammation, some of which are highlighted in the figure.
Chapter 1.2

Microbiota, metabolite production and host health
1.2.1 ABSTRACT

A healthy gut microbiota contributes towards the health and welfare of the host in a number of ways including participation in the correct development and functioning of the immune system, performance of a broad range of vital metabolic and biochemical functions and production of health-beneficial bioactive metabolites. Perturbations in the composition of the gut microbiota are highlighted in many disease states and are thought in some instances to render the host susceptible to infection. Metabolites produced by the gut microbiota include antimicrobials, conjugated polyunsaturated fatty acids (PUFA) and exopolysaccharides (EPS) which benefit host health. Evidence is also emerging of microbially-produced molecules with neuroactive functions that can have influences across the brain-gut axis. For example, gamma-aminobutyric acid (GABA), serotonin, catecholamines and acetylcholine, may modulate neural signalling within the enteric nervous system, when released in the intestinal lumen and consequently signal brain function and behaviour. Dietary supplementation with probiotics and prebiotics are fast becoming the most widely used dietary adjuncts to modulate the gut microbiota. Furthermore, evidence is emerging of the interactions between administered microbes and dietary substrates, i.e. fatty acids, which may promote neurological development in infants, as well as improved mental health status among adults.

1.2.2 INTRODUCTION

The microbial ecosystem residing in the human gut harbours over 100-fold more genes than the human genome (Backhed et al., 2004; Ley et al., 2006; Qin et al., 2010) and is tantamount to a virtual organ. To a large extent, intestinal ecological conditions
are set by the host and resident commensals must adapt to this environment. Host-microbe, environment-microbe and microbe-microbe interactions may also dictate the composition of this microbial community. A symbiotic relationship between the gut microbiota and host is the most favourable where both partners benefit; the host provides protection and nutrients for the microorganisms to flourish within (Maynard et al., 2012), while the microbiota contribute to food digestion, inhibit the growth of potential invading pathogens, convert harmful compounds to less toxic substances and produce bioactive molecules which play a role in host physiology (Marques et al., 2010). However, disruptions to this symbiotic relationship can occur, during which certain commensal gut microbes can adopt pathogenic potential such as small intestinal bacterial overgrowth (Bouhnik et al., 1999; Riordan et al., 2001) and/or translocation to other tissues and organs (Maes et al., 2013; Teltschik et al., 2012). The relationship between the gut microbiota and host is however important for health, and although it remains unclear whether disease development is causal or consequential of an altered microbiota, an increasing body of evidence describes a link between the two.

Microbial colonisation of the infant intestine begins at birth (Koenig et al., 2011). Extrinsic factors contribute to the initial colonisation of the infant gut (Butel et al., 2007; Scholtens et al., 2012), whereby feeding regime (Bezirtzoglou et al., 2011; Fallani et al., 2010), gestational age (Hallab et al., 2013) and antibiotic therapy to which the infant is exposed to (Fouhy et al., 2012; Hussey et al., 2011) can all significantly impact early colonisation. Intestinal establishment of a healthy microbiota in early life is believed to have a profound impact on the development and maturation of the immune system (Palmer et al., 2007). Vaginally born infants are initially colonised by
faecal and vaginal bacteria from the mother, whereas infants delivered by Caesarean-section render the gut susceptible to colonisation by maternal skin microbiota and bacteria from the hospital environment (Domínguez-Bello et al., 2010; Huurre et al., 2008; Penders et al., 2006). It has been shown that vaginally born babies have higher numbers of *Lactobacillus* and *Bifidobacterium*, compared with infants delivered by Caesarean-section (Domínguez-Bello et al., 2010). The weaning process determines the transition of an unstable infant microbiota to a more complex adult-like microbial ecosystem (Fallani et al., 2011; Koenig et al., 2011). The development and diversification of the gut microbiota continues into adulthood and is further influenced by several factors, including diet and environment (Yatsunenko et al., 2012). To a large extent, the gut microbiota remains relatively stable throughout adulthood, unless perturbed by extrinsic or host factors, for example, antibiotic treatment and inflammation, respectively.

The tools used to study the link between gut microbial diversity and health status have improved our knowledge of the host-microbe relationship significantly. Culture-independent analysis of the composition and functional capacity of the gut microbiome targets the 16S rRNA gene, due to its presence in all prokaryotes with the existence of variable domains that allow different taxa to be identified. Although compositional studies generate a large volume of data, they fail to provide direct information regarding the microbial viability or the functional potential of the populations present and so the knowledge generated is somewhat limited in these aspects. Metagenomic studies go beyond the 16S rRNA gene to sequence small fragments of metagenomic DNA at random to characterise the full genetic content and functional potential of the microbial community (Kurokawa et al., 2007; Qin et al., 2007).
The development of methods used to analyse gene expression (metatranscriptomics), protein products (metaproteomics) and metabolic profiles (metabolomics) of the gut microbiota have further enabled such studies to identify the microbial activity and to link this with compositional analysis, to determine host-microbe interactions (Guinane and Cotter, 2013).

Dietary interventions with probiotics and prebiotics, due to the dynamic nature of the gut microbial ecosystem, have fast become an attractive means of self-manipulating the microbiota to improve health status, and have been extensively reviewed (Sanders et al., 2013; Vyas and Ranganathan, 2012). Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” and have been shown to improve intestinal barrier function, modulate the immune system and enhance the host defence system by competing against pathogens for nutrients and binding sites. In addition, numerous probiotic intervention studies have revealed their functional capacity to improve certain gastrointestinal disorders, for example, irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) (Cappello et al., 2013; Whelan and Quigley, 2013) and antibiotic-associated diarrhoea (AAD) (Hempel et al., 2012; Hickson, 2011). *Bifidobacterium* and *Lactobacillus* are the main genera of microorganisms used as probiotics as many of them can survive gastrointestinal transit, have the capability to adhere to intestinal epithelial cells and are regarded as safe (Marques et al., 2013). Prebiotics on the other hand, are ‘non-digestible food ingredients that selectively stimulate the growth of beneficial indigenous microbes already established within the gut such as bifidobacteria and lactobacilli (Saulnier et al., 2009). Typically, prebiotics must reach the large intestine unaltered, resisting host digestion, absorption and adsorption to be
fermented by the gut microbiota. Commonly used prebiotics include inulin, fructooligosaccharides (FOS) and galactooligosaccharides (GOS) (Preidis and Versalovic, 2009). The fermentation of prebiotics by the gut microbiota generates short chain fatty acids (SCFA), such as butyric and acetic acids, which have demonstrated numerous health benefits in vivo (Preidis and Versalovic, 2009; Saulnier et al., 2009). This review will focus on the importance of the microbiota to host health and in establishing a healthy immune system and describes some of the known beneficial bioactive metabolites produced by the microbiota that impact on health.

1.2.3 ROLE OF THE MICROBIOTA IN ESTABLISHING A HEALTHY IMMUNE SYSTEM

During the first year of life, the immature developing gut microbial ecosystem rapidly shapes the maturation of the infant immune system, while the immune system also influences the gut microbiota (Hansen et al., 2012). From birth, breast milk provides passive transfer of maternal antibodies to the infant which shapes both the immature immune system and gut microbiota (Maynard et al., 2012). Much of the information regarding the influence of gut microbes on the host immune system are generated from studies using germ-free animals. Germ-free animals are those born and reared without exposure to microorganisms and so the immune responses of such animals have not been influenced by interactions with molecules of commensal and pathogenic microorganisms. Unsurprisingly, germ-free mice show defects in both the development of the immune system and in immune responses. One of the first immunological defects observed in these animals was a marked reduction in antibodies produced within the intestine (Moreau et al., 1978). Furthermore, germ-free animals show extensive defects in the development of gut-associated lymphoid
tissue (GALT) and have fewer and smaller Peyer’s patches and mesenteric lymph nodes (MLNs), compared with animals housed under specific pathogen-free conditions (Falk et al., 1998; Hoshi et al., 1992; Macpherson and Harris, 2004; Pollard and Sharon, 1970). Intestinal epithelial cells have many immunological functions; they secrete and respond to various cytokines and express molecules that directly interact with lymphocytes and line the gut to form a physical barrier between the luminal contents (including the microbiota) and the underlying cells of the immune system (Round and Mazmanian, 2009). Germ-free mice demonstrate a reduced number of these cells, whereby the toxicity of intestinal epithelial cells from germ-free mice is compromised (Imaoka et al., 1996; Umesaki et al., 1993) and demonstrate a decreased cell turnover rates of these cells (Abrams et al., 1963). Furthermore, animals lacking a gut microbiota are more susceptible to infection due to a poorly developed immune system; e.g. germ-free guinea pigs challenged with the enteric pathogen Shigella flexneri demonstrated a decrease in the immune resistance to infection coupled with an increase in mortality (Sprinz et al., 1961), while infection with the intracellular pathogen Listeria monocytogenes in germ-free mice resulted in decreased pathogen clearance, compared with conventionalised animals (Zachar and Savage, 1979).

Deliberate colonisation of the sterile gut of these animals either with a single microbial species or a defined species mixture, termed “gnotobiotics” is a powerful technological tool for determining which host immune functions are genetically encoded and which require interactions with microbes (Hooper and Macpherson, 2010). For example, colonisation of germ-free animals with a single bacterium, Bacteroides fragilis, has been shown to protect against inflammation in an animal model of experimental colitis (Mazmanian et al., 2005; Mazmanian et al., 2008). Collectively, these observations
suggest that developmental defects through the absence of a gut microbial ecosystem compromise immune function of the host at the tissue, cellular and molecular levels and highlight a role of the microbiota in the establishment of a functional immune system.

1.2.4 INTERRUPTION OF THE SYMBIOTIC HOST-MICROBE RELATIONSHIP WITH IMPLICATIONS FOR IMMUNE FUNCTION AND HEALTH

Disruptions in the development of the gut microbiota from infancy are common. Early antibiotic therapy of up to eight weeks after treatment has been shown to impact the gut microbiota composition of infants, which could have a negative impact on the development of the immune system, predisposing the infant to asthma, obesity and allergies (Fouhy et al., 2012; Hussey et al., 2011). Thus, disruptions in the host-microbe relationship can undoubtedly predispose to disease, from infancy to adulthood. Within this respect, there has been a rapid increase in the development of disorders such as IBS, asthma, rheumatoid arthritis, diabetes and obesity, particularly within developed, Western populations. Indeed, the role of a perturbed gut microbial ecosystem in such diseases is becoming more evident, although much future work is needed to decipher the mechanisms which link this relationship with disease.

1.2.4.1 IBD

IBD comprises a group of disorders characterised by severe inflammation in the intestine. The symptoms and inflammation pattern in the host characterise IBD as either Crohn’s disease (CD) or ulcerative colitis (UC). While the exact causes of IBD
remain unclear, the onset of both conditions is generally thought to be due to an overall disruption in the host-microbe relationship, and not by a single causal organism (Lepage et al., 2011; Martinez et al., 2008). Recently reviewed, numerous studies which indicate a role of the gut microbiota in the manifestation of IBD generally conclude that the gut microbiota are involved in the development of mucosal lesions causing intestinal inflammation in the host (Manichanh et al., 2012). Inflammatory damage in IBD has been linked to alterations in the relative abundances of *Enterobacteriaceae*, *Ruminococcaceae* and *Leuconostocaceae* (Morgan et al., 2012) and an overall decrease in bacterial diversity (Manichanh et al., 2006; Sokol et al., 2006). The incidence of *Clostridium difficile* carriage, an opportunistic pathogen frequently linked with AAD, has been reported to be over 8-fold higher in patients suffering from IBD, compared with healthy controls (Clayton et al., 2009).

**1.2.4.2 TYPE-1 AND TYPE-2 DIABETES**

The incidence of both type-1- (T1D) and type-2- (T2D) diabetes have become increasingly prevalent in recent decades. While genetic factors play a major role in disease onset, particularly in predisposing individuals to T1D, T2D is principally linked to obesity associated insulin resistance. Recent studies demonstrate disruptions to the host-microbe relationship and gut microbial composition and diversity associated with both T1D and T2D. Compositional sequencing studies have revealed a reduction in the relative proportions of Firmicutes, while Bacteroidetes were enriched in T2D subjects, compared with healthy controls (Larsen et al., 2010). Identification of gut microbial markers associated with the moderate degree of microbial disruption in patients with T2D could be useful in the future management of this disease (Qin et al., 2012).
Furthermore, increases in opportunistic pathogens such as *Clostridium* were also identified as contributing to disruption in the host-microbe interactions associated with T2D (Qin et al., 2012). Creating a link between the gut microbiota and T1D is much more difficult, since genetic factors play a more significant role in this disease. However, evidence indicates that alterations in the intestinal microbiota are associated with T1D and subsequent insulin dependence in various models of the disease. While one study demonstrated that the stool of bio-breeding diabetes-resistant (BBDR) rats contained higher relative abundances of *Lactobacillus* and *Bifidobacterium*, compared with bio-breeding diabetes-prone (BBDP) rats (Roesch et al., 2009), others have reported that lactate producing species such as *Lactobacillus*, *Lactococcus* and *Bifidobacterium* were increased in the stool of children who tested positive for T1D associated autoimmunity (Brown et al., 2011; Giongo et al., 2011). Furthermore, low relative abundances of two of the most common *Bifidobacterium* species, *B. adolescentis* and *B. pseudocatenulatum* have been associated with autoimmunity in children who tested positive for at least two T1D-associated autoantibodies (de Goffau et al., 2013).

1.2.4.3 OBESITY

Obesity develops from a prolonged imbalance of energy intake and expenditure. Undoubtedly, while lifestyle, genetic factors, diet and exercise contribute largely to this modern epidemic, an increasing body of evidence suggests that disruptions to the host-microbe relationship contributes towards disease (Ley, 2010; Ley et al., 2005; Tilg and Kaser, 2011; Turnbaugh et al., 2006). Identifying specific populations which may be associated with weight gain has been the subject of much debate, often differing
between the various models of obesity in both rodent and humans. Genetically (ob/ob) and diet-induced obese (DIO) mice have demonstrated an increase in the Firmicutes: Bacteroidetes ratio, compared with their lean counterparts (Ley et al., 2005). Furthermore, weight loss in human subjects has been linked with decreasing the Firmicutes: Bacteroidetes ratio (Ley et al., 2006). However, the relevance of the Firmicutes: Bacteroidetes ratio in obesity is still unclear (Schwiertz et al., 2010). The gut microbiota also increase the dietary energy harvesting capacity of the host (Murphy et al., 2010) and conventionally raised mice have 40% more body fat than their germ-free counterparts, whereby colonisation with what is considered a conventional gut microbiota induced hepatic lipogenesis and increased lipid storage in adipocytes (Backhed et al., 2004).

Obesity is also associated with low grade inflammation in the host which may be linked to host-microbe interactions. Data from several studies have revealed that the lipopolysaccharide (LPS) endotoxin derived from certain components of the gut microbiota contributes towards obesity associated inflammation. Endogenous LPS is continuously produced in the gut as a consequence of the death of Gram-negative bacteria, since LPS is a component of the Gram-negative bacterial cell wall and acts through the Toll-like receptor 4 (TLR4)/ MyD88/ NF-κB signalling pathway. LPS induced inflammation could also be an early factor which triggers high-fat diet-induced metabolic diseases, otherwise known as metabolic endotoxemia (Cani et al., 2007). It has been shown that high-fat feeding increased plasma LPS levels throughout the day, compared with controls, resulting in significant increases in fasting blood glucose, insulin, liver triglyceride content, body weight and proinflammatory cytokine mRNA expression, similar to mice that were infused with LPS (Cani et al., 2007). Further
studies examined the effect of changes in the gut microbiota leading to LPS induced metabolic endotoxemia in the host (Cani et al., 2008). It was revealed that while plasma LPS levels were increased following high-fat feeding relative to controls, this result was overturned in high-fat diet-fed mice following antibiotic treatment (Cani et al., 2008). Such studies reveal that disruptions to host-microbe interactions within the gut following obesity and high-fat dietary feeding may generate increased gastrointestinal levels of microbial-derived LPS endotoxin, associated with metabolic endotoxemia.

1.2.5 HOST-MICROBE INTERACTIONS, GENERATION OF LC-PUFA AND MICROBIAL METABOLITE PRODUCTION WITH HEALTH EFFECTS

The products of human enteric microbial metabolism often act as signalling molecules, developing ‘intelligent communication systems’ in the body. These microbial metabolites termed ‘pharmabiotics’, can exert beneficial health effects which directly impact host intestinal function but may also affect the liver and brain (Shanahan, 2009). Host-microbe interactions can together co-metabolise dietary components to produce a large array of molecules with beneficial impacts on health. Commensal bacteria have been shown to synthesise essential vitamins such as vitamin K2 and vitamin B12 (Said, 2011), can alter n-3 PUFA metabolism to generate increased levels of LC-PUFA metabolites such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Wall et al., 2012; Wall et al., 2009), can produce conjugated fatty acid derivatives of PUFA such as conjugated linoleic acid (CLA) and conjugated α-linolenic acid (CALA) (Barrett et al., 2012a; Hennessy et al., 2012) and can increase production of SCFA (Wall et al., 2012). The beneficial impacts of some of these...
bioactive compounds on host health are reviewed below and summarised in Table 1.2.1.

1.2.5.1 VITAMIN SYNTHESIS

Some commensals of the human gut microbiota possess the ability to synthesise menaquinone (vitamin K₂), as well as many of the water-soluble B vitamins, such as biotin, cobalamin, folate, nicotinic acid, panthotenic acid, pyridoxine, riboflavin and thiamine (LeBlanc et al., 2013). In particular, many *Bifidobacterium* strains have been shown to exhibit vitamin production capabilities (Deguchi et al., 1985; Noda et al., 1994; Pompei et al., 2007). Vitamin K is a lipophilic vitamin which acts as a co-factor for the enzyme γ-carboxylase which converts specific glutamyl residues in a limited number of proteins to γ-carboxyglutamyl (Gla) residues, responsible for high-affinity binding of calcium ions (LeBlanc et al., 2013). The daily requirement for vitamin K is fulfilled by dietary phylloquinone, present in plants and to an undermined extent, by bacterially produced vitamin K₂ (LeBlanc et al., 2013). Vitamin K is important for blood clotting, bone and vascular health and deficiencies have been associated with low bone mineral density (Knapen et al., 1998; Szulc et al., 1994), increased risk of fracture (Luukinen et al., 2000; Szulc et al., 1993) and cardiovascular disease (Geleijnse et al., 2004).

Vitamin B₁₂ is a type of cobalt corrinoid, particularly of the cobalamin group and is solely synthesised by some bacteria and archea. Vitamin B₁₂ biosynthesis was first identified in *Propionibacteriafreudenreichii*, now used in the commercial production of the vitamin (Roessner et al., 2002). Furthermore, *Lactobacillus reuteri* CRL1098 was shown to be the first lactic acid producing bacterial strain capable of producing a
cobalamin-like compound with an absorption spectrum resembling that of standard cobalamin (Taranto et al., 2003). The genetic pathway responsible for the de novo synthesis of vitamin B₁₂ by *L. reuteri* have previously been described for two *L. reuteri* strains (Saulnier et al., 2011). More recently, the presence of bifidobacterial genes, predicted to be involved in the biosynthesis of several B-vitamins have been identified in bifidobacteria residing in faecal samples of adult subjects (Gosalbes et al., 2011; Klaassens et al., 2011; Klaassens et al., 2009).

### 1.2.5.2 POLYUNSATURATED AND CONJUGATED FATTY ACID SYNTHESIS

PUFA contain two or more double bonds and are classified as either *n*-3, *n*-6 or *n*-9, based on the location of the last double bond relative to the terminal methyl end of the molecule. Linoleic acid (LA, 18:2n-6) (precursor to the *n*-6 series of fatty acids) and α-linolenic acid (ALA, 18:3n-3) (precursor to the *n*-3 series of fatty acids) are the simplest members of each family of PUFA and are essential fatty acids. PUFA regulate a wide variety of biological functions, ranging from blood pressure and blood clotting, to the development and functioning of the brain and nervous system. It has been shown that the gut microbiota not only affects fat quantity (Backhed et al., 2004) but also fat quality (Wall et al., 2010; Wall et al., 2009) in various models. CLA refers to a family of positional and geometric isomers of LA which have been associated with several health benefits. The CLA isomers cis-9, trans-11 and trans-10, cis-12 are most often studied for their beneficial in vitro and in some cases in vivo health effects associated with various types of cancer, atherosclerosis, obesity, diabetes, as well as an ability to improve immune function, body composition and bone formation (Belury, 2002; Benjamin and Spener, 2009; Bhattacharya et al., 2006; Brownbill et al., 2005; Chin et
al., 1994; Churrucua et al., 2009; Jaudszus et al., 2005; Kelley et al., 2007; Nagao and Yanagita, 2005; Pariza et al., 2001; Silveira et al., 2007; Valeille et al., 2006; Watras et al., 2007). It has recently been shown that plasma CLA metabolite concentrations in humans following dietary CLA supplementation were comparable with those previously observed in experimental animal models and sufficient enough to exert health benefits (Mele et al., 2013). Considerable species variations amongst bifidobacteria have been observed for PUFA and CLA production. While *Bifidobacterium breve* has been reported as one of the most efficient CLA producers among various strains tested (Coakley et al., 2003; Rosberg-Cody et al., 2004), *Bifidobacterium bifidum* (Rosberg-Cody et al., 2004) and *Bifidobacterium dentium* (Coakley et al., 2003) have also demonstrated good conversion rates *in vitro*. It has been shown that administration of CLA producing strains of bifidobacteria are metabolically active in the gastrointestinal tract of mice and pigs (Wall et al., 2009). Furthermore, administration of *B. breve* NCIMB 702258 in combination with LA resulted in modulation of tissue fatty acid composition, significantly increasing levels of *cis*-9, *trans*-11 CLA in the liver of both mice and pigs (Wall et al., 2009). Increased tissue concentrations of *n*-3 LC-PUFA, EPA and DHA were also found in the adipose tissue of both mice and pigs (Wall et al., 2009). Furthermore, the ratio of arachidonic acid to EPA in the liver and adipose tissue were reduced following *B. breve* supplementation, coupled with an anti-inflammatory cytokine profile in the host (Wall et al., 2009). Both EPA and DHA have previously been shown to exert anti-inflammatory properties (Nobre et al., 2013). In a related study, it was found that administration of *B. breve* NCIMB 702258 in combination with ALA was associated with alterations in the fatty acid composition of the brain, with elevated levels of EPA and
DHA (Wall et al., 2012). Although the mechanisms responsible for the bifidobacteria-mediated changes in host n-3 PUFA composition are largely unclear, such studies have demonstrated that manipulation of the gut microbiota with metabolically active strains may represent a therapeutic strategy for various disorders related to inflammation in the host, through the production of LC-PUFA and PUFA-derived conjugated fatty acids.

1.2.5.3 PRODUCTION OF SCFA

SCFA are the end products of anaerobic gut microbial fermentation of undigested dietary fibers and have important functions in host energy metabolism. Indeed, SCFA play a key role in the prevention and treatment of metabolic and bowel disorders and certain types of cancer (Blouin et al., 2011; Gao et al., 2009; Hu et al., 2010; Scharlau et al., 2009; Tang et al., 2011). The positive influence of SCFA treatment on UC and CD have been demonstrated in various clinical studies (Breuer et al., 1991; Di Sabatino et al., 2005; Harig et al., 1989; Scheppach, 1996; Vernia et al., 1995). Butyrate is the primary energy source for cellular metabolism in the colonic epithelium (den Besten et al., 2013). The colonic epithelial cells of germ-free mice are severely energy-deprived and are characterised by increased activation of AMP-activated protein kinase (AMPK), which senses cellular energy status (Donohoe et al., 2011). SCFA also regulate gene expression in the host by binding to the G-protein coupled receptors (GPCR), GPR41 and GPR43 to impact on several different cellular functions in the host, depending on the cell type (Tremaroli and Backhed, 2012). For example, SCFA suppress inflammation through GPR43 signalling in immune cells (Maslowski et al., 2009; Sina et al., 2009) and modulate secretion of the insulin secreting and antidiabetic hormone glucagon-like peptide-1 (GLP-1) in the distal small intestine and colon (Tolhurst et al., 2012).
1.2.5.4 MICROBIAL PRODUCTION OF EPS

Many organisms including some resident microbes of the gut microbial ecosystem have the ability to synthesise EPS with a large variation in composition, charge and molecular structure (Stack et al., 2010). EPS producing strains are responsible for a “ropy” phenotype and are beneficial in the food and health industries. Health benefits associated with EPS include immunostimulatory effects (Kitazawa et al., 1998; Vinderola et al., 2006), blood cholesterol lowering effects (Maeda et al., 2004; Nakajima et al., 1992) and prebiotic effects (Korakli et al., 2002; O’Connor et al., 2005). Beta-glucan is a water soluble fiber found in cereals, as well as in yeast, bacteria, algae and mushrooms (Theuwissen and Mensink, 2008). The EPS beta-glucan has been reported to have many health promoting properties including immunomodulatory effects (Akramiene et al., 2007; Hida et al., 2009; Tsoni and Brown, 2008; Volman et al., 2008), lowering serum cholesterol levels (Theuwissen and Mensink, 2008; Wilson et al., 2004), antiosteoporotic (Shin et al., 2007), antitumorigenic, anticytotoxic and antimutagenic effects (Gu et al., 2008; Mantovani et al., 2008). Furthermore, oat beta-glucan has also been associated with the ability to modulate satiety, thus controlling appetite (Beck et al., 2009a; Beck et al., 2009b). Heterologous expression of the pediococcal glycotransferase (gtf) gene responsible for the synthesis and secretion of the 2 substituted (1,3) beta-D-glucan in *Lactobacillus paracasei* NFBC 338 increased the stress tolerance of the probiotic, due to EPS production (Stack et al., 2010). Furthermore, *Bifidobacterium breve* UCC2003 has been shown to produce two EPS which have been associated with an increased resilience of this strain to tolerate acid and bile while reducing the intestinal colonisation levels of pathogenic *Citrobacter*
rodentium (Fanning et al., 2012). Thus, EPS production is thought to be important not only in host interactions but also for protection against pathogenic infection.

1.2.6 THE GUT-BRAIN AXIS: MICROBIAL METABOLITE PRODUCTION WITH IMPLICATIONS ON HOST PSYCHIATRIC HEALTH

The gut-brain axis is a bidirectional communication system between the brain and the gut, including the metabolically complex gut microbiota which integrates neural, hormonal and immunological signalling between the gut and the brain (Collins et al., 2012). The gut microbiota and the metabolites they produce may also modulate the peripheral (PNS) and central nervous system (CNS) to influence brain development and function (Forsythe et al., 2010). To date, numerous studies have demonstrated the importance of the gut microbiota in the stress response (Neufeld et al., 2011; Sudo et al., 2004) and neurodevelopmental disorders (de Theije et al., 2011; Desbonnet et al., 2013; Finegold et al., 2010). Commensal microbiota have demonstrated the ability to interact with the serotonergic system in the host by regulating the development of the hypothalamus-pituitary-adrenal (HPA) axis, a neuroendocrine system which controls reactions to stress (Sudo et al., 2004). Recent studies have demonstrated that germ-free mice display a reduction in anxiety like behaviour (Clarke et al., 2013; Heijtza et al., 2011), compared with conventionally colonised mice, possibly through an enhanced HPA response. Another study using germ-free mice described how in the absence of a gut microbiota, mice exhibited deficits in social motivation and preference for social novelty, behavioural characteristics indicative of disruptions in distinct normal social behaviours (Desbonnet et al., 2013). Probiotic intervention has proven successful for the treatment of psychiatric disorders such as anxiety (Bravo et
al., 2011; Messaoudi et al., 2011), depression (Desbonnet et al., 2008) and autism (Hsiao et al., 2013). Administration of *Bifidobacterium infantis* 35624 has displayed antidepressant properties in a maternal separation model of depression (Desbonnet et al., 2008), *Lactobacillus rhamnosus* JB-1 has also demonstrated antianxiety and antidepressant properties through activation of the vagus nerve in mice, compared with broth-fed controls (Bravo et al., 2011) and *Bacteroides fragilis* administration alleviated autistic-like behavioural impairments in communication, social behaviour, social abnormalities, and restricted/repetitive behaviour in mice symptomatic of this disorder, compared with autistic, untreated controls (Hsiao et al., 2013). Furthermore, administration of *Bifidobacterium breve* NCIMB 702258 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 higher concentrations of bioactive fatty acids, arachidonic acid and DHA, compared with unsupplemented controls (Wall et al., 2012), whereby these bioactive fatty acids have a role in neurotransmission and protection against oxidative stress (Henriksen et al., 2008; Yurko-Mauro et al., 2010).

A broad range of microbes, either probiotics or commensals can manufacture and secrete neurochemicals which can positively impact on mental health and thus, could be used for the treatment of CNS disorders, such as anxiety and depression. Recently defined, a psychobiotic is “a live microorganism that, when ingested in adequate amounts, produces a health benefit in patients suffering from psychiatric illness” (Dinan et al., 2013). Moreover, disruptions in the composition of the gut microbiota may lead to a deterioration of gastrointestinal, neuroendocrine and immune pathways, which could in turn lead to alterations in gut-brain interactions and consequently result in disease (Cryan and O'Mahony, 2011). The gut microbiota
produce a range of neurotransmitters and neuromodulators, bioactive metabolites which impact on host psychiatric health, only some of which have been demonstrated in vivo (Table 1.2.1).

1.2.6.1 GABA

GABA is a major inhibitory neurotransmitter of the vertebrate CNS and is the main inhibitory neurotransmitter in the brain. Dysfunctions of GABA have been linked with anxiety and depression (Cryan and Kaupmann, 2005; Schousboe and Waagepetersen, 2007). Certain strains of *Lactobacillus* and *Bifidobacterium* secrete GABA via the same biosynthetic pathway as in neuronal tissue involving conversion of glutamate by the action of the enzyme glutamate decarboxylase and vitamin co-factor pyridoxal phosphate (Komatsuzaki et al., 2008). Furthermore, the GABA producing capability of some bacterial strains is thought to protect the organism from the acidic environment of the stomach (Higuchi et al., 1997). Several human-derived lactobacilli and bifidobacteria were screened for their ability to produce GABA from monosodium glutamate (MSG), and it was found that five strains had this ability (Barrett et al., 2012b). Of these strains, *Lactobacillus brevis* and *Bifidobacterium dentium* were the most efficient GABA producers (Barrett et al., 2012b). Ko et al., (2013) recently demonstrated GABA production in black soybean milk by *L. brevis* FPA3709 and its administration to rats resulted in an antidepressant effect similar to that of fluoxetine, a common antidepressant drug, but without the side-effects such as appetite and weight loss (Ko et al., 2013). At the level of gene expression, ingestion of *L. rhamnosus* JB-1 altered the mRNA expression of both GABA_A and GABA_B, two GABA receptors which have been implicated in anxiety and depression (Bravo et al., 2011).
1.2.6.2 SEROTONIN

Serotonin (5-HT) is a metabolite of the amino acid tryptophan and plays an important role in the regulation of a number of brain functions, including mood (Dinan et al., 2013). The vast majority of antidepressant drugs work to increase serotonin levels in the brain and some studies have shown that bacteria can synthesise serotonin in vivo. For example, plasma serotonin levels were shown to be nearly 3-fold higher in conventional mice than in their germ-free counterparts (Wikoff et al., 2009). Oral ingestion of *Bifidobacterium infantis* 35624 increased the plasma levels of tryptophan, precursor to serotonin, suggesting that commensal bacteria have the ability to influence tryptophan metabolism and could potentially act as antidepressants (Desbonnet et al., 2008). This effect on tryptophan metabolism may be mediated by the impact of the microbiota on the expression of indoleamine-2,3-dioxygenase, a key enzyme in the physiologically dominant kynurenine pathway of tryptophan metabolism (Forsythe et al., 2010). Early life stress induces changes in the gut microbiota and is a risk factor for major depression in adulthood (O’Mahony et al., 2009). This phenomenon has been shown in rhesus monkeys, whereby prenatal stressors have been shown to alter the microbiome by reducing the overall numbers of bifidobacteria and lactobacilli (Bailey and Coe, 1999).

1.2.6.3 CATECHOLAMINES AND ACETYLCHOLINE

Catecholamines such as dopamine and norepinephrine are the major neurotransmitters that mediate a variety of CNS functions such as motor control,
cognition, memory processing, emotion and endocrine regulation. Tsavkelova et al., (2000) identified a wide range of bacteria which produce mmol quantities of dopamine (Tsavkelova et al., 2000) and which could be used for the treatment of Parkinson’s disease, Alzheimer’s disease and other major depressive disorders whereby dysfunctions in catecholamine neurotransmission are implicated. In addition, bacteria which constitute the normal gut microbiome in mice have been shown to be capable of the production of norepinephrine in vivo (Asano et al., 2012). Acetylcholine is a neurotransmitter found in the CNS and PNS which plays a critical role in cognitive function, particularly in memory and learning. Previous studies have shown that acetylcholine is both a component of bacterial strains and a microbial metabolite, including Lactobacillus plantarum and Bacillus subtilis (Girvin and Stevenson, 1954; Horiuchi et al., 2003; Rowatt, 1948).

1.2.7 CONCLUSION

Bioactive metabolites produced by the gut microbiota can induce local changes in the gut epithelium and the enteric nervous system, as well as the immune system with an impact on CNS signalling (Cryan and Dinan, 2012). Disturbances to the delicate host-microbe relationship may disrupt development of the immune system, which in turn may result in disease development. This inflammatory state has been implicated in a wide variety of disorders. The gut microbiota have the ability to produce a variety of metabolites which exert beneficial impacts on biological and neurological functions. Probiotics, prebiotics and dietary PUFA offer the potential to modulate the gut microbiota with knock-on health effects. Further future studies are needed to better
understand the mechanisms by which probiotic and prebiotic administration improve host health. Microbe manipulation to strengthen the host-microbe symbiotic relationship may be crucial for the future prevention of immune and psychiatric related disorders.

1.2.8 ACKNOWLEDGEMENTS

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1.2.9 REFERENCES


### TABLE 1.2.1 Effects of probiotic metabolite production on host metabolic and psychiatric health

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Probiotic Strain</th>
<th>Model</th>
<th>Trial Duration</th>
<th>Health Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metabolic health</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLA</td>
<td><em>Lactobacillus rhamnosus</em> PL60</td>
<td>C57Bl/6J mice</td>
<td>8 weeks</td>
<td>Reduced bodyweight, reduced white adipose tissue and no presence of liver steatosis. Anti-obesity effect. Reduced bodyweight, reduced serum and leptin levels. Anti-obesity effect.</td>
<td>(Lee et al., 2006)</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus plantarum</em> PL62</td>
<td>C57Bl/6J mice</td>
<td>8 weeks</td>
<td></td>
<td>(Lee et al., 2007)</td>
</tr>
<tr>
<td></td>
<td><em>Bifidobacterium breve</em> NCIMB 702258</td>
<td>BALB/c mice, SCID mice, swine</td>
<td>8-10 weeks</td>
<td>Reduced inflammatory profile and higher concentrations of n-3 fatty acids in the adipose tissue</td>
<td>(Wall et al., 2009)</td>
</tr>
<tr>
<td>n-3 LC-PUFA</td>
<td><em>Bifidobacterium breve</em> NCIMB 702258</td>
<td>BALB/c mice</td>
<td>8 weeks</td>
<td>Increased liver EPA and brain DHA concentrations</td>
<td>(Wall et al., 2010)</td>
</tr>
<tr>
<td>SCFA</td>
<td><em>Bifidobacterium breve</em> DPC 6330</td>
<td>C57BL/6J mice</td>
<td>8 weeks</td>
<td>Increased arachidonic acid and DHA concentrations in the brain</td>
<td>(Wall et al., 2012)</td>
</tr>
<tr>
<td>EPS</td>
<td><em>Lactococcus lactis subsp cremoris</em> SBT 0495</td>
<td>F-344 rats</td>
<td>7 days</td>
<td>Serum cholesterol level of rats fed the ropy fermented milk were the lowest among the three treatments</td>
<td>(Nakajima et al., 1992)</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus kefiranofaciens</em></td>
<td>BALB/c mice</td>
<td>2, 5 and 7 days</td>
<td>Positive influence of EPS on systemic immunity and maintenance of intestinal homeostasis</td>
<td>(Vinderola et al., 2006)</td>
</tr>
<tr>
<td><strong>Psychiatric health</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>GABA</td>
<td><em>Lactobacillus brevis</em> FPA3709</td>
<td>Sprague Dawley rats</td>
<td>28 days</td>
<td>Similar antidepressant effects to a commonly used antidepressant drug in the forced swim test Reduced stress and corticosterone and reduced anxiety and depression-related behaviour</td>
<td>(Ko et al., 2013)</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus rhamnosus</em> JB-1</td>
<td>BALB/c mice</td>
<td>28 days</td>
<td>Elevation of the plasma levels of the serotonergic precursor, tryptophan</td>
<td>(Bravo et al., 2011)</td>
</tr>
<tr>
<td>Serotonin</td>
<td><em>Bifidobacterium infantis</em> 35624</td>
<td>Sprague-Dawley rats</td>
<td>14 days</td>
<td>Elevation of the plasma levels of the serotonergic precursor, tryptophan</td>
<td>(Desbonnet et al., 2008)</td>
</tr>
</tbody>
</table>
Chapter 2

Impact of dietary fatty acids on metabolic activity and host intestinal microbiota composition in C57BL/6J mice

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2.1 ABSTRACT

Different dietary fat and energy subtypes have an impact on both the metabolic health and the intestinal microbiota population of the host. The present study assessed the impact of dietary fat quality, with a focus on dietary fatty acid compositions of varying saturation, on the metabolic health status and the intestinal microbiota composition of the host. C57BL/6J mice (n 9-10 mice per group) were fed high-fat (HF) diets containing either (1) palm oil, (2) olive oil, (3) safflower oil or (4) flaxseed/fish oil for 16 weeks and compared with mice fed low-fat (LF) diets supplemented with either high maize starch or high sucrose. Tissue fatty acid compositions were assessed by gas-liquid chromatography (GLC) and the impact of the diets on host intestinal microbiota populations was investigated using high-throughput 16S rRNA sequencing. Compositional sequencing analysis revealed that dietary palm oil supplementation resulted in significantly lower populations of Bacteroidetes at the phylum level, compared with dietary olive oil supplementation (P < 0.05). Dietary supplementation with olive oil was associated with an increase in the population of the family Bacteroidaceae, compared with dietary supplementation of palm oil, flaxseed/fish oil and high sucrose (P < 0.05). Ingestion of the HF-flaxseed/fish oil diet for 16 weeks led to significantly increased tissue concentrations of eicosapentaenoic acid (EPA), docosapentaenoic acid and docosahexaenoic acid (DHA), compared with ingestion of all the other diets (P < 0.05); furthermore, the diet significantly increased the intestinal population of Bifidobacterium at the genus level, compared with the LF-high-maize starch diet (P < 0.05). These data indicate that both quantity and quality of fat have an impact on host physiology with further downstream alterations to the
intestinal microbiota population, with a HF diet supplemented with flaxseed/fish oil positively shaping the host microbial ecosystem.

2.2 INTRODUCTION

Excessive dietary intakes of refined carbohydrates and fat strongly correlate with weight gain, obesity and associated metabolic diseases (Conterno et al., 2011), while also influencing the intestinal microbiota composition of the host (Hildebrandt et al., 2009; Turnbaugh et al., 2008; Turnbaugh et al., 2009b; Walker et al., 2011). The human intestine harbours trillions of microorganisms, containing over 100-fold more genes than the host genome, whereby the collective genome of these microorganisms has co-evolved with the host and contributes to biochemical and metabolic functions that the host could not otherwise perform (Backhed et al., 2004; Ley et al., 2006; Qin et al., 2010).

Recent advances in non-culture-based analysis, such as 16S rRNA sequencing technology, have provided extensive data in relation to the microbial composition of an ecosystem. Briefly, the 16S rRNA gene has been most frequently targeted as a means of identifying the microbial compositions due to its presence in all prokaryotes and the existence of variable domains that allow different taxa to be distinguished (Guinane and Cotter, 2013). Such technology has revealed clear alterations in the intestinal microbiota of obese mouse models (Murphy et al., 2010) and human subjects (Ley et al., 2006), compared with their lean counterparts. The obese phenotype harbours an intestinal microbial population capable of extracting energy efficiently from ingested food and superior ability to produce short chain fatty acids (SCFA) (Schwiertz et al., 2010; Turnbaugh et al., 2006). Recent studies have reported
the effects of specific dietary fatty acid subtypes, such as saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) on metabolic parameters and intestinal microbial populations. (Buettner et al., 2006; de Wit et al., 2012; Devkota et al., 2012; Liao et al., 2010; Mujico et al., 2013). For example, Buettner et al., (2006) showed a reduction in liver inflammation and triglyceride (TAG) levels following high levels of fish oil feeding (Buettner et al., 2006), while others have demonstrated that hamsters fed a low-MUFA diet with a low ratio of PUFA: SFA exhibited increased weight gain and body fat accumulation (Liao et al., 2010). Furthermore, while a diverse intestinal microbiota is preferable, ingestion of a high-fat diet containing palm oil has been reported to decrease bacterial diversity (de Wit et al., 2012; Mujico et al., 2013). While some studies have shown that subtle alterations caused by the ingestion of different dietary fatty acid subtypes indeed have an impact on the intestinal microbiota, the major difficulty arises in linking these changes in the microbiota to the metabolic health status of the host. Potential mechanisms that link how the diet alters the intestinal microbiota have been suggested, such as through changes to lipid metabolism-related genes in the distal small intestine or through changes in host bile composition caused by diet (de Wit et al., 2012; Devkota et al., 2012; Huang et al., 2013); however, it is imperative to further understand the influence that different fat qualities, as opposed to quantities and energy types, have on the intestinal microbiota for the future prevention of obesity.

Therefore, the aims of the present study were to investigate how different qualities of fat in the diet, achieved through altering dietary fatty acid compositions and different sources of energy in the diet, have an impact on the metabolic health status of the host, and furthermore, to investigate the influence of diets on the delicate
nature of host intestinal microbiota composition by employing 16S rRNA sequencing technology. For this purpose, mice were fed high-fat (HF, 45% energy from fat) diets containing either (1) palm oil (mainly SFA), (2) olive oil (MUFA), (3) safflower oil (n-6 PUFA) or (4) flaxseed/fish oil (n-3 PUFA). In parallel, to investigate metabolic parameters and the intestinal microbiota composition of the host, mice were fed low-fat (LF) diets rich in either maize starch (12% energy from fat and 41% maize starch) or sucrose (12% energy from fat and 65% sucrose) for 16 weeks.

2.3 MATERIALS AND METHODS

2.3.1 ANIMALS AND DIETS

Wild type C57BL/6J male mice (21 days old) were obtained from Harlan Limited (Briester, Oxon, UK) and housed under barrier-maintained conditions within the Biological Services Unit, Teagasc, Moorepark. All animal experiments were approved by University College Cork Animal Ethics Committee, and experimental procedures were conducted under appropriate license from the Irish Government in compliance with the institutional and national guidelines for the care and use of animals. Mice were allowed to acclimatise for 5 weeks before the commencement of the experiments and were subsequently randomly divided into the following six dietary groups (n 9-10 mice per group): (1) LF-high-maize starch diet (12% energy from fat and 41% maize starch; n 10); (2) LF-high-sucrose diet (12% energy from fat and 65% sucrose; n 10); (3) HF-palm oil (mainly SFA) diet (45% energy from fat; n 10); (4) HF-olive oil (extra virgin) (MUFA) diet (45% energy from fat; n 9); (5) HF-safflower oil (n-6 PUFA) diet (45% energy from fat; n 10); and (6) HF-flaxseed/fish oil (n-3 PUFA) diet (45% energy from fat; n 9). Mice were housed in groups of four to five per cage with
two cages per dietary treatment group, allowed free access to food and water at all
times and maintained under a 12 hr light-12 hr dark cycle. All diets were supplied by
Harlan Limited. Fatty acid compositions of the experimental diets are shown in Table
2.1. Body weight and food intake were recorded weekly. Following 16 weeks of dietary
treatment, fat and lean body masses were measured using a Minispec mq benchtop
NMR spectrometer (Bruker Instruments, Germany) and mice were killed by cervical
dislocation. Liver, brain, fat pads (epididymal, perirenal, mesenteric and subcutaneous)
and caecal contents were removed, blotted dry on filter paper, weighed, and flash
frozen immediately in liquid nitrogen. Blood samples were collected from fasted
animals into plasma collection tubes, containing ethylenediaminetetraacetic acid
(EDTA) (BD Diagnostics, Oxford, UK) and allowed to clot for at least 30 min at 4°C, then
centrifuged at 10,000g for 20 min to separate the plasma. All samples were stored at -
80 until processed.

2.3.2 BIOCHEMICAL MARKERS AND MEASUREMENTS OF PLASMA VARIABLES

Blood glucose was determined using a Contour glucose meter and blood glucose
strips. Plasma insulin and leptin concentrations were measured using the Ultra
Sensitive Mouse Insulin ELISA kit (Crystal Chem Inc., IL, USA) and the Mouse Leptin
ELISA kit (Crystal Chem Inc.), according to the manufacturer’s instructions. Plasma non-
esterified fatty acids (NEFA) were determined using the commercial NEFA-HR (2) kit
(Wako Diagnostics, Neuss, Germany), and plasma TAG levels were measured using
Infinity Triglyceride Liquid Stable Reagent (Thermo Scientific, Dublin, Ireland).

2.3.3 DETERMINATION OF LIVER TAG LEVELS
Lipids from 50 mg of frozen liver were extracted and purified according to the method of Folch (Folch, 1957). Liver lipids were extracted using chloroform-methanol (2:1, vol:vol; Thermo Scientific) and an aliquot of the organic phase was collected, dried and resuspended in Infinity Triglyceride Liquid Stable Reagent (Thermo Scientific). All samples and standards were analysed in duplicate and TAG content was determined as described previously (Murphy et al., 2010).

2.3.4 RNA EXTRACTION AND COMPLEMENTARY DNA SYNTHESIS

Total RNA was isolated from liver tissue using the commercial RNeasy Mini Kit (Qiagen, West Sussex, UK), according to the manufacturer’s instructions, and quantified using the Nanodrop (Thermo Scientific). Single-stranded complementary DNA (cDNA) was synthesised from 1μg of total RNA using 2.5ng/μL of random primers (Promega, WI, USA), 10mM PCR nucleotide mix (Promega), 40units/μL of RNasin Plus RNase Inhibitor (Promega) and Im-Prom II reverse transcriptase (Promega), according to the manufacturer’s instructions.

2.3.5 REAL-TIME PCR ANALYSIS

Amplification of generated cDNA was performed in the Lightcycler 480 system (Roche Diagnostics Limited, West Sussex, UK) using 0.25μM primers (Eurofins MWG Operon, Ebersberg, Germany), 1μL cDNA and the Lightcycler 480 SYBR Green I Master kit (Roche Diagnostics Limited), according to the manufacturer’s instructions. Real-time PCR conditions were set at: 95°C for 10 min followed by fifty cycles at 95°C for 10 s, 60°C for 5 s and 72°C for 15 s. Specific forward and reverse primers used to amplify cDNA were newly designed and are listed as follows: fatty acid synthase (Fas) cDNA:
forward, 5'-GGCCACCTCAGTTGATATCATG-3' and reverse, 5'-GCCCCGGAACCATAAGAC-3'; sterol regulatory element binding protein-1c (Srebp-1c) cDNA: forward, 5'-CTCCAGCTCAAAACCAAGAC-3' and reverse, 5'-AGAGGAGGCCAGAGAAGGAGAAGA-3'; peroxisome proliferator activated receptor α (Ppara) cDNA: forward, 5'-ATGGGGGTGATCGAGAGTAGTATAG-3' and reverse, 5'-GGGTGGCAGGAAGGGAACAGAC-3'; peroxisome proliferator activated receptor γ (Ppary) cDNA: forward, 5'-TCAGGTTTGGGCCGGAAGACTTATCGTATG-3'. All samples were analysed in duplicate and normalised to β-actin as a constitutively expressed control gene: forward, 5'-AGAGGAAATCGTGCGTGAC-3' and reverse, 5'-CAATAGTGACCTGGATG-3'. Melting curve analysis allowed the validation of the authenticity of the real-time PCR products. Basic relative quantification of expression was determined using the comparative 2^ΔΔCt method.

2.3.6 LIPID EXTRACTION AND FATTY ACID ANALYSIS

Lipids were extracted and purified with chloroform-methanol (2:1, vol:vol; Thermo Scientific) according to the method of Folch (Folch, 1957). Fatty acid methyl esters (FAME) were prepared by using first 10mL 0.5 M NaOH (Sigma, Wicklow, Ireland) in methanol for 10 min at 90°C followed by 10mL 14% BF₃ in methanol (Sigma) for 10 min at 90°C (Park and Goins, 1994). FAME were recovered with hexane (Fisher Scientific, Dublin, Ireland). Before GLC analysis, samples were dried over 0.5g anhydrous sodium sulphate (Sigma) for 1 hr and stored at -20°C. FAME were separated by GLC (Varian 3400; JVA Analytical) fitted with a flame-ionisation detector by using a Chrompack CP Sil 88 column (Chrompack, JVA Analytical; 100 m x 0.25 mm internal diameter, 0.20-
μm film thickness) and helium as carrier gas. The column oven was programmed 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 μL, with an automatic sample injection on a SRI 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., MN, USA). 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 data are presented as means ± standard errors of the mean (SEM) in g/100g FAME.

2.3.7 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 10,000g for 5 min to pellet bacteria and other solids. The supernatant fluid was collected, 3.0 mmol 2-ethylbutyric acid/L (Sigma) was added as internal standard, and samples were filtered before being transferred to clean vials. Standard solutions containing 10.0, 8.0, 6.0, 4.0, 2.0, 1.0 and 0.5 mmol/L of a mix of acetic acid, propionic acid, isobutyric acid and butyric acid (Sigma) were used for calibration. The concentration of SCFA was measured using a Varian 3800 GC flame-ionisation system, fitted with a ZB-FFAP column (30 m x 0.32 mm x 0.25 μm; Phenomenex, Cheshire, UK). Helium was used as the carrier gas at a flow rate of 1.3 mL/min. The initial oven temperature was 100°C for 0.5 min, raised to 180°C at 8°C/min and held for 1 min, then increased to 200°C at 20°C/min, and finally held at 200°C for 5 min. The temperature of the detector and
injector were set at 250°C and 240°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.

2.3.8 DNA EXTRACTION AND PYROSEQUENCING

Total DNA was extracted from the caecal contents of all mice using the QIAamp DNA Stool Mini Kit (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, as described previously (Murphy et al., 2010), were used for Taq based PCR amplification. Sequencing was performed on a Roche 454 GS-FLX using Titanium chemistry 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 bp 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) using default parameters. The resulting BLAST output was parsed 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 summarisation. A bit score of 86 was selected as previously used for 16S ribosomal sequence data (Urich et al., 2008). Phylum, family and genus counts for each sample were extracted from MEGAN. Sequences were clustered into operational taxonomical units (OTU), chimera checked and aligned using the default pipeline within Qiime, and subsequently α and β diversities were generated. A phylogenetic tree was calculated.
using the FastTree software (doi:10.1093/molbev/msp077). Resulting Principal Coordinate analysis was visualised within KiNG.

2.3.9 STATISTICAL ANALYSIS

All results are presented as means ± SEM (per group). To assess whether differences between the treatment groups were significant, data were analysed using one-way ANOVA followed by post hoc Tukey’s multiple comparison test using GraphPad Prism version 4.0 for Windows (GraphPad Software). Compositional data were statistically analysed using Minitab release 15.1.1.0 (www.minitab.com). A non-parametric Kruskal-Wallis test was used to estimate the relationships between different dietary groups. Statistical significance was accepted at $P < 0.05$.

2.4 RESULTS

2.4.1 EFFECT OF VARYING DIETARY FATTY ACID COMPOSITIONS ON WEIGHT GAIN, BODY COMPOSITION AND HOST FAT STORAGE

A higher percentage of weight gain was observed in the group fed the HF-palm oil diet for 16 weeks, compared with the HF-olive oil ($P < 0.05$)- and LF-high sucrose ($P < 0.05$)-fed groups, despite no differences in cumulative energy intake between these groups (Table 2.2). Furthermore, subcutaneous fat mass was higher following feeding of the HF-palm oil diet than after feeding of the HF-olive oil diet ($P < 0.05$; Table 2.2). The group supplemented with the HF-palm oil diet also had a higher percentage of fat mass ($P < 0.05$; Figure 2.1(A)) and a lower percentage of lean mass ($P < 0.05$; Figure 2.1(B)) than the HF-olive oil-fed group and the LF-high sucrose-fed group. The percentage lean mass was higher in the group fed the olive oil diet for 16 weeks than
in the other HF diet-fed groups \((P < 0.05; \text{Figure 2.1(B)})\). The percentage of weight gain was higher in the LF-high maize starch-fed group than in the LF-high sucrose-fed group \((P < 0.05; \text{Table 2.2})\), which was most probably due to the greater food intake \((P < 0.05; \text{Table 2.2})\), and therefore greater cumulative energy intake \((P < 0.05; \text{Table 2.2})\) in the former group. Visceral fat mass was also higher in the LF-high maize starch-fed group than in the LF-high sucrose-fed group after feeding for 16 weeks \((P < 0.05; \text{Table 2.2})\).

Since the energy content of the LF-high-carbohydrate diets \((16 \text{ kJ/g})\) was only approximately 3 kJ/g lower than that of the high-fat diets \((19 \text{ kJ/g})\), differences in total cumulative energy intake between the groups resulted from differences in food intake, due to dietary preference. The LF-high maize-starch fed group consumed more food over the study period than the groups supplemented with the HF-safflower oil and HF-flaxseed/fish oil diets and the LF-high-sucrose diet \((P < 0.05)\). However, no differences in the overall percentage of weight gain were observed between the groups fed the LF-high-maize starch, HF-safflower oil and HF-flaxseed/fish oil diets.

### 2.4.2 HIGHER PLASMA GLUCOSE AND LEPTIN CONCENTRATIONS ASSOCIATED WITH SATURATED FAT

No differences in plasma TAG, insulin or NEFA concentrations were found between the groups following 16 weeks of dietary feeding (Table 2.3). Fasting glucose levels were higher in the HF-palm oil-fed group than in both the HF-safflower oil- and LF-high sucrose-fed groups \((P < 0.05; \text{Table 2.3})\), while plasma glucose concentrations were lower in the LF-high-sucrose-fed group than in the groups fed the LF-high-maize starch, HF-palm oil and HF-olive oil diets \((P < 0.05; \text{Table 2.3})\). Circulating plasma leptin
concentrations were also higher in the HF-palm oil-fed group than in the HF-olive oil- and both LF-high carbohydrate-fed groups \((P < 0.05; \text{Table 2.3})\).

### 2.4.3 SATURATION OF FAT INFLUENCES HOST LIVER MASS, TAG LEVELS AND HEPATIC GENE EXPRESSION

Liver tissue weight (Figure 2.2(A)) and total liver TAG levels (Figure 2.2(B)) were higher \((P < 0.05)\) in the palm oil-fed group than in all the other dietary groups. Among the high-fat diets, as the degree of saturation shifted from the more SFA source of palm oil to the \(n-3\) PUFA source of flaxseed/fish oil, the levels of liver TAG steadily decreased in a coinciding manner (Figure 2.2(B)).

Briefly, the expression of certain genes related to fatty acid metabolism and inflammation in the liver were assessed in an effort to correlate their expression with liver mass and TAG levels. Hepatic expression of Fas was higher in the palm oil-fed group than in the flaxseed/fish oil-fed group \((P < 0.05; \text{Supplementary Figure 2.1})\). Furthermore, the olive oil-fed group had a higher hepatic expression of Fas than the flaxseed/fish oil-fed group \((P < 0.05; \text{Supplementary Figure 2.1})\), while Fas expression was higher in the LF-high-maize starch-fed group than in the high-fat diet-fed groups (except the olive oil-fed group) and the LF-high sucrose-fed group \((P < 0.05; \text{Supplementary Figure 2.1})\). Hepatic expression of Srebp-1c was higher in the LF-high-maize starch-fed group than in the high-fat diet-fed groups (except the olive oil-fed group) and the LF-high sucrose-fed group \((P < 0.05; \text{Supplementary Figure 2.1})\). Hepatic expression of Pparα was reduced in all the dietary groups, compared with the LF-high-maize starch-fed group \((P < 0.05; \text{Supplementary Figure 2.1})\), while Pparγ expression was higher in all the high-fat
diet-fed groups than in the LF-high sucrose-fed group (P < 0.05; Supplementary Figure 2.1).

2.4.4 IMPACT OF DIFFERENT SOURCES OF FAT AND ENERGY ON HOST TISSUE FATTY ACID COMPOSITIONS

The palm oil-fed group had higher levels of palmitic acid (16:0) in the liver and brain, compared with the other high-fat diet-fed groups and the LF-high sucrose-fed group (P < 0.05; Tables 2.4 and 2.6) and in epididymal adipose tissue, compared with all the other dietary groups (P < 0.05; Table 2.5). Additionally, the palm oil-fed group had higher levels of oleic acid (18:1 cis-9) in liver (P < 0.05; Table 2.4) and epididymal adipose tissue (P < 0.05; Table 2.5), compared with all the other dietary groups, except the olive oil-fed group, which had higher levels of oleic acid in the epididymal adipose tissue only, compared with all the other diet-fed groups (P < 0.05; Table 2.5). The safflower oil-fed group had higher levels of linoleic acid (18:2n-6), γ-linolenic acid (18:3n-6) and arachidonic acid (20:4n-6) in all tissues, compared with all the other diet-fed groups (P < 0.05; Tables 2.4-2.6). Both the LF-high carbohydrate-fed groups had higher levels of dihomoolinolenic acid (20:3n-6) in the brain (P < 0.05; Table 2.6) and higher levels of palmitoleic acid (16:1 cis-9) in the liver (P < 0.05; Table 2.4), compared with all the other dietary groups. α-linolenic acid (18:3n-3) levels were higher in all tissues (P < 0.05; Tables 2.4-2.6) of the flaxseed/fish oil-fed group than those of the other diet-fed groups, except in the brain of the LF-high sucrose-fed group. Furthermore, the flaxseed/fish oil-fed group had higher levels of EPA (20:5n-3), docosapentaenoic acid (22:5n-3) and DHA (22:6n-3) in all tissues (P < 0.05; Tables 2.4-2.6), lower levels of arachidonic acid in the brain (P < 0.05; Table 2.6) and higher levels
of stearic (18:0) and myristic (14:0) acids in epididymal adipose tissue \(P < 0.05\); Table 2.5), compared with the other dietary groups.

**2.4.5 SATURATED FAT IS ASSOCIATED WITH HIGHER CAECAL CONCENTRATIONS OF SCFA**

The mean total concentrations of SCFA (acetate, propionate, butyrate and isobutyrate) in caecal contents were found to be highest in the palm oil-fed group, compared with the other dietary groups \(P < 0.05\); Table 2.7). Of these SCFA, the concentrations of propionate, butyrate and isobutyrate were found to be higher in the palm oil-fed group than in the other diet-fed groups \(P < 0.05\); Table 2.7), while the concentration of acetate was found to be higher in the palm oil-fed group, compared with the other diet-fed groups \(P < 0.05\); Table 2.7), except the LF-high maize starch-fed group.

**2.4.6 FAT AND ENERGY SOURCES INFLUENCE DIFFERENTLY ON THE COMPOSITION OF THE INTESTINAL MICROBIOTA**

The microbial composition in the caecal contents of individual mice was elucidated through high-throughput DNA sequencing (Roche 454-Titanium) of 16S rRNA (V4) amplicons after 16 weeks of dietary intervention. A total of 355,743 reads were sequenced, corresponding to an average of 6,134 reads per mouse. Of these reads, 318,586 (89%) were assigned at the phylum level, 285,224 (80%) at the family level and 180,584 (51%) at the genus level. At the 97% similarity level, a total of five measures were used to indicate sufficient sampling for all the groups. Estimations for species richness, coverage and diversity were calculated for each data set.
(Supplementary Figures 2.2-2.6). All measures showed a high level of overall diversity within all samples and rarefaction curves for each group indicated a sufficient depth of sequencing. For the Chao1-α diversity metric (Supplementary Figure 2.2), olive oil and flaxseed/fish oil feeding resulted in the least and most diverse intestinal microbiota, respectively. This trend is somewhat reflected in all the other diversity metrics (Supplementary Figures 2.3-2.6). Furthermore, all diets were significantly different from each other. Principal coordinate analysis plots generated using an unweighted Unifrac distance matrix showed that mice clustered into relatively distinct groups based on dietary treatment (Figure 2.3). This suggests that exposure to different qualities of dietary fats or different energy sources (fat v. carbohydrate) can significantly alter intestinal microbial populations.

Taxonomy based analysis of the assigned sequences showed that at the phylum level, the mouse intestinal microbiota is dominated by Firmicutes and Bacteroidetes (together harbouring on average 92.3% of sequences; Figure 2.4). At the family level, the most dominant groups were Lachnospiraceae, Erysipelotrichaceae, Ruminococcaceae, Rikenellaceae and Deferrribacteraceae (Table 2.8 and Figure 2.5). Consistent with the high levels of Firmicutes and Bacteroidetes detected, the dominant bacteria detected at the genus level were Allobaculum, Ruminococcaceae Incertae Sedis, Bacteroides and Rikenella (Table 2.8 and Figure 2.6).

The palm oil-fed group had reduced caecal populations of Bacteroidetes at the phylum level ($P < 0.05$; Figure 2.4), at 10.5%, compared with the olive oil- and safflower oil-fed groups (both 20%). All the other phyla remained at relatively similar proportions across the groups. At the family level, the palm oil- and LF-high sucrose-fed groups had higher populations of Lachnospiraceae (46.4 and 49.6%, respectively, $P$
< 0.05; Figure 2.5), than the LF-high maize starch- and flaxseed/fish oil-fed groups (17.1 and 24.2%, respectively). Among the high-fat diets, as the degree of saturation shifted from the more SFA source of palm oil to the n-3 PUFA source of flaxseed/fish oil, the intestinal populations of *Lachnospiraceae* steadily decreased in a coinciding manner. Reduced proportions of *Lachnospiraceae* were also found in the safflower oil-fed group, relative to the LF-high sucrose-fed group (*P* < 0.05; Figure 2.5). Within the LF-high carbohydrate-fed groups, contrasting results were found. The relative proportions of *Ruminococcaceae* were higher in the LF-high sucrose-fed group than in the high maize starch-fed group (*P* < 0.05; Figure 2.5), while the high maize starch-fed group had a higher population of *Erysipelotrichaceae* than the high sucrose-fed group (*P* < 0.05; Figure 2.5). The olive oil- and flaxseed/fish oil-fed groups had a greater abundance of *Erysipelotrichaceae*, compared with the LF-high sucrose-fed group (*P* < 0.05; Figure 2.5). Again, among the high-fat dietary groups, as the degree of saturation shifted from the more SFA source of palm oil to the n-3 PUFA source of flaxseed/fish oil, the intestinal populations of *Erysipelotrichaceae* steadily increased in a coinciding manner. Thus, the saturation or quality of fat can shift the intestinal microbiota population in numerous ways. Interestingly, higher proportions of *Bacteroidaceae* and *Bacteroides* were found in the olive oil-fed group, compared with the palm oil-, flaxseed/fish oil- and LF-high sucrose-fed groups (*P* < 0.05; Figures 2.5-2.6). Additionally, the n-3 PUFA-rich flaxseed/fish oil diet was the only diet to increase intestinal populations of *Bifidobacteriaceae* and *Bifidobacterium*, compared with the LF-high maize starch diet (*P* < 0.05; Figures 2.5-2.6).

At the genus level, *Allobaculum* was found to be higher in the LF-high maize starch-, HF- flaxseed/fish oil- and HF-olive oil-fed groups than in the LF-high sucrose-fed group.
High populations of uncultured *Lachnospiraceae, Oscillibacter, Odoribacter* and *Anaerotruncus* were detected in the LF-high sucrose-fed group, reaching significance compared with a variety of different groups within each population ($P < 0.05$; Table 2.8 and Figure 2.6).

### 2.5 DISCUSSION

The data presented herein reveal that different dietary fatty acids and qualities of dietary fat from different sources (palm oil, olive oil, safflower oil and flaxseed/fish oil) and high-carbohydrate diets (maize starch and sucrose) significantly influenced both metabolic parameters and the composition of the intestinal microbiota in mice. The present study has further highlighted that consumption of MUFA and PUFA are generally healthful in comparison with SFA, whereby chronic dietary SFA intake (palm oil) for 16 weeks resulted in significant increases in the percentage of body weight gain, the percentage of body fat mass, subcutaneous fat mass and plasma glucose and leptin concentrations, compared with the other groups. In contrast, consumption of MUFA (olive oil) for 16 weeks was associated with a significant decrease in the percentage of body weight gain and the percentage of body fat mass, compared with mice ingesting a palm oil-rich diet. Current nutritional recommendations suggest that a reduction in SFA consumption with an increase in plant oils containing MUFA is desirable; however, the mechanism by which MUFA may be responsible for a reduction in body weight is largely unknown. Recently, a study has reviewed numerous health benefits associated with the polyphenol fraction of olive oil, such as anti-inflammatory and antioxidant activities, which may possibly provide further evidence...
for the positive impact the olive oil diet has on body weight gain observed in the present study (Martin-Pelaez et al., 2013).

The group that ingested the LF-high sucrose diet had the lowest percentage of weight gain over the 16 week study period, while the LF-high maize starch-fed group exhibited a higher percentage of weight gain and plasma blood glucose levels, compared with the high sucrose-fed group. This difference can partly be explained by the greater food intake of the high maize starch-fed group, compared with the high sucrose-fed group. The maize starch diet had a greater impact on food intake, glucose intolerance and weight gain, compared with dietary sucrose as the primary carbohydrate source in the diet. While a combination of high dietary fat with high sucrose has been reported to induce insulin resistance and glucose intolerance (Yang et al., 2012), it appears that dietary sucrose itself has differential effects depending on the amount of fat in the diet (Surwit et al., 1995).

Dietary SFA is known to play a substantial clinical role in the onset and progression of non-alcoholic fatty liver disease (NAFLD) (Tetri et al., 2008; van den Berg et al., 2010; Wang et al., 2006). Supplementation with palm oil significantly increased both liver weight and TAG levels in mice after 16 weeks, compared with all the other groups. Interestingly, it was shown herein that as the degree of saturation shifted from SFA (palm oil), to MUFA (olive oil), to n-6 PUFA (safflower oil) and finally to n-3 PUFA (flaxseed/fish oil), the levels of liver TAG steadily decreased in a coinciding manner. This indicates that it is not only the quantity of fat but also the quality of fat consumed that has an impact on the tissue distribution of fat in the host. Furthermore, the increased hepatic expression of Fas and Srebp-1c, which are involved in de novo
lipogenesis correlated with the increase in hepatic TAG observed for the palm oil-fed group.

The clinical implications of n-3 PUFA in preventing the progression of NAFLD have been described (Parker et al., 2012). The reduced levels of liver TAG observed following dietary intake of flaxseed/fish oil for 16 weeks may be partly explained by the increase in hepatic expression of Ppary found for this group, given that Ppary is associated with a reduction in inflammatory responses in the liver (Galli et al., 2002; Marra et al., 2005; Marra et al., 2000). Furthermore, flaxseed/fish oil feeding increased the levels of α-linolenic acid, EPA, docosapentaenoic acid and DHA in the liver, epididymal adipose tissue and brain. Dietary EPA and DHA exert anti-inflammatory properties (Mori and Beilin, 2004) by altering cytokine production (Browning, 2003; Calder, 2001), often at the expense of arachidonic acid, whereby proinflammatory eicosanoids derived from arachidonic acid, such as prostaglandin E₂ and leukotriene B₄, are replaced by the anti-inflammatory eicosanoids derived from EPA and DHA (Calder, 2009). The present study has demonstrated favourable increases in tissue EPA, docosapentaenoic acid and DHA and decreases in tissue arachidonic acid for the flaxseed/fish oil-fed group in this study, whereby DHA has been shown to play important roles in neurogenesis, neurotransmission, and protection against oxidative stress (Hashimoto et al., 2002; Innis, 2007; Lim et al., 2005). Importantly, the present study has demonstrated the ability of flaxseed/fish oil-derived fatty acids EPA, docosapentaenoic acid and DHA to cross the blood-brain barrier where it is understood that DHA may influence cognitive processes such as learning and memory in the brain (Henriksen et al., 2008; Yurko-Mauro et al., 2010). This further highlights the importance of n-3 PUFA-derived dietary fatty acids to the brain-gut axis.
Diet composition significantly altered intestinal microbial populations and diversity after 16 weeks of feeding. A key finding was that all measures of α diversity demonstrated a high level of overall diversity between the dietary groups, whereby mice clustered into distinct groups based on dietary treatment. Decreases in the populations of Bacteroidetes and Bacteroidaceae at the phylum and family levels, respectively, and increases in the population of Lachnospiraceae at the family level were observed for the palm oil-fed group. Interestingly, other studies have demonstrated similar trends in the Bacteroidetes population that positively correlate with the development of obesity (Ley et al., 2006; Turnbaugh et al., 2009a). Based on previous studies, it is generally understood that the gut microbiota can indeed increase the energy-harvesting capacity of the host through the fermentation of non-digestible complex polysaccharides in the large intestine and thus producing SCFA (Jumpertz et al., 2011; Turnbaugh et al., 2006). In the present study, the palm oil-fed group increased total caecal SCFA produced, compared with all the other groups. While the positive impact these SCFA have on human gastrointestinal health have previously been described (D'Argenio et al., 1996; Emenaker et al., 2001; Galvez et al., 2005; Topping and Clifton, 2001), the process by which they drive energy production has also been portrayed as a potential mechanism involved in the increase in host fat mass storage (Turnbaugh et al., 2006). Furthermore, it is unclear whether the beneficial effect of SCFA is somehow compromised in obese subjects, or whether the effect is simply not strong enough to compensate for an adverse diet.

The group that ingested the olive oil-rich diet had increased populations of Bacteroidaceae in the caecum at the family level, compared with the palm oil- and flaxseed/fish oil-fed groups. Interestingly, these bacteria maintain a complex and
generally beneficial relationship with the host when retained in the gut and their role as commensals has been extensively reviewed (Xu and Gordon, 2003). In the present study, between the two LF-high carbohydrate-fed groups, the proportions of *Allobaculum* were reduced while *Lachnospiraceae* were increased in the caecum of the high sucrose-fed group, compared with the maize starch-fed group. Interestingly, a recent study by Ravussin et al., (2012) reported an association between low-fat feeding and increases in the genus *Allobaculum*, thus suggesting that high dietary sucrose may be responsible for a reduction in the caecal numbers of *Allobaculum* (Ravussin et al., 2012).

The flaxseed/fish oil diet was the only diet to significantly increase the intestinal populations of *Bifidobacteriaceae* and *Bifidobacterium*, at the family and genus levels, respectively, possibly through the increased ability of flaxseed/fish oil to increase the adhesion of bifidobacteria to the intestinal wall.

The results from the present study indicate that dietary ingestion of different fatty acids in a high-fat diet, or a chronic intake of high levels of maize starch or sucrose significantly influences the distribution of fat in the host and also the intestinal microbiota. While subtle differences in the intestinal microbiota did exist between the two LF-high carbohydrate-fed groups, it is apparent that dietary maize starch has a greater impact on weight gain and glucose intolerance than sucrose. We demonstrated that dietary SFA (palm oil) had a negative impact on host metabolic parameters often associated with obesity and the Metabolic Syndrome, while also shifting the intestinal microbiota population to one similar to that seen in an obese phenotype. In contrast, dietary MUFA (olive oil) may beneficially alter the bacterial population in the intestine by increasing the populations of commensal bacteria. Finally, the present study has
demonstrated that a rich dietary source of \( n-3 \) PUFA (flaxseed/fish oil) may have a \textit{bifidogenic} effect on the intestinal microbiota composition of the host by increasing the levels of \textit{Bifidobacterium}, while also positively influencing the composition of host tissues with \( n-3 \) PUFA-derived health-promoting fatty acids.

Indeed, the mechanism by which dietary fat types alter the intestinal microbiota remains largely to be elucidated. Promisingly, recent reports by Devkota et al., (2012) and deWit et al., (2012) suggest that certain saturated fat types can alter conditions for gut microbial assemblage by promoting changes in host bile composition (Devkota et al., 2012) and that an overflow of palm oil to the distal small intestine rather than obesity itself may trigger an elevation of lipid metabolism-related genes in the distal small intestine, thus altering the intestinal microbiota (de Wit et al., 2012). Further future studies will confirm a precise mechanism by which diet alters the intestinal microbiota. In conclusion, the present study has demonstrated some novel findings on how different qualities of fat and energy subtypes have an impact on the metabolic health and intestinal microbiota composition of the host.

\textbf{2.6 ACKNOWLEDGEMENTS}

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2.7 REFERENCES


attributable to early supplementation of human milk with docosahexaenoic acid and arachidonic acid. *Pediatrics*. **121**:1137-1145.


### TABLE 2.1 Dietary components and fatty acid compositions of experimental oils.

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<tr>
<th></th>
<th>LF-high</th>
<th>LF-high</th>
<th>HF-palm</th>
<th>HF-olive</th>
<th>HF-safflower oil</th>
<th>HF-flaxseed/fish</th>
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% of total fat as:

<p>| | | | | | | |</p>
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<td>Oleic acid</td>
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<td>33.9</td>
<td>38.5</td>
<td>70.5</td>
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<td>DHA</td>
<td>1.3</td>
<td>1.3</td>
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</table>
TABLE 2.2 Body mass, fat mass, food intake and cumulative energy intake of mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with those fed low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. (Mean values ± SEM; n 9-10 mice per group).

<table>
<thead>
<tr>
<th></th>
<th>LF-high maize starch</th>
<th>LF-high sucrose</th>
<th>HF-palm oil</th>
<th>HF-olive oil</th>
<th>HF-safflower oil</th>
<th>HF-flaxseed/fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight (g)</td>
<td>Mean: 21.67 SEM: 0.66</td>
<td>Mean: 21.03 SEM: 0.53</td>
<td>Mean: 21.8 SEM: 0.69</td>
<td>Mean: 20.96 SEM: 0.52</td>
<td>Mean: 20.91 SEM: 0.46</td>
<td>Mean: 20.5 SEM: 0.41</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>Mean: 31.00 SEM: 0.91</td>
<td>Mean: 26.10 SEM: 0.86</td>
<td>Mean: 34.49 SEM: 0.82</td>
<td>Mean: 28.93 SEM: 1.56</td>
<td>Mean: 32.17 SEM: 1.5</td>
<td>Mean: 31.50 SEM: 0.92</td>
</tr>
<tr>
<td>Weight gain (%)</td>
<td>Mean: 43.35 SEM: 2.96</td>
<td>Mean: 24.32 SEM: 3.5</td>
<td>Mean: 58.63 SEM: 1.99</td>
<td>Mean: 37.56 SEM: 5.48</td>
<td>Mean: 53.33 SEM: 4.85</td>
<td>Mean: 53.82 SEM: 4.08</td>
</tr>
<tr>
<td>Visceral fat mass (g)</td>
<td>Mean: 2.38 SEM: 0.15</td>
<td>Mean: 1.44 SEM: 0.15</td>
<td>Mean: 3.00 SEM: 0.13</td>
<td>Mean: 2.18 SEM: 0.28</td>
<td>Mean: 2.91 SEM: 0.27</td>
<td>Mean: 2.56 SEM: 0.14</td>
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<td>Subcutaneous fat mass (g)</td>
<td>Mean: 0.75 SEM: 0.04</td>
<td>Mean: 0.47 SEM: 0.06</td>
<td>Mean: 1.15 SEM: 0.05</td>
<td>Mean: 0.71 SEM: 0.11</td>
<td>Mean: 0.94 SEM: 0.1</td>
<td>Mean: 1.03 SEM: 0.09</td>
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<td>Food Intake (g/day/mouse)</td>
<td>Mean: 3.67 SEM: 0.11</td>
<td>Mean: 3.25 SEM: 0.11</td>
<td>Mean: 2.92 SEM: 0.06</td>
<td>Mean: 2.98 SEM: 0.09</td>
<td>Mean: 2.71 SEM: 0.06</td>
<td>Mean: 2.52 SEM: 0.08</td>
</tr>
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</table>

^a,b,c,d^ Mean values within a row with unlike superscript letters were significantly different (P < 0.05; ANOVA followed by post hoc Tukey’s multiple comparison test).
**TABLE 2.3** Plasma variables in mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks*. (Mean values ± SEM; n 9-10 mice per group).

<table>
<thead>
<tr>
<th></th>
<th>LF-high maize starch</th>
<th>LF-high sucrose</th>
<th>HF-palm oil</th>
<th>HF-olive oil</th>
<th>HF-safflower oil</th>
<th>HF-flaxseed/fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>45.1</td>
<td>7.8</td>
<td>43.7</td>
<td>9.4</td>
<td>34.9</td>
<td>4.3</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>160.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.1</td>
<td>121.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.4</td>
<td>167.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.3</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>0.2</td>
<td>0.04</td>
<td>0.25</td>
<td>0.06</td>
<td>0.36</td>
<td>0.06</td>
</tr>
<tr>
<td>Leptin (ng/mL)</td>
<td>25.4&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>3.2</td>
<td>11.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.9</td>
<td>44.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.4</td>
</tr>
<tr>
<td>NEFA (mmol/L)</td>
<td>0.57</td>
<td>0.06</td>
<td>0.54</td>
<td>0.08</td>
<td>0.56</td>
<td>0.05</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Mean values within a row with unlike superscript letters were significantly different (P < 0.05; ANOVA followed by post hoc Tukey’s multiple comparison test). *Blood was collected after animals were fasted.
TABLE 2.4 Fatty acid profile (g/100g FAME) in the liver of mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with those fed low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. (Mean values ± SEM; n 9-10 mice per group).

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>LF-high maize starch g/100g FAME</th>
<th>LF-high sucrose g/100g FAME</th>
<th>HF-palm oil g/100g FAME</th>
<th>HF-olive oil g/100g FAME</th>
<th>HF-safflower oil g/100g FAME</th>
<th>HF-flaxseed/fish oil g/100g FAME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>14:0</td>
<td>0.51&lt;sup&gt;a&lt;/sup&gt; 0.02</td>
<td>0.45&lt;sup&gt;ad&lt;/sup&gt; 0.02</td>
<td>0.44&lt;sup&gt;ad&lt;/sup&gt; 0.01</td>
<td>0.37&lt;sup&gt;cd&lt;/sup&gt; 0.01</td>
<td>0.29&lt;sup&gt;c&lt;/sup&gt; 0.02</td>
<td>0.56&lt;sup&gt;b&lt;/sup&gt; 0.04</td>
</tr>
<tr>
<td>16:0</td>
<td>24.02&lt;sup&gt;ab&lt;/sup&gt; 0.18</td>
<td>22.51&lt;sup&gt;ad&lt;/sup&gt; 0.50</td>
<td>25.02&lt;sup&gt;b&lt;/sup&gt; 0.29</td>
<td>18.38&lt;sup&gt;c&lt;/sup&gt; 0.28</td>
<td>19.55&lt;sup&gt;c&lt;/sup&gt; 0.67</td>
<td>22.05&lt;sup&gt;d&lt;/sup&gt; 0.33</td>
</tr>
<tr>
<td>16:1 cis-9</td>
<td>4.68&lt;sup&gt;a&lt;/sup&gt; 0.18</td>
<td>4.11&lt;sup&gt;a&lt;/sup&gt; 0.20</td>
<td>3.24&lt;sup&gt;b&lt;/sup&gt; 0.10</td>
<td>1.67&lt;sup&gt;c&lt;/sup&gt; 0.10</td>
<td>1.23&lt;sup&gt;c&lt;/sup&gt; 0.13</td>
<td>2.49&lt;sup&gt;d&lt;/sup&gt; 0.10</td>
</tr>
<tr>
<td>18:0</td>
<td>6.83&lt;sup&gt;a&lt;/sup&gt; 0.53</td>
<td>7.37&lt;sup&gt;ac&lt;/sup&gt; 0.50</td>
<td>3.62&lt;sup&gt;b&lt;/sup&gt; 0.26</td>
<td>5.12&lt;sup&gt;ab&lt;/sup&gt; 0.68</td>
<td>6.51&lt;sup&gt;a&lt;/sup&gt; 0.83</td>
<td>9.74&lt;sup&gt;c&lt;/sup&gt; 0.43</td>
</tr>
<tr>
<td>18:1 cis-9</td>
<td>38.95&lt;sup&gt;a&lt;/sup&gt; 1.77</td>
<td>36.80&lt;sup&gt;a&lt;/sup&gt; 1.79</td>
<td>51.73&lt;sup&gt;b&lt;/sup&gt; 1.11</td>
<td>56.29&lt;sup&gt;b&lt;/sup&gt; 1.85</td>
<td>19.10&lt;sup&gt;c&lt;/sup&gt; 1.86</td>
<td>15.79&lt;sup&gt;c&lt;/sup&gt; 0.58</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>7.91&lt;sup&gt;a&lt;/sup&gt; 0.47</td>
<td>8.44&lt;sup&gt;a&lt;/sup&gt; 0.45</td>
<td>4.46&lt;sup&gt;b&lt;/sup&gt; 0.25</td>
<td>4.40&lt;sup&gt;b&lt;/sup&gt; 0.32</td>
<td>34.87&lt;sup&gt;c&lt;/sup&gt; 0.79</td>
<td>8.58&lt;sup&gt;a&lt;/sup&gt; 0.17</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.71&lt;sup&gt;a&lt;/sup&gt; 0.05</td>
<td>0.72&lt;sup&gt;a&lt;/sup&gt; 0.06</td>
<td>0.03&lt;sup&gt;a&lt;/sup&gt; 0.001</td>
<td>0.18&lt;sup&gt;a&lt;/sup&gt; 0.09</td>
<td>0.18&lt;sup&gt;a&lt;/sup&gt; 0.01</td>
<td>12.14&lt;sup&gt;b&lt;/sup&gt; 0.47</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>0.07&lt;sup&gt;a&lt;/sup&gt; 0.01</td>
<td>0.06&lt;sup&gt;a&lt;/sup&gt; 0.004</td>
<td>0.07&lt;sup&gt;a&lt;/sup&gt; 0.01</td>
<td>0.06&lt;sup&gt;a&lt;/sup&gt; 0.01</td>
<td>0.58&lt;sup&gt;b&lt;/sup&gt; 0.02</td>
<td>0.09&lt;sup&gt;a&lt;/sup&gt; 0.01</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.76&lt;sup&gt;ad&lt;/sup&gt; 0.04</td>
<td>0.86&lt;sup&gt;ad&lt;/sup&gt; 0.05</td>
<td>0.36&lt;sup&gt;b&lt;/sup&gt; 0.03</td>
<td>0.54&lt;sup&gt;ab&lt;/sup&gt; 0.07</td>
<td>1.98&lt;sup&gt;c&lt;/sup&gt; 0.09</td>
<td>0.45&lt;sup&gt;b&lt;/sup&gt; 0.02</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>2.80&lt;sup&gt;a&lt;/sup&gt; 0.23</td>
<td>3.69&lt;sup&gt;a&lt;/sup&gt; 0.31</td>
<td>2.29&lt;sup&gt;a&lt;/sup&gt; 0.26</td>
<td>3.48&lt;sup&gt;a&lt;/sup&gt; 0.58</td>
<td>7.25&lt;sup&gt;b&lt;/sup&gt; 0.82</td>
<td>3.36&lt;sup&gt;a&lt;/sup&gt; 0.15</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.88&lt;sup&gt;a&lt;/sup&gt; 0.08</td>
<td>1.10&lt;sup&gt;a&lt;/sup&gt; 0.12</td>
<td>0.01&lt;sup&gt;b&lt;/sup&gt; 0.001</td>
<td>0.03&lt;sup&gt;b&lt;/sup&gt; 0.004</td>
<td>0.02&lt;sup&gt;b&lt;/sup&gt; 0.003</td>
<td>5.10&lt;sup&gt;c&lt;/sup&gt; 0.12</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.53&lt;sup&gt;a&lt;/sup&gt; 0.05</td>
<td>0.49&lt;sup&gt;a&lt;/sup&gt; 0.04</td>
<td>0.01&lt;sup&gt;b&lt;/sup&gt; 0.001</td>
<td>0.05&lt;sup&gt;b&lt;/sup&gt; 0.01</td>
<td>0.13&lt;sup&gt;b&lt;/sup&gt; 0.01</td>
<td>2.27&lt;sup&gt;c&lt;/sup&gt; 0.06</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>4.87&lt;sup&gt;a&lt;/sup&gt; 0.50</td>
<td>5.60&lt;sup&gt;a&lt;/sup&gt; 0.47</td>
<td>0.42&lt;sup&gt;b&lt;/sup&gt; 0.04</td>
<td>1.78&lt;sup&gt;b&lt;/sup&gt; 0.30</td>
<td>1.81&lt;sup&gt;b&lt;/sup&gt; 0.30</td>
<td>12.77&lt;sup&gt;c&lt;/sup&gt; 0.37</td>
</tr>
<tr>
<td>Others</td>
<td>5.60&lt;sup&gt;a&lt;/sup&gt; 0.17</td>
<td>6.73&lt;sup&gt;c&lt;/sup&gt; 0.17</td>
<td>6.50&lt;sup&gt;cd&lt;/sup&gt; 0.29</td>
<td>6.22&lt;sup&gt;ac&lt;/sup&gt; 0.17</td>
<td>5.86&lt;sup&gt;ad&lt;/sup&gt; 0.21</td>
<td>3.84&lt;sup&gt;b&lt;/sup&gt; 0.17</td>
</tr>
</tbody>
</table>

FAME, fatty acid methyl ester. <sup>a,b,c,d</sup> Mean values within a row with unlike superscript letters were significantly different (P < 0.05 ANOVA followed by post hoc Tukey’s multiple comparison test).
TABLE 2.5 Fatty acid profile (g/100g FAME) in the epididymal adipose tissue of mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. (Mean values ± SEM; n 9-10 mice per group).

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>LF-high maize starch g/100g FAME</th>
<th>LF-high sucrose g/100g FAME</th>
<th>HF-palm oil g/100g FAME</th>
<th>HF-olive oil g/100g FAME</th>
<th>HF-safflower oil g/100g FAME</th>
<th>HF-flaxseed/fish oil g/100g FAME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>14:0</td>
<td>1.01 ± 0.03</td>
<td>1.20 ± 0.02</td>
<td>0.68 ± 0.02</td>
<td>0.38 ± 0.01</td>
<td>0.46 ± 0.02</td>
<td>2.47 ± 0.06</td>
</tr>
<tr>
<td>16:0</td>
<td>20.04 ± 0.44</td>
<td>19.02 ± 0.16</td>
<td>22.35 ± 0.30</td>
<td>10.42 ± 0.20</td>
<td>11.54 ± 0.22</td>
<td>20.14 ± 0.25</td>
</tr>
<tr>
<td>16:1 cis-9</td>
<td>7.28 ± 0.21</td>
<td>8.53 ± 0.19</td>
<td>7.08 ± 0.26</td>
<td>3.01 ± 0.14</td>
<td>2.60 ± 0.28</td>
<td>6.13 ± 0.26</td>
</tr>
<tr>
<td>18:0</td>
<td>1.65 ± 0.06</td>
<td>1.35 ± 0.03</td>
<td>1.35 ± 0.02</td>
<td>1.28 ± 0.05</td>
<td>1.69 ± 0.09</td>
<td>3.04 ± 0.06</td>
</tr>
<tr>
<td>18:1 cis-9</td>
<td>48.40 ± 0.67</td>
<td>49.12 ± 0.50</td>
<td>55.21 ± 0.48</td>
<td>74.55 ± 0.39</td>
<td>22.57 ± 0.25</td>
<td>27.95 ± 0.31</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>13.13 ± 0.16</td>
<td>12.43 ± 0.25</td>
<td>8.29 ± 0.15</td>
<td>5.63 ± 0.12</td>
<td>56.27 ± 0.43</td>
<td>12.22 ± 0.14</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>1.45 ± 0.05</td>
<td>1.61 ± 0.07</td>
<td>0.10 ± 0.01</td>
<td>0.22 ± 0.01</td>
<td>0.35 ± 0.02</td>
<td>17.51 ± 0.26</td>
</tr>
<tr>
<td>18:3n-6</td>
<td>0.03 ± 0.001</td>
<td>0.03 ± 0.001</td>
<td>0.02 ± 0.001</td>
<td>0.01 ± 0.004</td>
<td>0.10 ± 0.01</td>
<td>0.07 ± 0.003</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.12 ± 0.003</td>
<td>0.12 ± 0.003</td>
<td>0.09 ± 0.003</td>
<td>0.08 ± 0.001</td>
<td>0.39 ± 0.02</td>
<td>0.12 ± 0.002</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.16 ± 0.01</td>
<td>0.17 ± 0.006</td>
<td>0.23 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>0.62 ± 0.04</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.10 ± 0.01</td>
<td>0.11 ± 0.005</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.98 ± 0.05</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.14 ± 0.01</td>
<td>0.15 ± 0.004</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.63 ± 0.02</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.35 ± 0.03</td>
<td>0.38 ± 0.02</td>
<td>ND</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.004</td>
<td>1.94 ± 0.06</td>
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<tr>
<td>Others</td>
<td>6.16 ± 0.10</td>
<td>4.95 ± 0.15</td>
<td>3.43 ± 0.10</td>
<td>3.49 ± 0.10</td>
<td>2.44 ± 0.10</td>
<td>3.79 ± 0.15</td>
</tr>
</tbody>
</table>

FAME, fatty acid methyl ester. ND, not detected. *Mean values in a row with unlike superscript letters were significantly different (P < 0.05 ANOVA followed by post hoc Tukey's multiple comparison test).
TABLE 2.6  Fatty acid profile (g/100g FAME) in the brain of mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. (Mean values ± SEM; n 9-10 mice per group).

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>LF-high maize starch g/100g FAME Mean</th>
<th>SEM</th>
<th>LF-high sucrose g/100g FAME Mean</th>
<th>SEM</th>
<th>HF-palm oil g/100g FAME Mean</th>
<th>SEM</th>
<th>HF-olive oil g/100g FAME Mean</th>
<th>SEM</th>
<th>HF-safflower oil g/100g FAME Mean</th>
<th>SEM</th>
<th>HF-flaxseed/fish oil g/100g FAME Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.57&lt;sup&gt;a&lt;/sup&gt; 0.07</td>
<td></td>
<td>0.19&lt;sup&gt;b&lt;/sup&gt; 0.03</td>
<td></td>
<td>0.18&lt;sup&gt;b&lt;/sup&gt; 0.02</td>
<td></td>
<td>0.12&lt;sup&gt;b&lt;/sup&gt; 0.002</td>
<td></td>
<td>0.12&lt;sup&gt;b&lt;/sup&gt; 0.01</td>
<td></td>
<td>0.25&lt;sup&gt;b&lt;/sup&gt; 0.04</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>24.31&lt;sup&gt;a&lt;/sup&gt; 0.28</td>
<td></td>
<td>22.18&lt;sup&gt;bc&lt;/sup&gt; 0.11</td>
<td></td>
<td>23.84&lt;sup&gt;a&lt;/sup&gt; 0.15</td>
<td></td>
<td>22.21&lt;sup&gt;bc&lt;/sup&gt; 0.13</td>
<td></td>
<td>21.78&lt;sup&gt;b&lt;/sup&gt; 0.17</td>
<td></td>
<td>22.84&lt;sup&gt;c&lt;/sup&gt; 0.17</td>
<td></td>
</tr>
<tr>
<td>16:1 cis-9</td>
<td>1.18&lt;sup&gt;a&lt;/sup&gt; 0.07</td>
<td></td>
<td>0.78&lt;sup&gt;de&lt;/sup&gt; 0.10</td>
<td></td>
<td>0.75&lt;sup&gt;bd&lt;/sup&gt; 0.03</td>
<td></td>
<td>0.49&lt;sup&gt;bd&lt;/sup&gt; 0.01</td>
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<td>0.49&lt;sup&gt;c&lt;/sup&gt; 0.02</td>
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<td>0.82&lt;sup&gt;de&lt;/sup&gt; 0.07</td>
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</tr>
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<td>21.59&lt;sup&gt;ac&lt;/sup&gt; 0.31</td>
<td></td>
<td>21.20&lt;sup&gt;ac&lt;/sup&gt; 0.17</td>
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<td>21.99&lt;sup&gt;bc&lt;/sup&gt; 0.15</td>
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<td>21.67&lt;sup&gt;ac&lt;/sup&gt; 0.25</td>
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</tr>
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<td></td>
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<td>20.29&lt;sup&gt;ad&lt;/sup&gt; 0.35</td>
<td></td>
<td>17.53&lt;sup&gt;b&lt;/sup&gt; 0.16</td>
<td></td>
<td>19.99&lt;sup&gt;ac&lt;/sup&gt; 0.26</td>
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</tr>
<tr>
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<td>0.64&lt;sup&gt;a&lt;/sup&gt; 0.15</td>
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<td>0.49&lt;sup&gt;a&lt;/sup&gt; 0.04</td>
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<td></td>
<td>1.95&lt;sup&gt;b&lt;/sup&gt; 0.22</td>
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<td>0.73&lt;sup&gt;a&lt;/sup&gt; 0.16</td>
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</tr>
<tr>
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<td></td>
<td>0.20&lt;sup&gt;ab&lt;/sup&gt; 0.15</td>
<td></td>
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<td>0.02&lt;sup&gt;a&lt;/sup&gt; 0.002</td>
<td></td>
<td>0.60&lt;sup&gt;b&lt;/sup&gt; 0.23</td>
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</tr>
<tr>
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<td></td>
<td>ND</td>
<td></td>
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<td>ND</td>
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<tr>
<td>20:3n-6</td>
<td>0.52&lt;sup&gt;a&lt;/sup&gt; 0.01</td>
<td></td>
<td>0.51&lt;sup&gt;a&lt;/sup&gt; 0.01</td>
<td></td>
<td>0.26&lt;sup&gt;b&lt;/sup&gt; 0.002</td>
<td></td>
<td>0.33&lt;sup&gt;c&lt;/sup&gt; 0.01</td>
<td></td>
<td>0.39&lt;sup&gt;d&lt;/sup&gt; 0.01</td>
<td></td>
<td>0.36&lt;sup&gt;e&lt;/sup&gt; 0.004</td>
<td></td>
</tr>
<tr>
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<td>6.97&lt;sup&gt;a&lt;/sup&gt; 0.21</td>
<td></td>
<td>7.39&lt;sup&gt;a&lt;/sup&gt; 0.21</td>
<td></td>
<td>8.84&lt;sup&gt;b&lt;/sup&gt; 0.09</td>
<td></td>
<td>8.71&lt;sup&gt;b&lt;/sup&gt; 0.09</td>
<td></td>
<td>8.96&lt;sup&gt;b&lt;/sup&gt; 0.08</td>
<td></td>
<td>6.15&lt;sup&gt;c&lt;/sup&gt; 0.09</td>
<td></td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.10&lt;sup&gt;a&lt;/sup&gt; 0.00</td>
<td></td>
<td>0.10&lt;sup&gt;a&lt;/sup&gt; 0.01</td>
<td></td>
<td>0.01&lt;sup&gt;b&lt;/sup&gt; 0.001</td>
<td></td>
<td>0.01&lt;sup&gt;b&lt;/sup&gt; 0.001</td>
<td></td>
<td>0.01&lt;sup&gt;b&lt;/sup&gt; 0.002</td>
<td></td>
<td>0.42&lt;sup&gt;c&lt;/sup&gt; 0.01</td>
<td></td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.29&lt;sup&gt;a&lt;/sup&gt; 0.01</td>
<td></td>
<td>0.29&lt;sup&gt;a&lt;/sup&gt; 0.01</td>
<td></td>
<td>0.05&lt;sup&gt;b&lt;/sup&gt; 0.001</td>
<td></td>
<td>0.10&lt;sup&gt;c&lt;/sup&gt; 0.003</td>
<td></td>
<td>0.07&lt;sup&gt;b&lt;/sup&gt; 0.001</td>
<td></td>
<td>0.77&lt;sup&gt;d&lt;/sup&gt; 0.01</td>
<td></td>
</tr>
<tr>
<td>22:6n-3</td>
<td>13.44&lt;sup&gt;a&lt;/sup&gt; 0.30</td>
<td></td>
<td>13.79&lt;sup&gt;a&lt;/sup&gt; 0.28</td>
<td></td>
<td>11.88&lt;sup&gt;b&lt;/sup&gt; 0.10</td>
<td></td>
<td>13.26&lt;sup&gt;a&lt;/sup&gt; 0.14</td>
<td></td>
<td>12.12&lt;sup&gt;b&lt;/sup&gt; 0.12</td>
<td></td>
<td>15.26&lt;sup&gt;c&lt;/sup&gt; 0.19</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>9.95&lt;sup&gt;a&lt;/sup&gt; 0.39</td>
<td></td>
<td>10.22&lt;sup&gt;a&lt;/sup&gt; 0.31</td>
<td></td>
<td>11.65&lt;sup&gt;b&lt;/sup&gt; 0.10</td>
<td></td>
<td>10.30&lt;sup&gt;ad&lt;/sup&gt; 0.11</td>
<td></td>
<td>11.28&lt;sup&gt;bd&lt;/sup&gt; 0.22</td>
<td></td>
<td>8.48&lt;sup&gt;c&lt;/sup&gt; 0.17</td>
<td></td>
</tr>
</tbody>
</table>

FAME, fatty acid methyl ester. ND, not detected. Mean values in a row with unlike superscript letters were significantly different (P < 0.05 ANOVA followed by post hoc Tukey’s multiple comparison test).
<table>
<thead>
<tr>
<th>SCFA</th>
<th>LF-high maize starch</th>
<th>LF-high sucrose</th>
<th>HF-palm oil</th>
<th>HF-olive oil</th>
<th>HF-safflower oil</th>
<th>HF-flaxseed/fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umol/g</td>
<td>umol/g</td>
<td>umol/g</td>
<td>umol/g</td>
<td>umol/g</td>
<td>umol/g</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Acetate</td>
<td>31.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.55</td>
<td>24.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.36</td>
<td>38.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.03</td>
</tr>
<tr>
<td>Propionate</td>
<td>14.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.93</td>
<td>11.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.46</td>
<td>19.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.03</td>
</tr>
<tr>
<td>Butyrate</td>
<td>14.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.06</td>
<td>11.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.56</td>
<td>21.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.00</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>10.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.03</td>
<td>8.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62</td>
<td>16.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.45</td>
</tr>
<tr>
<td>Total</td>
<td>72.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.56</td>
<td>55.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.01</td>
<td>96.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.52</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Mean values in a row with unlike superscript letters were significantly different (P < 0.05 ANOVA followed by post hoc Tukey’s multiple comparison test).
TABLE 2.8 Intestinal microbiota composition (% reads) in the caecal contents of mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with those fed low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. (n 9-10 mice per group). a,b Mean values within a row with unlike superscript letters were significantly different (P < 0.05; Kruskal-Wallis algorithm).

<table>
<thead>
<tr>
<th>Phylum</th>
<th>LF-high maize starch</th>
<th>LF-high sucrose</th>
<th>HF-palm oil</th>
<th>HF-olive oil</th>
<th>HF-safflower oil</th>
<th>HF-flaxseed/fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spirochaetes</td>
<td>0.01</td>
<td>0.00</td>
<td>0.04</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04</td>
</tr>
<tr>
<td>Candidate Division TM7</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Family</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfovibrionaceae</td>
<td>0.91</td>
<td>0.68</td>
<td>0.96</td>
<td>1.00</td>
<td>0.85</td>
<td>0.52</td>
</tr>
<tr>
<td>Rikenellaceae</td>
<td>9.18</td>
<td>6.20</td>
<td>3.37</td>
<td>6.02</td>
<td>6.88</td>
<td>5.88</td>
</tr>
<tr>
<td>Lactobacillaceae</td>
<td>2.41</td>
<td>1.95</td>
<td>2.24</td>
<td>1.45</td>
<td>2.25</td>
<td>0.95</td>
</tr>
<tr>
<td>Deferribacteraceae</td>
<td>5.98</td>
<td>4.28</td>
<td>5.83</td>
<td>7.93</td>
<td>7.01</td>
<td>6.23</td>
</tr>
<tr>
<td>Others</td>
<td>0.94</td>
<td>0.93</td>
<td>2.27</td>
<td>0.55</td>
<td>0.87</td>
<td>0.72</td>
</tr>
<tr>
<td>Genus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alistipes</td>
<td>5.50</td>
<td>3.62</td>
<td>1.83</td>
<td>3.05</td>
<td>3.87</td>
<td>4.17</td>
</tr>
<tr>
<td>Rikenella</td>
<td>3.35</td>
<td>2.22</td>
<td>1.24</td>
<td>2.37</td>
<td>2.48</td>
<td>1.42</td>
</tr>
<tr>
<td>Odoribacter</td>
<td>1.06</td>
<td>1.99</td>
<td>0.60</td>
<td>0.09</td>
<td>0.83</td>
<td>2.33</td>
</tr>
<tr>
<td>Lachnospiraceae Incertae Sedis</td>
<td>0.59</td>
<td>1.28</td>
<td>1.12</td>
<td>0.71</td>
<td>0.47</td>
<td>0.62</td>
</tr>
<tr>
<td>uncultured (Lachnospiraceae)</td>
<td>0.21</td>
<td>0.72</td>
<td>0.34</td>
<td>0.07</td>
<td>0.16</td>
<td>0.38</td>
</tr>
<tr>
<td>Coprococcus</td>
<td>0.24</td>
<td>0.37</td>
<td>0.61</td>
<td>0.16</td>
<td>0.17</td>
<td>0.24</td>
</tr>
<tr>
<td>Anaerotruncus</td>
<td>0.34</td>
<td>0.99</td>
<td>0.66</td>
<td>0.48</td>
<td>0.55</td>
<td>0.42</td>
</tr>
<tr>
<td>Peptococcus</td>
<td>0.16</td>
<td>0.22</td>
<td>0.31</td>
<td>0.07</td>
<td>0.25</td>
<td>0.19</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>2.41</td>
<td>1.95</td>
<td>2.24</td>
<td>1.45</td>
<td>2.25</td>
<td>0.95</td>
</tr>
<tr>
<td>Mucispirillum</td>
<td>5.98</td>
<td>4.28</td>
<td>5.83</td>
<td>7.93</td>
<td>7.01</td>
<td>6.23</td>
</tr>
<tr>
<td>Others</td>
<td>1.58</td>
<td>0.97</td>
<td>2.14</td>
<td>1.28</td>
<td>1.20</td>
<td>0.95</td>
</tr>
</tbody>
</table>
FIGURE 2.1(A) Body composition as determined by NMR showing the percentage of fat mass for mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with those fed low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. Values are means (n 9-10) ± SEM, represented by vertical bars. a,b,c Mean values with unlike letters were significantly different (P < 0.05; ANOVA followed by post hoc Tukey’s multiple comparison test).

(B) Body composition as determined by NMR showing the percentage of lean mass for mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with those fed low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. Values are means (n 9-10) ± SEM, represented by vertical bars. a,b,c,d Mean values with unlike letters were significantly different (P < 0.05; ANOVA followed by post hoc Tukey’s multiple comparison test).
**FIGURE 2.2(A)** Total liver weight of mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. Values are means (n 9-10) ± SEM, represented by vertical bars. a,b Mean values with unlike letters were significantly different (P < 0.05; ANOVA followed by post hoc Tukey’s multiple comparison test). **(B)** Total liver triglyceride levels in mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with low-fat (HF) diets supplemented with either high sucrose or high maize starch for 16 weeks. Values are means (n 9-10) ± SEM, represented by vertical bars. a,b,c Mean values with unlike letters were significantly different (P < 0.05; ANOVA followed by post hoc Tukey’s multiple comparison test).
FIGURE 2.3 Principal co-ordinate analysis using unweighted UniFrac distances for mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil compared with those fed low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. ● LF-high maize starch, ○ HF-palm oil, ○ HF-olive oil, ● HF-safflower oil, ○ HF-flaxseed/fish oil and ○ LF-high sucrose.
FIGURE 2.4 Phylum level distributions of the microbial communities in caecal contents, expressed as a percentage of the total population of assignable tags, in mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with those fed low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. a,b,c Values with unlike letters were significantly different ($P < 0.05$; Kruskal-Wallis algorithm).
FIGURE 2.5 Family level taxonomic distributions of the microbial communities in caecal contents, expressed as a percentage of total tags assignable at family level, in mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with those fed low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. Values with unlike letters were significantly different ($P < 0.05$; Kruskal-Wallis algorithm).
FIGURE 2.6 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 fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with those fed low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. \( a, b, c \) Values with unlike letters were significantly different \( (P < 0.05; \text{Kruskal-Wallis algorithm}) \).
SUPPLEMENTARY FIGURE 2.1 Relative mRNA expression of metabolism genes within the liver of mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. Values are means (n 9-10) ± SEM, represented by vertical bars. \(^{a,b,c}\) Mean values with unlike letters were significantly different (\(P < 0.05\); ANOVA followed by post hoc Tukey’s multiple comparison test).
**SUPPLEMENTARY FIGURE 2.2** Chao1 richness estimation of α diversity of intestinal microbiota compositions of mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. The nonparametric Kruskal-Wallis test was used to estimate the relationship between different groups. Values in the same row which do not share a common superscript letter are significantly different, $P < 0.05$. 

![Chao1 richness estimation](image-url)
SUPPLEMENTARY FIGURE 2.3 Simpson index of α diversity of intestinal microbiota compositions of mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. The nonparametric Kruskal-Wallis test was used to estimate the relationship between different groups. Values in the same row which do not share a common superscript letter are significantly different, P < 0.05.
SUPPLEMENTARY FIGURE 2.4 Shannon index of α diversity of intestinal microbiota compositions of mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. The nonparametric Kruskal-Wallis test was used to estimate the relationship between different groups. Values in the same row which do not share a common superscript letter are significantly different, P < 0.05.
SUPPLEMENTARY FIGURE 2.5 Phylogenetic diversity measurement of α diversity of intestinal microbiota compositions of mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. The nonparametric Kruskal-Wallis test was used to estimate the relationship between different groups. Values in the same row which do not share a common superscript letter are significantly different, P < 0.05.
SUPPLEMENTARY FIGURE 2.6 Observed species measurement of α diversity of intestinal microbiota compositions of mice fed high-fat (HF) diets supplemented with either palm oil, olive oil, safflower oil or flaxseed/fish oil, compared with low-fat (LF) diets supplemented with either high sucrose or high maize starch for 16 weeks. The nonparametric Kruskal-Wallis test was used to estimate the relationship between different groups. Values in the same row which do not share a common superscript letter are significantly different, P < 0.05.
Chapter 3

*Bifidobacterium breve* and α-linolenic acid impact on hepatic and intestinal lipid metabolism in the host
3.1 ABSTRACT

The aim of this study was to address the mechanisms by which an α-linolenic acid (ALA)-enriched diet supplemented with either *Bifidobacterium breve* NCIMB 702258 or *B. breve* DPC 6330 impacted on fat distribution and composition in the host, as previously reported (Wall et al., 2010). This was approached by assessing gene expression of key enzymes involved in *n*-3 polyunsaturated fatty acid (PUFA) metabolism in the liver and fatty acid uptake enzymes in the distal small intestine and/or liver, following dietary supplementation with the strains. Female BALB/c mice (*n* 10 mice per group) received a diet enriched with 3% (w/w) ALA either alone or supplemented with either *B. breve* NCIMB 702258 or *B. breve* DPC 6330 (10⁹ CFU/day) for 6 weeks and compared with unsupplemented controls. Tissue fatty acid composition was assessed by gas-liquid chromatography (GLC) and gene expression levels were determined using real-time PCR. Supplementation of the ALA-enriched diet with *B. breve* DPC 6330 increased mRNA expression of fatty acid binding protein (FABP)-1 (*P* < 0.05) and diacylglycerol acyltransferase (DGAT)-2 (*P* < 0.05) in the ileum, compared with unsupplemented controls. In addition, *B. breve* DPC 6330 increased ileal mRNA expression of CD-36, compared with all groups (*P* < 0.05). Liver triglyceride (TAG) levels were reduced in all ALA-enriched dietary groups, compared with unsupplemented controls (*P* < 0.05). Supplementation of *B. breve* in an ALA-enriched diet further reduced liver TAG levels (*P* < 0.05), compared with the ALA-enriched diet alone and this was associated with a reduced hepatic CD-36 and FABP-1 mRNA expression (*P* < 0.05). As expected, (Wall et al., 2012), supplementation of the ALA-enriched diet with *B. breve* NCIMB 702258 increased docosapentaenoic acid (precursor to docosahexaenoic acid (DHA)) (*P* < 0.05), with a tendency for increased DHA.
concentrations in the liver \((P=0.077)\), compared with mice fed the ALA-enriched diet alone. This strain-specific effect of \textit{B. breve} NCIMB 702258 on tissue fatty acid composition was not associated with an impact on mRNA expression of hepatic \(n\)-3 PUFA metabolism enzymes. In conclusion, \textit{B. breve} NCIMB 702258 and \textit{B. breve} DPC 6330 both exhibited strain-specific effects on liver TAG levels associated with a decrease in fatty acid uptake by the liver.

### 3.2 INTRODUCTION

It has previously been shown that dietary supplementation with \textit{B. breve} NCIMB 702258 and \textit{B. breve} DPC 6330 positively influenced fatty acid profiles of the liver, adipose tissue and brain in different animal species and models, as well as influencing host intestinal microbiota composition (Barrett et al., 2012; Wall et al., 2012; Wall et al., 2010; Wall et al., 2009). These studies suggest that dietary supplementation with a probiotic bacteria can significantly influence health through the production of bioactive fatty acids such as conjugated derivatives of PUFA (conjugated linoleic acid (CLA) and conjugated ALA (CALA), for example) and by increasing tissue concentrations of bioactive long-chain (LC) members of the \(n\)-3 PUFA family (eicosapentaenoic acid (EPA; 20:5\(n\)-3), docosapentaenoic acid (22:5\(n\)-3) and DHA (22:6\(n\)-3), for example). The interaction between resident gut microbes, dietary derived fatty acids, probiotic supplementation and implications for health have recently been reviewed (Marques et al., 2013). However, the mechanisms by which probiotic bacteria such as \textit{B. breve} can increase the production of bioactive LC-PUFA in host tissues are unclear.

Optimal dietary intakes of \(n\)-6: \(n\)-3 PUFA should be in the ratio of \(~1\)-4:1, however recent shifts in dietary PUFA consumption has increased this ratio to \(~15\)-16:1.
Coinciding increases in the incidence of diseases linked to inflammatory processes such as cardiovascular disease (CVD), obesity, inflammatory bowel disease (IBD) and cancer have all been linked with a low dietary intake of $n$-3 PUFA (Calder, 2009). In contrast, the benefits of ALA consumption on cardiovascular health, immune regulation and inflammation have all been described (Pan et al., 2012). Other functions of ALA require its further metabolism to more highly unsaturated, members of the $n$-3 PUFA family. Flaxseed is thus emerging as an important functional food ingredient since it is one of the richest sources of ALA (Singh et al., 2011).

ALA is converted to EPA, docosapentaenoic acid and DHA through a series of desaturation and elongation reactions, primarily in the liver (Patterson et al., 2012). Dietary supplementation with ALA has previously been shown to increase tissue concentrations of EPA and DHA (Ander et al., 2010; Ren et al., 2012; Wall et al., 2010). Furthermore, dietary supplementation with an ALA-enriched diet in combination with *B. breve* can increase tissue concentrations of EPA, docosapentaenoic acid and DHA, compared with an ALA-enriched diet alone (Barrett et al., 2012; Wall et al., 2010). It was hypothesised that the strains may alter hepatic $n$-3 PUFA metabolism/uptake or fatty acid uptake in the distal small intestine (Wall et al., 2012). Thus, the aim of this study was to determine whether supplementing an ALA-enriched diet with *B. breve* NCIMB 702258 or *B. breve* DPC 6330 could impact on mRNA expression of genes involved in lipid metabolism in both the ileum and liver, and/or $n$-3 PUFA metabolism in the liver.

### 3.3 MATERIALS AND METHODS
3.3.1 PREPARATION AND ADMINISTRATION OF *B. breve* NCIMB 702258 AND *B. breve* DPC 6330

It was previously shown 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 cis-9, trans-11 CLA when grown in 0.5 mg/mL linoleic acid 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 MRS agar (de Man, Rogosa & Sharpe; Difco Laboratories, Detroit, MI, USA), supplemented with 0.05% (w/v) L-cysteine hydrochloride (Sigma, Wicklow, Ireland) (mMRS), containing 500 µg/mL rifampicin (Sigma). Following anaerobic incubation (anaerobic jars with Anaerocult A gas packs; Merck, Darmstadt, Germany) at 37°C for 3 days, colonies were stocked in mMRS broth containing 40% (v/v) glycerol and stored at -80°C. To confirm that the rifampicin resistant variants were identical to the parent strain, molecular fingerprinting using pulse-field gel electrophoresis was employed.

Prior to freeze drying, *B. breve* NCIMB 702258 and *B. breve* DPC 6330 were grown in mMRS by incubating for 48 hr at 37°C under anaerobic conditions. The culture was washed twice in phosphate buffered saline (PBS) and resuspended at a concentration of ~2×10^10 cells/mL in 15% (w/v) trehalose (Sigma) in dH_2O. One millilitre aliquots were freeze-dried using a 24 hr programme (freeze temp. -40°C, condenser set point -60, vacuum set point 600 m Torr). Each mouse that received the bacterial strains consumed approximately 1×10^9 live microorganisms per day. 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% (w/v) trehalose in dH2O). Water containing either the bacterial strains or placebo freeze-dried powder was the only water supplied to the mice throughout the trial. Freeze dried powders with the bacterial strains underwent continuous quality control checks of cell counts for the duration of the trial, by plating serial dilutions on mMRS agar supplemented with 100 µg/mL of mupirocin (Oxoid) and 100 µg/mL rifampicin (Sigma) and incubating plates anaerobically for 72 hr at 37°C.

3.3.2 ANIMALS AND TREATMENT

All animal experiments were approved by the University College Cork (UCC) Animal Ethics Committee and experimental procedures were conducted under the appropriate license from the Irish Government. Female BALB/c mice were purchased from Harlan Limited (Briester, Oxon, UK) at 8 weeks of age and housed under barrier-maintained conditions within the Biological Services Unit, UCC. Mice were allowed to acclimatise for 1 week prior to commencement of the study and were fed ad libitum with Teklad Global Rodent Standard Diet (Harlan Laboratories, Madison, WI, USA, #2018S), with free access to water at all times. Mice were housed in groups of five per cage and kept in a controlled environment at 25°C under a 12-hr-light/12-hr-dark cycle. After 1 week of acclimatisation, the mice were randomly divided into four groups (n 10 mice per group): (1) a FXO supplemented dietary group (enriched in ALA) with B. breve NCIMB 702258 (approximate daily dose of 10^9 microorganisms); (2) a FXO supplemented dietary group (enriched in ALA) with B. breve DPC 6330 (approximate daily dose of 10^9 microorganisms); (3) a flaxseed-oil (FXO) supplemented dietary group (enriched in ALA) with placebo freeze-dried powder (15% w/v trehalose in drinking
water); (4) a control- unsupplemented group fed a standard rodent diet with placebo freeze-dried powder (15% w/v trehalose in drinking water).

The unsupplemented control diet contained the following nutrient composition: corn starch (32.45%), casein (20.0%), sucrose (15.0%), maltodextrin (12.0%), cellulose (5.0%), mineral mix (3.5%), vitamin mix (1.5%), L-cysteine (0.3%), choline bitartrate (0.25%), TBHQ antioxidant (0.002%) and the following composition of fat: palm oil (3.0%), safflower oil (3.0%), olive oil (3.0%), FXO (1.0%). The n-6 : n-3 ratio of this diet was ~5.3 and the diet contained ~0.5% ALA. The FXO supplemented diet contained the following nutrient composition: corn starch (32.45%), casein (20.0%), sucrose (15.0%), maltodextrin (12.0%), cellulose (5.0%), mineral mix (3.5%), vitamin mix (1.5%), L-cysteine (0.3%), choline bitartrate (0.25%), TBHQ antioxidant (0.002%) and the following composition of fat: FXO (5.5%), palm oil (1.5%), safflower oil (1.5%), olive oil (1.5%). The n-6 : n-3 ratio of this diet was ~0.75 and the diet contained ~3% ALA.

Body weight and food intake were assessed weekly. Following 6 weeks on experimental diets, the animals were killed by cervical dislocation. 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°C until processed. Blood samples were collected in serum collection tubes (BD Diagnostics, Oxford, UK) from fasted animals, and allowed to clot for at least 30 min at 4°C before centrifugation for 20 min at 10,000g to separate the serum.

3.3.3 CULTURE DEPENDENT MICROBIAL ANALYSIS
Fresh faecal samples were taken from BALB/c mice every second week for microbial analysis. Microbial analysis of the faecal samples involved enumeration of the \textit{B. breve} strains by plating serial dilutions on MRS agar supplemented with 100 µg/mL of mupirocin (Oxoid), 100 µg/mL of rifampicin (Sigma) and 50 units/mL of nystatin (Sigma). Agar plates were incubated anaerobically for 72 hr at 37°C.

3.3.4 LIPID EXTRACTION AND FATTY ACID ANALYSIS

Lipids were extracted with chloroform-methanol (2:1, vol:vol; Thermo Scientific, Dublin, Ireland) according to the method of Folch (Folch, 1957). Fatty acid methyl esters (FAME) were prepared by using first 10mL 0.5 M NaOH (Sigma) in methanol for 10 min at 90°C followed by 10mL 14% BF$_3$ in methanol (Sigma) for 10 min at 90°C (Park and Goins, 1994). FAME were recovered with hexane (Thermo Scientific). Before GLC analysis, samples were dried over 0.5g anhydrous sodium sulphate (Sigma) for 1 hr and stored at -20°C. FAME were separated by GLC (Varian 3800; Varian) fitted with a flame-ionisation detector by using a Chrompack CP Sil 88 column (Chrompack; 100 m x 0.25 mm internal diameter, 0.20-µm 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 µL, with an 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, MN, USA). 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 data are presented as means ± standard errors of the mean (SEM) in g/100g FAME.

### 3.3.5 Measurement of TAG in the Liver and Serum

The lipids from 50 mg of frozen liver were extracted and purified according to the method of Folch (Folch, 1957). Liver lipids were extracted using chloroform-methanol (2:1, vol:vol; Thermo Scientific) and an aliquot of the organic phase was collected, dried and resuspended in duplicate in Infinity-TAG lipid-stable reagent (Thermo Scientific). TAG levels in the samples were determined according to the manufacturer’s instructions and lipids were quantified using a TAG chemistry calibrator (Pointe Scientific Inc., MI, USA). Serum TAG levels were analysed in duplicate using the commercial L-Type TAG M kit (Wako Diagnostics, Neuss, Germany).

### 3.3.6 RNA Extractions and Complementary DNA Synthesis

Total RNA was isolated from the liver and ileum using the commercial RNeasy Mini-Kit (Qiagen, West Sussex, UK), according to the manufacturer’s instructions. Total isolated RNA was quantified using the Nanodrop (Thermo Scientific). Single stranded complementary DNA (cDNA) was synthesised from 1μg of total RNA using 2.5ng/μL random primers (Promega, WI, USA), 10mM PCR nucleotide mix (Promega), 40units/μL RNasin Plus RNase inhibitor (Promega) and the Im-Prom II reverse transcriptase (Promega).

### 3.3.7 Real-time PCR Analysis
Amplification of generated cDNA was performed in the Lightcycler 480 system (Roche Diagnostics Ltd., West Sussex, UK) using 0.25μM primers (MWG Eurofins, Ebersberg, Germany), 1μL cDNA and the Lightcycler 480 SYBR Green I Master kit (Roche Diagnostics Ltd). Real-time PCR conditions were set at: 95°C for 10 min followed by 50 cycles at 95°C for 10 sec, 60°C for 5 sec and 72°C for 15 sec. Specific forward and reverse primers used to amplify cDNA were newly designed and are listed in Table 3.1. All samples were analysed in duplicate and normalised to β-actin, as a constitutively expressed control gene. Melting curve analysis allowed the validation of the authenticity of the real-time PCR products. Basic relative quantification of expression was determined using the comparative $2^{-\Delta\Delta Ct}$ method.

3.3.8 STATISTICAL ANALYSIS

All results are presented as means ± SEM (per group). To assess whether differences between the treatment groups were significant, data were analysed using one-way ANOVA followed by post hoc Tukey’s multiple comparison test using GraphPad Prism version 4.0 for Windows (GraphPad Software). Statistical significance was accepted at $P < 0.05$.

3.4 RESULTS

3.4.1 SURVIVAL AND TRANSIT OF B. breve DPC 6330 AND B. breve NCIMB 702258 IN BALB/c MICE

Quantification of the numbers of administered B. breve strains in the faeces of mice confirmed gastrointestinal transit and survival. Stool recovery of B. breve NCIMB 702258 and B. breve DPC 6330 were approximately 1x10^7 CFU/g faeces and 5x10^6
CFU/g faeces, respectively after 1 week of feeding and remained at similar numbers at weeks 2 and 4. At week 6, there was a decline in the numbers of excreted *B. breve* strains, with stool recovery of *B. breve* NCIMB 702258 and *B. breve* DPC 6330 being 9x10⁵ CFU/g faeces and 8x10⁵ CFU/g faeces, respectively.

3.4.2 DIETARY SUPPLEMENTATION WITH *B. breve* NCIMB 702258 AND *B. breve* DPC 6330 REDUCED LIVER TAG LEVELS

No differences in food intake, body weight gain, visceral fat mass or serum TAG levels were observed between the groups (Table 3.2). Analysis of total liver TAG levels showed that ALA-supplementation, either alone or in combination with either *B. breve* strain significantly reduced liver TAG levels, compared with unsupplemented controls (*P* < 0.05; Table 3.2). Supplementation of *B. breve* NCIMB 702258 and *B. breve* DPC 6330 in an ALA-enriched diet further reduced liver TAG, compared with feeding the ALA-enriched diet alone (*P* < 0.05; Table 3.2).

3.4.3 EFFECT OF DIETARY ENRICHMENT WITH ALA ALONE OR SUPPLEMENTED WITH *B. breve* NCIMB 702258 AND *B. breve* DPC 6330 ON TISSUE FATTY ACID COMPOSITION

To investigate the effects of the ALA-enriched diet on tissue fatty acid composition, fatty acid profiling was performed on liver, epididymal adipose tissue and brain. As expected, following the 6 week feeding period, enrichment of the diet with ALA significantly increased tissue ALA concentrations, by 5- and 6-fold in the epididymal adipose tissue (*P* < 0.05; Table 3.4) and liver (*P* < 0.05; Table 3.3) respectively, compared with unsupplemented controls. Furthermore, the ALA-enriched dietary
groups exhibited significantly higher concentrations of EPA in the liver (by ~8-fold; P < 0.05; Table 3.3), in the epididymal adipose tissue (by ~6-fold; P < 0.05; Table 3.4) and in the brain (by ~2-fold; P < 0.05; Table 3.5), compared with unsupplemented controls. Additionally, docosapentaenoic acid concentrations were significantly higher in the ALA-enriched groups in the liver (by ~2-fold; P < 0.05; Table 3.3), in the epididymal adipose tissue (by ~4-fold; P < 0.05; Table 3.4) and in the brain (by 2-fold; P < 0.05; Table 3.5), compared with unsupplemented controls. Supplementation of the ALA-enriched diet with *B. breve* NCIMB 702258 significantly increased concentrations of EPA in the brain, compared with *B. breve* DPC 6330 supplementation (P < 0.05; Table 3.5). Moreover, docosapentaenoic acid concentrations in the liver were significantly higher following supplementation of the ALA-enriched diet with *B. breve* NCIMB 702258, compared with the ALA-enriched diet (P < 0.05; Table 3.3). In addition, supplementation of the ALA-enriched diet with *B. breve* NCIMB 702258 led to significantly increased DHA in the liver, compared with unsupplemented controls (P < 0.05; Table 3.3) and resulted in a tendency for increased DHA in the liver, compared with mice fed the ALA-enriched diet (P=0.077; Table 3.3). Dietary ALA enrichment regardless of microbial supplementation, significantly increased epididymal adipose tissue concentrations of DHA, compared with unsupplemented controls (P < 0.05; Table 3.4) while in the brain, the ALA-enriched diet increased DHA concentrations, compared with unsupplemented controls (P < 0.05; Table 3.5).

All groups fed the ALA-enriched diet exhibited lower oleic acid (18:1 *cis*-9) in the liver and epididymal adipose tissue (P < 0.05; Tables 3.3 and 3.4) and higher dihomoy-γ-linolenic acid (20:3n-6) in the brain (P < 0.05; Table 3.5), compared with unsupplemented controls. In addition, concentrations of arachidonic acid (20:4n-6)
were significantly lower in the liver \((P < 0.05; \text{Table } 3.3)\) and epididymal adipose tissue \((P < 0.05; \text{Table } 3.4)\) in all ALA-enriched dietary groups, compared with unsupplemented controls. Both groups that received \textit{B. breve} supplementation with the ALA-enriched diet exhibited higher palmitoleic acid \((16:1 \text{ cis}-9)\) and oleic acid in the brain \((P < 0.05; \text{Table } 3.5)\) and higher stearic acid \((18:0)\) in the liver \((P < 0.05; \text{Table } 3.3)\), compared with the ALA-enriched dietary group. Supplementation with \textit{B. breve} DPC 6330 also resulted in higher palmitic acid \((16:0)\) and lower linoleic acid and \(\gamma\)-linolenic acid in the liver \((P < 0.05; \text{Table } 3.3)\), compared with the ALA-enriched dietary group.

### 3.4.4 Impact on mRNA gene expression levels of enzymes involved in n-3 PUFA metabolism within the liver and the uptake of fatty acids to the liver following dietary enrichment with ALA either alone or supplemented with \textit{B. breve} NCIMB 702258 or \textit{B. breve} DPC 6330

Following the 6 week feeding period, mRNA expression of enzymes involved in n-3 PUFA metabolism within the liver (Figure 3.1(A)) and genes involved in the transport and uptake of fatty acids to the liver (Figure 3.1(B)) were determined using real-time PCR.

An increase in the mRNA expression of \(\Delta-6\)-desaturase was found in all ALA-enriched dietary groups, compared with unsupplemented controls \((P < 0.05; \text{Figure } 3.1(A))\). Hepatic mRNA expression of elongation of very long chain fatty acids (ELOVL)-5 was significantly reduced in mice fed the ALA-enriched diet, compared with unsupplemented controls and the ALA-enriched dietary group supplemented with \textit{B. breve} DPC 6330 \((P < 0.05; \text{Figure } 3.1(A))\). In addition, supplementation of the ALA-
enriched diet with *B. breve* DPC 6330 significantly increased mRNA expression of ELOVL-6, compared with the ALA-enriched diet (*P* < 0.05; Figure 3.1(A)). Both ALA-enriched dietary groups supplemented with *B. breve* and the unsupplemented control group exhibited significantly reduced hepatic mRNA expression of Δ-5-desaturase, compared with the ALA-enriched dietary group (*P* < 0.05; Figure 3.1(A)). No significant differences were observed in hepatic mRNA expression of stearyl-CoA desaturase (SCD)-1 or ELOVL-2 (Figure 3.1(A)).

Both groups fed the ALA-enriched diet supplemented with *B. breve* NCIMB 702258 and *B. breve* DPC 6330 exhibited significantly decreased hepatic mRNA expression of CD-36, compared with mice that received the ALA-enriched diet and unsupplemented controls (*P* < 0.05; Figure 3.1(B)). In addition, supplementation of the ALA-enriched diet with both *B. breve* strains was associated with significantly decreased hepatic mRNA expression of FABP-1, compared with the ALA-enriched dietary group (*P* < 0.05; Figure 3.1(B)). The unsupplemented controls exhibited ~5-fold increase in hepatic mRNA expression of fatty acid synthase (FAS), compared with all ALA-enriched groups (*P* < 0.05; Figure 3.1(B)). Hepatic mRNA expression of sterol regulatory element binding protein (SREBP)-1c was significantly increased following supplementation with ALA either alone or in combination with *B. breve* DPC 6330, compared with unsupplemented controls (*P* < 0.05; Figure 3.1(B)). No differences in hepatic mRNA expression of fatty acid transport protein (FATP)-5 were observed between the groups (Figure 3.1(B)).

### 3.4.5 Impact on mRNA Gene Expression Levels of Enzymes Involved in the Transport and Uptake of Fatty Acids to the Ileum Following Dietary
Supplementation of the ALA-enriched diet with *B. breve* DPC 6330 significantly increased ileal mRNA gene expression of FABP-1 and DGAT-2, compared with unsupplemented controls (*P* < 0.05; Figure 3.2). No differences in the expression of FABP-1, DGAT-2, FABP-2 or FATP-4 were observed between the ALA-enriched dietary groups (Figure 3.2). Mice supplemented with *B. breve* DPC 6330 with the ALA-enriched diet exhibited a significant increase in CD-36 mRNA expression, compared with all other groups (*P* < 0.05; Figure 3.2). No differences were observed in mRNA expression of FABP-2 or FATP-4 between the groups (Figure 3.2).

3.5 DISCUSSION

The mechanisms by which both dietary substrates and ingested probiotics influence tissue fatty acid composition with implications for health are unclear. The present study investigated the mechanisms involved in modulation of tissue fatty acid composition by *B. breve* NCIMB 702258 and *B. breve* DPC 6330 following dietary supplementation. In particular, we focused on the impact of *B. breve* in an ALA-enriched diet on lipid metabolism in the distal small intestine and liver.

Supplementation of the ALA-enriched diet with *B. breve* DPC 6330 significantly increased hepatic mRNA expression levels of ELOVL-5 and ELOVL-6, involved in the elongation of fatty acids to more unsaturated members of the n-3 PUFA family. This indicates a strain-specific effect of *B. breve* DPC 6330 on hepatic n-3 PUFA metabolism. Despite an increase in hepatic ELOVL-5 and ELOVL-6 expression following supplementation of the ALA-enriched diet with *B. breve* DPC 6330, no additional
increases in tissue EPA, docosapentaenoic acid or DHA concentrations were observed in this group. All ALA-enriched dietary groups exhibited increased hepatic mRNA expression of Δ-6-desaturase. This enzyme is involved in the biosynthetic pathway of LC-PUFA (Stoffel et al., 2008) and thus, may partly explain the increased EPA and docosapentaenoic acid concentrations in the liver, epididymal adipose tissue and brain exhibited for all ALA-enriched dietary groups. Beyond docosapentaenoic acid formation, Δ-6-desaturase also metabolises tetracosapentaenoic acid (precursor to DHA), however since Δ-6-desaturase is used by two substrates in the same pathway of n-3 PUFA metabolism, there is intrinsic potential for competitive substrate inhibition to occur (Gregory et al., 2011). Previous studies have revealed that high dietary ALA up regulates hepatic Δ-6-desaturase mRNA expression levels in different models (Li et al., 2008; Ren et al., 2012). One recent study demonstrating the impact of ALA supplementation on Δ-6-desaturase and ELOVL-5 expression in the common carp showed that after 42 days of feeding, while Δ-6-desaturase activity decreased, there was an increase in ELOVL-5 expression (Ren et al., 2012). This suggests that while the activity of one rate-limiting enzyme involved in the metabolism of n-3 PUFA increases, there may not be a corresponding increase in the activity of the other at the same time, thus the sequence of enzyme activity is time dependent. This may explain why we did not observe an increase in ELOVL-5 expression in all ALA-enriched dietary groups since Δ-6-desaturase activity was increased at this time.

Supplementation of an ALA-enriched diet with B. breve DPC 6330 had a significant effect on increasing the mRNA expression of genes involved in the transport and uptake of fatty acids to the ileum. Significant increases in mRNA expression of FABP-1, DGAT-2 and CD-36 in the ileum were found following supplementation of the ALA-
enriched diet with *B. breve* DPC 6330. The increase in mRNA expression of CD-36 following supplementation of the ALA-enriched diet with *B. breve* DPC 6330 was significant compared with the group fed the ALA-enriched diet alone, thus indicating a strain-specific effect of *B. breve* DPC 6330 on lipid metabolism in the ileum. FABP-1 and CD-36 glycoprotein, which play a role in cellular adhesion and in the regulation of fatty acid transport and uptake, have previously been shown to be increased in the intestine following *in vivo* exposure of human intestinal mucosa to the probiotic species *Lactobacillus plantarum* WCFSI (Troost et al., 2008). Furthermore, the role of the microbiota in stimulating dietary fatty acid absorption in the intestinal epithelium have previously been demonstrated (Semova et al., 2012).

Hepatic TAG levels were significantly reduced following 6 weeks of dietary intervention with an ALA-enriched diet, thus indicating an n-3 PUFA impact on hepatic TAG reduction. It has been reported that EPA may be a promising novel therapy to decrease hepatic TAG (Masterton et al., 2010; Shapiro et al., 2011) and may therefore be of importance in the treatment of non-alcoholic fatty liver disease (NAFLD). Coincidently, all ALA-enriched diets in this study increased liver EPA concentrations. In addition, all groups fed the ALA-enriched diets exhibited reduced hepatic FAS mRNA expression. Supplementation of the ALA-enriched diet with either *B. breve* NCIMB 702258 or *B. breve* DPC 6330 further decreased hepatic TAG levels, compared with the ALA-enriched dietary group. These data demonstrate a potential for *B. breve* to reduce liver TAG levels in the host. The decrease in liver TAG following supplementation of the ALA-enriched diet with *B. breve* correlated with a significant decrease in hepatic mRNA expression levels of CD-36 and FABP-1, compared with the ALA-enriched dietary group, whereby CD-36 and FABP-1 are involved in transport and uptake of fatty acids to the
liver (Garcia-Monzon et al., 2011; Newberry et al., 2003). This indicates a strain-specific effect of *B. breve* NCIMB 702258 and *B. breve* DPC 6330 toward reducing hepatic TAG levels which correlates with a reduction in the uptake of fatty acids to the liver. Recent studies have described a potential therapeutic role for certain probiotic strains in the treatment of NAFLD (Iacono et al., 2011). For example, using a cholesterol enriched diet, administration of *Lactobacillus plantarum* MA2 to rats reduced liver TAG and increased the numbers of faecal lactobacilli and bifidobacteria (Wang et al., 2009). Furthermore, the potential of VSL#3, a multistrain preparation composed of *Streptococcus thermophilus* and several strains of *Lactobacillus* and *Bifidobacterium* at reducing inflammation and oxidative damage to the liver while also ameliorating liver lipid profiles has been demonstrated (Esposito et al., 2009).

Dietary ALA-supplementation increased liver EPA and docosapentaenoic acid concentrations, epididymal adipose tissue EPA, docosapentaenoic acid and DHA concentrations and brain EPA and docosapentaenoic acid concentrations. In addition, the increases in liver and epididymal adipose tissue EPA and docosapentaenoic acid concentrations following dietary ALA-supplementation were at the expense of arachidonic acid. EPA replaces arachidonic acid as a eicosanoid substrate in cell membranes (Simopoulos, 2003), resulting in decreased production of arachidonic-acid derived proinflammatory eicosanoids such as prostaglandin E₂ and leukotriene B₄ (Calder, 2006) thus, increases in tissue EPA concentrations would be beneficial in a variety of chronic inflammatory settings such as NAFLD and IBD. Moreover, dietary ALA-supplementation increased docosapentaenoic acid concentrations in the brain. Since docosapentaenoic acid is the precursor to DHA, which has been shown as a vital fatty acid for brain development, influencing cognitive processes such as memory and
learning (Innis, 2007; Yurko-Mauro et al., 2010), the observed increases in brain docosapentaenoic acid concentrations following dietary ALA supplementation is beneficial. Supplementation of the ALA-enriched diet with *B. breve* NCIMB 702258 increased liver docosapentaenoic acid with a tendency towards increased liver DHA, compared with mice fed the ALA-enriched diet. This is consistent with previous observations, *i.e.* that the response of fatty acid metabolism to administration of bifidobacteria is strain-dependent (Wall et al., 2012).

The data presented in this study have demonstrated strain-specific effects of *B. breve* DPC 6330 supplementation in an ALA-enriched diet on lipid metabolism in the ileum and strain-specific effects of both *B. breve* NCIMB 702258 and *B. breve* DPC 6330 on lipid metabolism in the liver. Supplementation with either *B. breve* strains in an ALA-enriched diet reduced liver TAG levels and this was associated with reduced hepatic CD-36 and FABP-1 expression, involved in the uptake of fatty acids to the liver. The effect of *B. breve* NCIMB 702258 and *B. breve* DPC 6330 on reducing hepatic TAG levels may be of therapeutic importance for the future treatment of NAFLD. Dietary ALA-supplementation positively impacted on tissue fatty acid compositions, increased hepatic n-3 PUFA metabolism enzymes and reduced hepatic TAG levels, hepatic fatty acid synthesis enzymes and hepatic uptake of fatty acids. In conclusion, this study provided some promising data on the potential mechanisms through which the probiotic strains *B. breve* NCIMB 702258 and *B. breve* DPC 6330 may influence fat distribution in the host by altering lipid metabolism at the molecular level.
3.6 ACKNOWLEDGEMENTS

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3.7 REFERENCES


acid uptake in mice with deletion of the liver fatty acid-binding protein gene. *The Journal of Biological Chemistry.* **278:**51664-51672.


### TABLE 3.1 Primer sequences used for real-time PCR

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<th>Liver Gene Symbol</th>
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<td>5’-CAATAGTGATGACCTGGCGT-3’</td>
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**TABLE 3.2** Body mass, fat mass, liver mass, liver and serum triglyceride (TAG) levels in mice fed an ALA-enriched diet either alone or in combination with *Bifidobacterium breve* NCIMB 702258 or *B. breve* DPC 6330 or an unsupplemented diet for 6 weeks. (Mean values ± SEM; *n* 10 mice per group).

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<td>0.61</td>
<td>0.05</td>
<td>0.75</td>
<td>0.06</td>
</tr>
<tr>
<td>Liver TAG (mg/g)</td>
<td>16.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.79</td>
<td>12.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.54</td>
</tr>
<tr>
<td>Serum TAG (mg/dL)</td>
<td>80.21</td>
<td>10.02</td>
<td>58.31</td>
<td>5.18</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Mean values within a row with unlike superscript letters were significantly different (*P* < 0.05 ANOVA followed by post hoc Tukey’s multiple comparison test).

Includes epididymal, perirenal and mesenteric fat pads.
TABLE 3.3 Fatty acid profile (g/100g FAME) in the liver of mice fed an ALA-enriched diet either alone or in combination with *Bifidobacterium breve* NCIMB 702258 or *B. breve* DPC 6330 or an unsupplemented diet for 6 weeks. (Mean values $\pm$ SEM; $n$ 10 mice per group).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Unsupplemented diet</th>
<th>ALA-enriched diet</th>
<th>ALA-enriched diet and <em>B. breve</em> NCIMB 702258</th>
<th>ALA-enriched diet and <em>B. breve</em> DPC 6330</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100g FAME</td>
<td>g/100g FAME</td>
<td>g/100g FAME</td>
<td>g/100g FAME</td>
</tr>
<tr>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>16:0</td>
<td>26.51$^{a,b}$</td>
<td>25.68$^{a}$</td>
<td>26.42$^{a,b}$</td>
<td>27.78$^{b}$</td>
</tr>
<tr>
<td>16:1 cis-9</td>
<td>2.36</td>
<td>2.73</td>
<td>2.55</td>
<td>2.72</td>
</tr>
<tr>
<td>18:0</td>
<td>6.85$^{a}$</td>
<td>7.41$^{a}$</td>
<td>8.67$^{b}$</td>
<td>8.60$^{b}$</td>
</tr>
<tr>
<td>18:1 cis-9</td>
<td>35.45$^{b}$</td>
<td>31.68$^{a}$</td>
<td>29.85$^{a}$</td>
<td>29.50$^{a}$</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>15.34$^{a}$</td>
<td>15.35$^{a}$</td>
<td>13.39$^{a,b}$</td>
<td>13.84$^{b}$</td>
</tr>
<tr>
<td>18:3 n-3</td>
<td>0.76$^{b}$</td>
<td>5.09$^{a}$</td>
<td>4.89$^{a}$</td>
<td>4.72$^{a}$</td>
</tr>
<tr>
<td>18:3 n-6</td>
<td>0.25$^{b}$</td>
<td>0.17$^{a}$</td>
<td>0.13$^{a,c}$</td>
<td>0.10$^{c}$</td>
</tr>
<tr>
<td>20:3 n-6</td>
<td>0.44</td>
<td>0.43</td>
<td>0.50</td>
<td>0.49</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>4.10$^{b}$</td>
<td>2.82$^{a}$</td>
<td>2.85$^{a}$</td>
<td>2.79$^{a}$</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>0.12$^{b}$</td>
<td>0.91$^{a}$</td>
<td>1.11$^{a}$</td>
<td>1.09$^{a}$</td>
</tr>
<tr>
<td>22:5 n-3</td>
<td>0.09$^{b}$</td>
<td>0.23$^{a}$</td>
<td>0.32$^{c}$</td>
<td>0.29$^{a,c}$</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>2.04$^{a}$</td>
<td>2.21$^{a,b}$</td>
<td>2.81$^{b}$</td>
<td>2.64$^{a,b}$</td>
</tr>
</tbody>
</table>

FAME, fatty acid methyl ester. $^{a,b,c}$ Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$ ANOVA followed by *post hoc* Tukey's multiple comparison test).
TABLE 3.4 Fatty acid profile (g/100g FAME) in the epididymal adipose tissue of mice fed an ALA-enriched diet either alone or in combination with *Bifidobacterium breve* NCIMB 702258 or *B. breve* DPC 6330 or an unsupplemented diet for 6 weeks. (Mean values ± SEM; n 10 mice per group).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Unsupplemented diet</th>
<th>ALA-enriched diet</th>
<th>ALA-enriched diet and <em>B. breve</em> NCIMB 702258</th>
<th>ALA-enriched diet and <em>B. breve</em> DPC 6330</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100g FAME</td>
<td>g/100g FAME</td>
<td>g/100g FAME</td>
<td>g/100g FAME</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>16:0</td>
<td>19.83</td>
<td>0.42</td>
<td>18.35</td>
<td>0.23</td>
</tr>
<tr>
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<td>3.57</td>
<td>0.19</td>
<td>4.03</td>
<td>0.25</td>
</tr>
<tr>
<td>18:0</td>
<td>3.17</td>
<td>0.21</td>
<td>2.68</td>
<td>0.12</td>
</tr>
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<td>47.84</td>
<td>0.34</td>
<td>43.34</td>
<td>0.64</td>
</tr>
<tr>
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<td>17.49</td>
<td>0.49</td>
<td>18.53</td>
<td>0.62</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>1.13</td>
<td>0.03</td>
<td>6.07</td>
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<tr>
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<td>0.06</td>
<td>0.00</td>
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<td>0.00</td>
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<td>0.12</td>
<td>0.00</td>
<td>0.11</td>
<td>0.00</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.25</td>
<td>0.01</td>
<td>0.21</td>
<td>0.01</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.01</td>
<td>0.00</td>
<td>0.06</td>
<td>0.00</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.04</td>
<td>0.00</td>
<td>0.15</td>
<td>0.01</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.14</td>
<td>0.01</td>
<td>0.25</td>
<td>0.01</td>
</tr>
</tbody>
</table>

FAME, fatty acid methyl ester. a,b Mean values within a row with unlike superscript letters were significantly different (P < 0.05 ANOVA followed by post hoc Tukey’s multiple comparison test).
<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Unsupplemented diet</th>
<th>ALA-enriched diet</th>
<th>ALA-enriched diet and B. breve NCIMB 702258</th>
<th>ALA-enriched diet and B. breve DPC 6330</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100g FAME</td>
<td>g/100g FAME</td>
<td>g/100g FAME</td>
<td>g/100g FAME</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>16:0</td>
<td>31.61</td>
<td>0.53</td>
<td>30.44</td>
<td>0.69</td>
</tr>
<tr>
<td>16:1 cis-9</td>
<td>0.75(^{a,b})</td>
<td>0.02</td>
<td>0.71(^a)</td>
<td>0.02</td>
</tr>
<tr>
<td>18:0</td>
<td>23.31</td>
<td>0.19</td>
<td>23.18</td>
<td>0.20</td>
</tr>
<tr>
<td>18:1 cis-9</td>
<td>20.26(^{a,b})</td>
<td>0.22</td>
<td>20.01(^a)</td>
<td>0.13</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>0.88</td>
<td>0.03</td>
<td>0.88</td>
<td>0.02</td>
</tr>
<tr>
<td>18:3 n-3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>18:3 n-6</td>
<td>0.27</td>
<td>0.01</td>
<td>0.30</td>
<td>0.02</td>
</tr>
<tr>
<td>20:3 n-6</td>
<td>0.34(^b)</td>
<td>0.01</td>
<td>0.42(^a)</td>
<td>0.01</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>6.03</td>
<td>0.13</td>
<td>5.90</td>
<td>0.15</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>0.03(^b)</td>
<td>0.00</td>
<td>0.08(^a,c)</td>
<td>0.00</td>
</tr>
<tr>
<td>22:5 n-3</td>
<td>0.05(^b)</td>
<td>0.01</td>
<td>0.15(^a)</td>
<td>0.01</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>5.75(^b)</td>
<td>0.34</td>
<td>7.20(^a)</td>
<td>0.44</td>
</tr>
</tbody>
</table>

FAME, fatty acid methyl ester. \(^{a,b,c}\) Mean values within a row with unlike superscript letters were significantly different \((P < 0.05\) ANOVA followed by post hoc Tukey’s multiple comparison test).
FIGURE 3.1(A) Expression of fatty acid metabolism enzymes in the livers of mice fed ALA-enriched diets either alone or in combination with *Bifidobacterium breve* NCIMB 702258 or *B. breve* DPC 6330, or an unsupplemented diet, relative to β-actin. Expression in the unsupplemented and ALA-enriched diets supplemented with either *B. breve* NCIMB 702258 or *B. breve* DPC 6330 is relative to the ALA-enriched diet, which was set to 1. Values are means (n 10) ± SEM, represented by vertical bars. Mean values within a row with unlike superscript letters were significantly different (P < 0.05 ANOVA followed by post hoc Tukey’s multiple comparison test).
FIGURE 3.1(B) Expression of fatty acid uptake enzymes in the livers of mice fed ALA-enriched diets either alone or in combination with *Bifidobacterium breve* NCIMB 702258 or *B. breve* DPC 6330, or an unsupplemented diet, relative to β-actin. Expression in the unsupplemented diet and ALA-enriched diets supplemented with either *B. breve* NCIMB 702258 or *B. breve* DPC 6330 is relative to the ALA-enriched diet, which was set to 1. Values are means (n 10) ± SEM, represented by vertical bars. a,b Mean values within a row with unlike superscript letters were significantly different (P < 0.05 ANOVA followed by post hoc Tukey’s multiple comparison test).
FIGURE 3.2 Expression of fatty acid uptake and transport enzymes in the ileum of mice fed ALA-enriched diets either alone or in combination with *Bifidobacterium breve* NCIMB 702258 or *B. breve* DPC 6330, or an unsupplemented diet, relative to β-actin. Expression in the unsupplemented diet and ALA-enriched diets supplemented with either *B. breve* NCIMB 702258 or *B. breve* DPC 6330 is relative to the ALA-enriched diet, which was set to 1. Values are means (n 10) ± SEM, represented by vertical bars.  

a,b Mean values within a row with unlike superscript letters were significantly different (P < 0.05 ANOVA followed by post hoc Tukey’s multiple comparison test).

Please note that Chapter 4 (pp.177-215) is unavailable due to a restriction requested by the author.

CORA Cork Open Research Archive [http://cora.ucc.ie](http://cora.ucc.ie)
Chapter 5

Streptozotocin induced type-1-diabetes disease onset in Sprague-Dawley rats is associated with an altered intestinal microbiota composition, coupled with decreased diversity
5.1 ABSTRACT

The aim of this study was to assess the impact of streptozotocin (STZ) induced type-1-diabetes (T1D) on intestinal microbiota composition and diversity in Sprague-Dawley rats, compared with healthy controls over time. T1D was induced by injection of a single dose (60 mg/Kg) of STZ, administered via the intraperitoneal cavity. Total DNA was isolated from faecal pellets at week 0 (pre- STZ injection), week 1, week 2, week 4 and from caecal content at week 5 from both healthy and T1D groups. High throughput 16S rRNA sequencing was employed to investigate intestinal microbiota composition. The data revealed that while intestinal microbiota composition between the groups was similar at week 0, a dramatic impact of T1D development and progression on microbiota composition was apparent post- STZ injection and up to 5 weeks. Most notably, T1D onset was associated with a shift in the Bacteroidetes: Firmicutes ratio \( (P < 0.05) \), while at the genus level, increased proportions of lactic acid producing bacteria such as *Lactobacillus* and *Bifidobacterium* were associated with the later stages of T1D progression \( (P < 0.05) \). Coincidently, T1D increased caecal lactate levels \( (P < 0.05) \). Microbial diversity was also reduced following T1D \( (P < 0.05) \). Principle co-ordinate analyses demonstrated temporal clustering in T1D and control groups over time with separation of the T1D clusters at each time point from healthy controls. The results provide a comprehensive account of how T1D is associated with an altered intestinal microbiota composition and reduced microbial diversity over time.
5.2 INTRODUCTION

T1D is an organ-specific autoimmune disease involving the selective destruction of insulin-producing pancreatic β-cells. While the pathophysiology of T1D is not yet entirely clear, it is the genetic predisposition of an individual combined with environmental factors which initiate the process of autoimmune destruction of pancreatic β-cells (Boerner and Sarvetnick, 2011). The human leukocyte antigen (HLA) complex allows the immune system to distinguish between the body’s own proteins and foreign proteins; however, certain combinations of various genes belonging to the HLA complex are associated with a higher genetic risk of developing T1D (Ziegler and Nepom, 2010). This process, involving both the innate and adaptive immune systems, remains subclinical, until approximately 80% of the β-cell mass is destroyed (Notkins and Lernmark, 2001). Over time, β-cell destruction results in insulin deficiency, worsening hyperglycemia and an eventual call for insulin therapy. As the incidence of T1D is increasing at a rate far beyond the rate of population growth, additional emphasis has been placed on the contribution of environmental factors towards disease onset (Ehehalt et al., 2010; Patterson et al., 2001; Vaarala et al., 2008). Indeed, the intestinal microbiota is an environmental factor which cannot be overlooked as contributing towards T1D development.

Many studies have linked alterations in the intestinal microbiota composition with conditions such as obesity (Ley et al., 2005; Ley et al., 2006; Murphy et al., 2010; Turnbaugh et al., 2006), inflammatory bowel disease (Greenblum et al., 2012), colitis (Bellavia et al., 2013; Klimesova et al., 2013) and T1D (Giongo et al., 2011; Murri et al., 2013; Roesch et al., 2009). For example, risk of T1D onset in childhood is higher in children delivered by Caesarean section (Cardwell et al., 2008). Previous studies which
have examined the role of the intestinal microbiota in the development of T1D are often limited due to the models used (i.e. genetically pre-disposed animals and humans who develop T1D). While it has been reported that stool from bio-breeding diabetes-resistant (BBDR) rats contained higher abundances of Lactobacillus and Bifidobacterium, compared with bio-breeding diabetes-prone (BBDP) rats (Roesch et al., 2009), others have reported that lactic acid producing species such as Lactobacillus, Lactococcus and Bifidobacterium were increased in the stool of children who tested positive for β-cell autoimmunity (Brown et al., 2011; Giongo et al., 2011). In contrast, de Goffau et al., (2013) observed a low relative abundance of two of the most common Bifidobacterium species, B. adolescentis and B. pseudocatenulatum associated with β-cell autoimmunity in children who had tested positive for at least two diabetes-associated auto-antibodies (de Goffau et al., 2013). Giongo et al., (2011) identified a reduction in the stability and diversity of the intestinal microbiota present in autoimmune infants, which worsens with age (Giongo et al., 2011). In addition, a high ratio of Firmicutes: Bacteriodetes was observed as early as six months post birth in autoimmune case children, compared with healthy age-matched controls (Giongo et al., 2011). Murri et al., (2013) highlighted a negative correlation between the numbers of Bifidobacterium and Lactobacillus with the plasma glucose levels of diabetic children, while Clostridium correlated positively with glycaemic levels in case children, compared with healthy controls (Murri et al., 2013). Recently, Brown et al., (2012) linked T1D with microbial fermentation products and mucin synthesis, important for maintaining intestinal integrity, often implicated in T1D (Brown et al., 2011).

Administration of the probiotic strain Lactobacillus johnsonii N6.2, previously isolated from the stool of BBDR rats, to BBDP rats, inhibited the onset of T1D
(Valladares et al., 2010), while early oral administration of the probiotic compound VSL#3 to non-obese diabetic (NOD) mice prevented T1D development (Calcinaro et al., 2005). Antibiotics have also been shown to prevent autoimmune diabetes in BBDP rats (Brugman et al., 2006), while the intestinal microflora have demonstrated a capacity to influence the development of T1D (King and Sarvetnick, 2011). Finally, specific pathogen-free NOD mice lacking MyD88, an adaptor for multiple innate immune receptors that recognise microbial stimuli, do not develop T1D (Wen et al., 2008) and this effect is dependent on commensal microbes. The role of the intestinal microbiota in the development of T1D or indeed modulating the resident intestinal ecosystem as a therapeutic strategy in T1D has only very recently been investigated and extensively reviewed (Atkinson and Chervonsky, 2012).

STZ is a glucosamine–nitrosourea compound, originally derived from cultures of Streptomyces achromogenes and is used clinically as a chemotherapeutic agent in the treatment of pancreatic β-cell carcinoma. STZ produces rapid pancreatic β-cell destruction, resulting in hypoinsulinemia and hyperglycemia. The selectivity for β-cells is associated with preferential accumulation of the chemical in these cells after entry through glucose transporter 2 (GLUT2), whereby chemical structural similarity with glucose allows STZ to bind to this receptor. Diabetes was induced in male Sprague-Dawley rats in this study through a single intraperitoneal injection of STZ. The impact of T1D disease onset and progression on intestinal microbiota composition and microbial fermentation products was investigated. DNA was isolated from faecal pellets at week 0, (pre-STZ injection in one of the groups), then at week 1, week 2 and week 4 and from caecal content at week 5 from T1D and healthy control rats and prepared for 16S rRNA pyrosequencing.
5.3 MATERIALS AND METHODS

5.3.1 ASSESSMENT OF ANTIMICROBIAL ACTIVITY OF STZ

Bioactivity of STZ (Sigma, Wicklow, Ireland) was assessed by well-diffusion assays. For this purpose, molten agar was cooled to 48°C and seeded with fresh overnight cultures of various Gram-positive (*Bifidobacterium, Lactobacillus* and *Listeria*) and Gram-negative (*Escherichia coli* and *Cronobacter*) strains. The inoculated medium was dispensed into sterile Petri dishes in 20 mL volumes, allowed to solidify and dried. Wells (4.6 mm in diameter) were then bored in the seeded agar plates and 50 μL volumes of various concentrations (7.5, 4, 2 and 1 mg/mL) of freshly prepared STZ in filter sterilised sodium citrate buffer (50mM; pH 4.5) to be assayed were dispensed into the wells. Petri dishes were incubated accordingly for each of the strains used.

5.3.2 ANIMALS AND TREATMENT

All animal experiments were approved by the University College Cork (UCC) Animal Ethics Committee and experimental procedures were conducted under the appropriate license from the Irish Government. Male Sprague-Dawley rats were purchased from Harlan Limited (Briester, Oxon, UK) at 5 weeks of age and housed under barrier-maintained conditions within the Biological Services Unit, UCC. Rats were allowed to acclimatise for 1 week before the start of the study and were fed *ad libitum* with Teklad Global Standard Rodent Diet (Harlan Laboratories, Madison, WI, USA, #2018S), with free access to water at all times. Rats were housed in groups of 5 per cage and kept in a controlled environment at 25°C under a 12-hr-light/12-hr-dark cycle. The rats were divided into 2 groups (*n* 15 rats per group): a non-diabetic healthy control group and an STZ induced T1D group. After 1 week of acclimatisation, T1D was
induced in the latter group by injection of a single dose (60mg/Kg) of freshly prepared STZ (Sigma) administered via the intraperitoneal cavity, according to the method described by (Wu and Huan, 2008). Glucose levels were measured in triplicate using a Contour Next glucometer (Bayer, UK) in blood samples collected from a tail vein each week. Rats with blood glucose higher than 200 mg/dL were considered diabetic and STZ induced rats with lower glucose levels were excluded from the rest of the study. Body weight was assessed and fresh faecal pellets were collected weekly. At the end of the 5 weeks, the rats were killed by decapitation. Caecal content was removed from individual rats and flash-frozen immediately on dry ice. All samples were stored at -80°C until processed.

5.3.3 DNA ISOLATION FROM FAECAL PELLETS AND CAECAL CONTENT AND HIGH-THROUGHPUT AMPICLON SEQUENCING

Total DNA was isolated from fresh individual rat faecal pellets collected at week 0, pre-STZ induced diabetes in the T1D group, then 1 week, 2 weeks and 4 weeks post-STZ injection and from the individual caecal content of all rats, 5 weeks following STZ injection, using the QIAmp DNA Stool Mini Kit (Qiagen, West Sussex, UK). The extraction was coupled with an initial bead-beating step (30 sec x 3). Isolated DNA was then stored at -20°C (n 10 per group), until further processing. The microbiota composition of the samples was established by amplicon sequencing; universal 16S rRNA primers estimated to bind to 94.6% of all 16S genes (i.e. the forward primer F1 (5'-AYTGGGYDTAAAGNG-3’) and a combination of four reverse primers, R1 (5’-TACCRGGGHTCTAATCC-3’), R2 (5’-TACCAGAGTATCTAATTC-3’), R3 (5’-CTACDSRGGTGTTCTAATC-3’) and R4 (5’-TACNVGGGTATCTAATC) (RDP’S Pyrosequencing
Pipeline: [http://pyro.cme.msu.edu/pyro/help.jsp](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 prior to being sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd., West Sussex, UK), in line with 454 protocols at the Teagasc high throughput sequencing centre.

### 5.3.4 BIOINFORMATICS SEQUENCE ANALYSIS

Quality trimming of sequence reads was performed using the Qiime suite of tools (version 1.7) (Caporaso et al., 2010); reads shorter than 200 bp, failing to reach a quality score of 25 and not exact matches to barcoded tags and primer sequence were discarded. A locally installed version of SILVA 16S rRNA database (version 106) (Pruesse et al., 2007) was used to BLAST (Altschul et al., 1997) the trimmed sequence using default parameters. Resulting BLAST output files were parsed through MEGAN (Huson et al., 2007); similar to previous studies a bit-score cut-off of 86 was selected (Rea et al., 2011; Urich et al., 2008). Clustering of sequence reads into operational taxonomical units (OTUs) at 97% identity and subsequent alignments were achieved using QIIME suite software tools (Caporaso et al., 2010). ChimeraSlayer was used to remove chimeric OTUs from aligned OTUs and the FastTreeMP tool generated a phylogenetic tree (Haas et al., 2011; Price et al., 2010). α and β diversities were calculated on the sequence reads. Principal co-ordinate analyses were performed on the samples and viewed within KiNG viewer (Chen et al., 2009).
5.3.5 SHORT CHAIN FATTY ACID, LACTIC AND FORMIC ACID ANALYSIS OF CAECAL CONTENT

Short chain fatty acid (SCFA) analysis was performed as previously described (Wall et al., 2012). Briefly, caecal content was vortex-mixed with Milli-Q water, incubated at room temperature for 10 min and centrifuged to pellet bacteria and other solids. The supernatant was filtered, transferred to a clear GC vial and 2-ethylbutyric acid (Sigma) was added as internal standard. The concentrations of SCFA was measured using a Varian 3800 GC flame-ionization system, fitted with a ZB-FFAP column (30 m x 0.32 mm x 0.25 um; Phenomenex, Macclesfield, Cheshire, UK). The initial oven temperature was set at 100°C for 0.5 min, raised to 180°C at 8°C/min and held for 1 min, then increased to 200°C at 20°C/min, and finally held at 200°C for 5 min. The temperature of the injector and the detector were set at 240°C and 250°C, respectively. Helium was used as the carrier gas at a flow rate of 1.3 mL/min. A standard curve was built with different concentrations of a standard mix containing acetic acid, propionic acid and N-butyric acid (Sigma). Peaks were integrated by using the Varian Star Chromatography Workstation version 6.0 software. Formic acid and lactic acid were measured using formic acid and D/L lactate commercial kits (Megazyme, Wicklow, Ireland).

5.3.6 STATISTICAL ANALYSIS

All results are presented as means ± standard errors of the mean (SEM) (per group). To assess whether differences between treatment groups were significant, the unpaired student t-test was used with the use of GraphPad Prism version 5.0 for Windows (GraphPad Software). Statistical significance was accepted at $P < 0.05$. Compositional data were statistically analysed using the nonparametric Mann-Whitney
and Kruskal-Wallis tests (Kruskal and Wallis, 1952) to estimate the relationships between different groups using GraphPad Prism version 5.0. Statistical significance was accepted at $P < 0.05$.

5.4 RESULTS

5.4.1 ANTIMICROBIAL ACTIVITY OF STZ

Initially, the antimicrobial activity of different concentrations of STZ was tested against Gram-positive (*Bifidobacterium, Lactobacillus* and *Listeria*) and Gram-negative (*Escherichia coli* and *Cronobacter*) strains. At a concentration of 7.5 mg/mL, STZ produced a clear zone of inhibition of strains *Lb. bulgaricus*, *Lb. plantarum*, *Lb. gasseri* and *B. infantis* which decreased at concentrations of 4 mg/mL, 2 mg/mL and 1 mg/mL (data not shown). At a concentration of 7.5 mg/mL, STZ had a bacteriostatic effect on *B. breve*, *Lb. acidophilus*, *E. coli* and *C. sakazakii* which decreased at concentrations of 4 mg/mL, 2 mg/mL and 1 mg/mL (data not shown). No zone of inhibition was observed for any concentration of STZ on *B. bifidus* and *L. monocytogenes* (data not shown).

5.4.2 INDUCTION OF T1D BY STZ INJECTION IN RATS

One week after STZ injection, rats were severely diabetic as indicated by elevated blood glucose levels (572.6 ± 10.3 mg/dL), compared with the normoglycemic range observed in healthy controls (104.2 ± 2.1 mg/dL) ($P < 0.05$; Figure 5.1(B)). The chronic increase in blood glucose levels were maintained throughout the study period (Figure 5.1(B)). Diabetic rats exhibited polyuria, polydipsia, hyperphagia and excessive faecal output (data not shown). Diabetic rats displayed significant decreases in percentage
body weight gain from week 3 onwards (following STZ injection), compared with healthy controls ($P < 0.05$; Figure 5.1(A)).

### 5.4.3 STZ INDUCED T1D DECREASES $\alpha$ DIVERSITY OF THE RAT INTESTINAL MICROBIOTA OVER TIME

A total of 777,796 reads were sequenced, corresponding to a total of 388,898 reads per group and an average of 7,857 reads per rat. At the 97% similarity level, a total of five metrics were used to estimate $\alpha$ diversity; Shannon, Simpson, Chao1 (Supplementary Figures 5.1-5.3), phylogenetic diversity and observed species (Figures 5.2-5.4). At week 0, pre-STZ injection and T1D induction in one of the groups, no differences were observed across any of the five metrics calculated between the groups (Supplementary Figures 5.1-5.3 and Figures 5.2-5.3). This indicated that pre-STZ injection in the T1D group, all rats had similar levels of diversity. However, 1, 2, 4 and 5 weeks post-STZ injection in the T1D group, Chao1, phylogenetic diversity and observed species were all significantly reduced in the T1D group, compared with healthy controls ($P < 0.05$; Figures 5.2-5.4 and Supplementary Figure 5.1). While Shannon diversity data revealed no change to the biodiversity of the microbiota within the healthy controls and the T1D group at week 2, significant reductions to the diversity profile were found between the T1D group and the healthy controls at all other time points taken ($P < 0.05$; Supplementary Figures 5.2(A) and 5.2(B)). Similarly, the Simpson diversity index-based analysis, which also takes into account the number of species present and the relative abundance of each species, highlighted a significant reduction in diversity in animals induced with T1D at 1, 4 and 5 weeks following STZ injection, compared with healthy controls ($P < 0.05$; Supplementary Figures 5.3(A) and
5.3(B). Rarefaction curves were seen to be parallel (Supplementary Figure 5.4) indicating that additional sampling would yield a limited increase in species richness.

5.4.4 TAXONOMICAL ANALYSIS HIGHLIGHTS THE EFFECT OF STZ INDUCED T1D ON INTESTINAL MICROBIAL COMPOSITION OVER TIME

Taxonomy based analysis of the assigned sequences highlighted few differences in the intestinal microbiota populations between the groups at week 0, pre-STZ injection. While no significant differences were observed at the phylum level at week 0 (Figure 5.5(A)), significantly lower proportions of *Clostridiaceae* (*P* < 0.05; Figure 5.6(A)) and *Clostridium* (*P* < 0.05; Figure 5.7(A)) were found at the family and genus levels, respectively while the proportions of *Parabacteroides* were significantly increased in the group yet to be induced with T1D, compared with the control group (*P* < 0.05; Figure 5.7(A)).

Analysis of the microbial profile of the intestine at week 1 (post-STZ injection in one of the groups) revealed a greater number of statistically significant differences between the groups at the phylum, family and genus levels. While the relative abundance of the Tenericute population was significantly increased in the T1D group (*P* < 0.05; Figure 5.5(A)), the proportions of Actinobacteria and Deferribacteres were significantly reduced (*P* < 0.05; Figure 5.5(A)), compared with healthy controls. Most notably, just 1 week after STZ injection, the proportions of Bacteroidetes were significantly increased (*P* < 0.05; Figure 5.5(A)) in the T1D group, while the Firmicutes population was significantly reduced (*P* < 0.05; Figure 5.5(A)), compared with healthy controls. At the family level, the proportions of *S24-7*, *Prevotellaceae*, *Erysipelotrichaceae*, *Porphyromonadaceae* and *Anaeroplasmataceae* were significantly
increased in the T1D group ($P < 0.05$; Figure 5.6(A)), while *Bifidobacteriaceae* and *Peptococcaceae* were significantly reduced, compared with healthy controls ($P < 0.05$; Figure 5.6(A)). At the genus level, both the proportions of *Parabacteroides* and *Mucispirillium* were significantly increased ($P < 0.05$; Figure 5.7(A)) while the proportions of *Ruminococcus* were significantly reduced ($P < 0.05$; Figure 5.7(A)) in the T1D group, compared with healthy controls.

While the significant changes in the proportions of certain intestinal microbiota populations at week 2 remained the same as they were at week 1, many more significant changes to the intestinal microbiota, at the family and genus levels were observed 2 weeks post-STZ induced T1D, compared with healthy controls. At the phylum level, the proportions of Firmicutes remained significantly reduced ($P < 0.05$; Figure 5.5(A)), while the proportions of Deferribacteres became significantly increased, ($P < 0.05$; Figure 5.5(A)) in the T1D group, compared with healthy controls. Similar to week 1, the proportions of *Prevotellaceae* and *Erysipelotrichaceae* remained significantly higher while *Peptococcaceae* remained significantly lower in the T1D group, compared with healthy controls at week 2 ($P < 0.05$; Figure 5.6(A)), while the proportions of *Porphyromonadaceae* shifted from being significantly higher at week 1 to significantly lower in the T1D group at week 2 ($P < 0.05$; Figure 5.6(A)). Progression of T1D increased the proportions of *Lactobacillaceae*, *Peptostreptococcaceae* and *Clostridiaceae* ($P < 0.05$; Figure 5.6(A)), while reducing the proportions of *RF-9* group ($P < 0.05$; Figure 5.6(A)), after 2 weeks in the T1D group, compared with healthy controls. At the genus level, the proportions of *Ruminococcus* and *Mucispirillium* remained significantly lower ($P < 0.05$; Figure 5.7(A)) and higher ($P < 0.05$; Figure 5.7(A)), respectively, in the T1D group, as they had at week 1, compared with healthy controls.
Significant increases in the proportions of *Bacteroides, Lactobacillus, Turicibacter* and *Clostridium* were also observed 2 weeks after STZ induced diabetes in the T1D group (*P* < 0.05; Figure 5.7(A)), compared with healthy controls. The proportions of *Parabacteroides*, previously observed as significantly higher in the T1D group, 1 week following STZ injection, were significantly lower 2 weeks after STZ induced T1D, compared with healthy controls (*P* < 0.05; Figure 5.7(A)). The proportions of *Ruminococcaceae Incertae Sedis*, one of the most dominant populations at the genus level, was significantly reduced at week 2 in the T1D group, compared with healthy controls (*P* < 0.05; Figure 5.7(A)).

Four weeks after STZ injection in the T1D group, the proportions of Tenericutes were significantly higher in the T1D group (*P* < 0.05; Figure 5.5(A)), as they had been at week 1, compared with healthy controls. The proportions of Actinobacteria and Proteobacteria (*P* < 0.05; Figure 5.5(A)) were significantly higher, compared with healthy controls. Similar to week 1 and 2, the proportions of Firmicutes remained significantly lower in the T1D group, compared with healthy controls (*P* < 0.05; Figure 5.5(A)). At the family level, the proportions of *Prevotellaceae* recovered in the healthy controls, while *Lactobacillaceae* and *Clostridiaceae* remained significantly higher (*P* < 0.05; Figure 5.6(A)), and *Peptococcaceae* and *RF9* group remained significantly lower in the T1D group, compared with healthy controls (*P* < 0.05; Figure 5.6(A)), as they had been at week 2. At week 4, the abundance of *Porphyromonadaceae* was significantly higher in the T1D group, compared with healthy controls (*P* < 0.05; Figure 5.6(A)). *Bacteroidaceae, Bifidobacteriaceae* and *Alcaligenaceae* proportions were significantly higher (*P* < 0.05; Figure 5.6(A)), while *Ruminococcaceae* and *Lachnospiraceae* proportions were significantly lower (*P* < 0.05; Figure 5.6(A)), 4 weeks after T1D
induction, compared with healthy controls. Correlating with the changes seen at the family level, the abundances of *Parasuterella*, *Bifidobacterium*, *Lactobacillus* and *Clostridium* were all significantly higher (*P* < 0.05; Figure 5.7(A)), while the proportions of *Alistipes*, *Ruminococcus* and *Ruminococcaceae Incertae Sedis* were all significantly lower (*P* < 0.05; Figure 5.7(A)) in the T1D group, at week 4, compared with healthy controls.

Data highlighting the intestinal microbiota changes at week 5 following T1D induction, compared with healthy controls correspond to microbiota populations from the caecal content of the rats after they had been killed. Thus, week 5 data is graphed separately from the other time points as variations between the microbiota of faecal and caecal content may skew results. The proportions of Actinobacteria and Proteobacteria remained significantly higher, compared with healthy controls (*P* < 0.05; Figure 5.5(B)). The Bacteroidetes: Firmicutes ratio appeared to equilibrate as no significant differences were seen between the groups in either of these phyla from caecal content (Figure 5.5(B)), similarly, the proportion of Tenericutes were no longer significant between the groups (Figure 5.5(B)). The abundance of Deferrribacteres were significantly decreased (*P* < 0.05; Figure 5.5(B)), as they had been at week 1, in the T1D group, compared with healthy controls. Again, similar to week 4, the proportions of *Bacteroidaceae*, *Bifidobacteriaceae* and *Alcaligenaceae* were significantly increased (*P* < 0.05; Figure 5.6(B)), while *Ruminococcaceae* and *Peptococcaceae* were significantly decreased (*P* < 0.05; Figure 5.6(B)) in the T1D group, compared with healthy controls. The proportions of *Rikenellaceae* and *Peptostreptococcaceae* were also significantly reduced (*P* < 0.05; Figure 5.6(B)) in the T1D group, compared with healthy controls, while *Erysipelotrichaceae* was significantly increased (*P* < 0.05; Figure 5.6(B)), as in
week 2, in the T1D group, compared with healthy controls. At the genus level, *Parasuterella* and *Bifidobacterium* were found at significantly higher proportions (*P* < 0.05; Figure 5.7(B)), while the proportions of *Alistipes*, *Ruminococcus* and *Ruminococcaceae Incertae Sedis* were all significantly reduced (*P* < 0.05; Figure 5.7(B)) in the T1D group, compared with healthy controls. The proportions of *Bacteroides* were significantly higher while the abundance of *Phascolarctobacterium*, not previously highlighted at any other week to be significantly different was also higher and *Peptostreptococcaceae Incertae Sedis* and *Mucispirillum* abundances were both significantly lower in the T1D group, compared with healthy controls (*P* < 0.05; Figure 5.7(B)). The abundance of *Mucispirillum* was previously shown as significantly higher both 1 (*P* < 0.05; Figure 5.7(A)) and 2 weeks (*P* < 0.05; Figure 5.7(A)) following STZ induced diabetes in the T1D group, compared with healthy controls.

Throughout the study, from week 0 to week 5, the relative populations of the intestinal microbiota at phylum, family and genus levels fluctuated only slightly within the healthy control group (Supplementary Figures 5.5-5.7). In contrast, the proportions of the intestinal microbiota at all levels in the group induced with T1D fluctuated significantly at all weeks following T1D induction (Supplementary Figures 5.8-5.10).

### 5.4.5 β DIVERSITY HIGHLIGHTS AN STZ INDUCED T1D EFFECT ON MICROBIAL POPULATION VARIATION OVER TIME AS DISEASE PROGRESSES

Principal co-ordinate analyses, based on unweighted Unifrac distances of the 16S rRNA sequences, illustrated changes to the microbial populations following STZ induced T1D, compared with healthy controls. Figure 5.8(A) shows data points corresponding to healthy controls (red for weeks 0, 1, 2 and 4 and orange for week 5).
cluster closely together, irrelevant of time point, between week 0, 1, 2, 4 and 5 (different shades of red for weeks 0, 1, 2 and 4 and orange for week 5; Figure 5.8(B)). Similarly, Figure 5.8(A) also shows data points corresponding to T1D rats (blue for weeks 0, 1, 2 and 4 and green for week 5) initially cluster close together with healthy controls (red) at week 0, since T1D had not been induced at this time point. However, subsequent data points corresponding to the T1D rats (different shades of blue for weeks 0, 1, 2 and 4 and green for week 5), as the trial progressed from week 1 to week 5 following T1D induction became increasingly distant from the healthy control data points (red). Individual data points within particular time points for the T1D group (blue and green) become much more dispersed and fail to form distinct clusters with each other (Figure 5.8(B)). These results are in line with the α diversity and taxonomical data presented since data points corresponding to T1D rats cluster away from those corresponding to healthy controls in all time points from week 1 onwards (post-STZ injection).

5.4.6 STZ INDUCED T1D ALTERED THE PRODUCTION OF SCFA AND LACTIC ACID

T1D was associated with a significant decrease in caecal propionate ($P < 0.05$) and butyrate ($P < 0.05$) and a significant increase in caecal acetate ($P < 0.05$) and lactate levels ($P < 0.05$), compared with healthy controls (Figure 5.9). No differences in formate were exhibited between the groups (Figure 5.9).
5.5 DISCUSSION

This study provides a comprehensive account of the impact of STZ induced T1D on the intestinal microbiota composition, diversity and microbial fermentation metabolite production over time. The antimicrobial effect of STZ has previously been described (Vavra et al., 1959), however, very little is understood of the antimicrobial impact a single intraperitoneal injection of STZ could have on intestinal microbiota composition. A circulating half-life of only 15 minutes has been reported in man, following intravenous infusion (Schein et al., 1973) or 30-40 minutes following intravenous bolus injection (Adolphe et al., 1975). In addition, approximately 70% of $^{14}$C-labelled STZ administered intravenously to rats in single doses of 70 mg/Kg was recovered in the urine over the first 6 hours, thus indicating rapid excretion (Karunanayake et al., 1976). It is therefore unlikely that the changes to microbiota composition found in this study were caused by the antimicrobial activity of STZ and were most likely a result of T1D onset and progression.

The data revealed that STZ induced T1D altered the Bacteroidetes: Firmicutes ratio, increasing the abundance of Bacteroidetes and depleting the Firmicutes population following disease onset, as previously demonstrated in various T1D models (de Goffau et al., 2013; Giongo et al., 2011; Murri et al., 2013). Initially, pre- T1D induction, no difference in the Bacteroidetes: Firmicutes ratio was observed between the groups. In contrast, the intestinal relative proportions of Actinobacteria and Proteobacteria were significantly increased at week 4 and 5 following T1D induction, compared with healthy controls. Overall bacterial diversity was also significantly reduced in the T1D group, compared with healthy controls, creating an opportunity for the members of the phyla Actinobacteria and Proteobacteria to thrive. The data correlate with previous
observations (Giongo et al., 2011), that T1D was associated with decreased abundances of *Ruminococcaceae* and *Lachnospiraceae* and increased abundances of *Bacteroidaceae*, compared with healthy controls at the later stages of diabetes development. However, in contrast to this study which found *Porphyromonadaceae* significantly more abundant in healthy control children, the data generated in this study reveal that the proportions of *Porphyromonadaceae* were higher in T1D rats, 1 and 4 weeks after T1D induction and lower at week 2, compared with healthy controls. This data highlights the impact of STZ induced T1D onset on intestinal microbiota composition and furthermore, the time-dependent changes to intestinal microbiota composition in response to T1D progression in an altered intestinal environment. The proportions of *Bifidobacteriaceae*, *Lactobacillaceae* and *Clostridiaceae* were all increased in T1D rats at the later stages of T1D progression, compared with healthy controls.

The intestinal surface barrier is one of the most important components of the innate immune system (Vaarala et al., 2008) and several studies report that T1D compromises intestinal integrity (Carratu et al., 1999; Kuitunen et al., 2002; Sapone et al., 2006). Abnormalities of the intestinal barrier; the so called “leaky gut”, exposes the intestinal immune system to antigens in the setting of T1D (Vaarala et al., 2008). Brown et al., (2011) in a follow up study to Giongo et al., (2011) revealed knock-on effects of an impaired intestinal microbiota composition in children pre-diagnosed with autoimmune diabetes on microbial fermentation metabolite production (Brown et al., 2011). Butyrate producers such as *Eubacterium*, *Fusobacterium*, *Anaerostipes* and *Roseburia* were higher in the faeces of control, healthy children and lactic acid producers such as *Lactobacillus*, *Lactococcus*, *Bifidobacterium* and *Streptococcus* were
higher in the faeces of children pre-diagnosed with autoimmune diabetes (Brown et al., 2011). The fate of lactate is crucial in determining intestinal health as conversion to butyrate results in mucin synthesis and tighter junctions, while conversion to other SCFA, such as propionate, acetate and succinate reduces mucin synthesis and tight junction assembly (Brown et al., 2011). Mucin is a glycoprotein made by the host that is believed to maintain the integrity of intestine epithelium (Brown et al., 2011).

Interestingly, the data in this study revealed increases in the abundances of lactic acid producing bacteria such as *Bifidobacterium* and *Lactobacillus* in T1D rats, as were the concentrations of lactate and acetate, while butyrate was decreased in the caecum of T1D rats, compared with healthy controls. Butyrate contributes to colonic health due to its anti-inflammatory properties (Hamer et al., 2008; Louis and Flint, 2009; Pryde et al., 2002). In addition, butyrate induces mucin synthesis (Barcelo et al., 2000; Burger-van Paassen et al., 2009; Finnie et al., 1995; Shimotoyodome et al., 2000), decreases bacterial transport across metabolically stressed epithelia (Lewis et al., 2010) and improves the intestinal barrier by increasing tight junction assembly (Peng et al., 2007; Peng et al., 2009). The mucin synthesis induced by butyrate is believed to maintain the integrity of the intestinal epithelia (Brown et al., 2011), thus preventing the development of the so-called “leaky gut” associated with T1D. Acetate, on the other hand does not induce mucin synthesis, and so renders the intestinal epithelia of the T1D animals more susceptible to developing “leaky gut” based on the production of SCFA by the intestinal microbiota of T1D rats. Furthermore, the proportions of *Bacteroides* were higher 2 and 5 weeks after T1D onset, compared with healthy controls. *Bacteroides* have recently been implicated in both diabetic animal and human studies (Brown et al., 2011; Brugman et al., 2006; de Goffau et al., 2013; Giongo et al.,
Such studies have highlighted an association between *Bacteroides* and enhanced intestinal bacterial translocation, resulting in β-cell autoimmunity, associated with T1D (de Goffau et al., 2013). Bifidobacteria have previously been associated with an inhibition of bacterial translocation (Duffy, 2000; Romond et al., 2008; Wang et al., 2004) and it has recently been hypothesised (de Goffau et al., 2013) that bifidobacteria may inhibit the translocation and growth of *Bacteroides* as they compete for space and/or adherence (Stecher and Hardt, 2008) and nutrients (Gibson et al., 1996). Furthermore, bifidobacteria enhance the intestinal epithelial barrier function (Lievin et al., 2000) by increasing the thickness of the mucous layer (Kleessen and Blaut, 2005; Kleessen et al., 2003). The data generated in this study demonstrate increased proportions of *Bifidobacterium* in the T1D group at the later stages of diabetes progression which may have a protective role for the host by enhancing intestinal epithelial barrier function to prevent “leaky-gut”.

Recent advances in sequencing technology have highlighted that a diverse intestinal microbiota is positively associated with human health and that a decrease in the number and abundance distribution of distinct intestinal microbes has been linked to intestinal diseases (Manichanh et al., 2006; Sartor, 2008; Turnbaugh et al., 2009) and in particular, T1D (Brown et al., 2011; de Goffau et al., 2013; Giongo et al., 2011; Knip et al., 2011). Recently, the importance of a diverse microbiota to the health status of the host has been demonstrated in an elderly population whereby community dwelling elderly populations, with a more diverse diet had a greater microbial diversity, compared with elderly in long-stay care (Claesson et al., 2012). The data herein found that intestinal microbiota composition diversity was significantly decreased just 1 week post-STZ induced T1D and did not recover for the duration of the trial following
disease onset. In addition, the principal co-ordinate analyses plots further demonstrated a temporal decrease in diversity. As disease progressed over the 5 weeks, the individual microbiota populations of T1D rats became less diverse, compared with healthy controls and failed to cluster together distinctly, suggesting vast alterations in the intestinal microbiota composition between T1D rats.

This study highlighted temporal variations in the intestinal microbiota of T1D rats over time coinciding with disease progression. The data describes a comprehensive account of the impact T1D onset and progression had on intestinal microbiota diversity and composition. The proportions of lactic acid producing bacteria such as *Lactobacillus* and *Bifidobacterium*, particularly within the later stages of T1D progression correlated with increased caecal lactate concentrations. The intestinal microbiota composition further impacted on microbial fermentation metabolites produced and T1D was associated with increased acetate and total SCFA concentrations in the caecum. Butyrate, associated with mucin synthesis, involved in maintaining intestinal integrity was reduced in T1D rats, compared with healthy controls. Furthermore, the intestinal proportions of bifidobacteria were increased in T1D rats which could have a protective role in the diabetic host. Importantly, this study highlights the importance of a diverse microbiota for health as T1D temporally reduced diversity over the five time points taken. The changes observed in the intestinal microbiota of T1D rats resulted from disease development only and did not have any causative role in the onset of diabetes. Although this study did not provide any data on functional changes related to the observed bacterial diversity, the results provide a comprehensive account of the effect of T1D onset and progression on the intestinal microbiota composition, diversity and microbial metabolite production in the host.
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5.7 REFERENCES


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FIGURE 5.1(A) Percentage body weight gain over the 8 week trial. At week 3, T1D was induced in one of the groups by injection of a single dose of STZ, administered via the intra-abdominal cavity (B) Blood glucose levels (mg/dL) of healthy control and T1D rats.
FIGURE 5.2 Phylogenetic diversity metric of α diversity between healthy control and T1D rats at weeks 0, 1, 2 and 4. T1D was induced in the T1D group at week 1. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at P < 0.05. ** P < 0.01, ***P < 0.001.
FIGURE 5.3 Observed species measurement of α diversity between healthy control and T1D rats at weeks 0, 1, 2 and 4. T1D was induced in the T1D group at week 1. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$. ** $P < 0.01$, ***$P < 0.001$. 
FIGURE 5.4 Phylogenetic diversity and observed species measurements of α diversity between healthy control and T1D rats from caecal content at week 5. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$. ** $P < 0.01$. 
FIGURE 5.5(A) Phylum level distributions of the microbial communities which were either significantly increased or decreased in DNA extracted from the faecal content of T1D rats, compared with healthy controls between weeks 1, 2 and 4. No significant differences were observed at the phylum level at week 0 between the groups. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$. 
**FIGURE 5.5(B)** Phylum level distributions of the microbial communities which were either significantly increased or decreased in DNA extracted from the caecal content of T1D rats, compared with healthy controls at week 5. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$. 

![Graph showing relative abundance of Phyla Actinobacteria, Proteobacteria, and Firmicutes at Week 5.](image)
FIGURE 5.6(A) Family level distributions of the microbial communities which were either significantly increased or decreased in DNA extracted from the faecal content of T1D rats, compared with healthy controls between weeks 0, 1, 2 and 4. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$. 
FIGURE 5.6(B) Family level distributions of the microbial communities which were either significantly increased or decreased in DNA extracted from the caecal content of T1D rats, compared with healthy controls at week 5. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$. 

![Graph showing relative abundance of microbial communities](image)
FIGURE 5.7(A) Genus level distributions of the microbial communities which were either significantly increased or decreased in DNA extracted from the faecal content of T1D rats, compared with healthy controls between weeks 0, 1, 2 and 4. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$. 

(A)
**FIGURE 5.7(B)** Genus level distributions of the microbial communities which were either significantly increased or decreased in DNA extracted from the caecal content of T1D rats, compared with healthy controls at week 5. The nonparametric Mann-Whitney test was used to estimate the relations between the groups. Statistical significance was accepted at $P < 0.05$. 
**FIGURE 5.8(A)** Principal co-ordinate analysis based on unweighted UniFrac distances in T1D and healthy control rats over the 5 time points measured (week 0 to week 5).

- DNA extracted from the faecal pellets of healthy control rats at weeks 0, 1, 2 and 4.
- DNA extracted from the caecal content of healthy control rats at week 5.
- DNA extracted from the faecal pellets of T1D rats at weeks 0, 1, 2 and 4.
- DNA extracted from the caecal content of T1D rats at week 5.
**FIGURE 5.8(B)** Principal co-ordinate analysis based on unweighted UniFrac distances in T1D and healthy control rats over the 5 time points measured (week 0 to week 5). Data point’s coloured different shades of red correspond to DNA extracted from the faecal pellets of healthy control rats at weeks 0, 1, 2 and 4. Orange data points correspond to DNA extracted from the caecal content of healthy control rats at week 5. Data point’s coloured different shades of blue correspond to DNA extracted from the faecal pellets of T1D rats at weeks 0, 1, 2 and 4. Green data points refer to DNA extracted from the caecal content of T1D rats at week 5. The blue data points which cluster together with the data points coloured different shades of red for the healthy control group, corresponds to DNA extracted from the T1D group before STZ injection.
FIGURE 5.9 Caecal content SCFA, lactate and formate concentrations (umol/g) in T1D and healthy control rats. The unpaired student t-test was used to compare the levels of caecal acetate, propionate, butyrate, lactate and formate between the groups. Values are means ± SEM, represented by vertical bars. Statistical significance was accepted at $P < 0.05$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 
SUPPLEMENTARY FIGURE 5.1(A) Chao1 richness estimation of $\alpha$ diversity between healthy control and T1D rats at weeks 0, 1, 2 and 4. T1D was induced in the T1D group at week 1. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$. ** $P < 0.01$, ***$P < 0.001$. 
SUPPLEMENTARY FIGURE 5.1(B) Chao1 richness estimation of $\alpha$ diversity between healthy control and T1D rats at week 5. T1D was induced in the T1D group at week 1. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$. ** $P < 0.01$. 
SUPPLEMENTARY FIGURE 5.2(A) Shannon index of $\alpha$ diversity between healthy control and T1D rats at weeks 0, 1, 2 and 4. T1D was induced in the T1D group at week 1. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$. ***$P < 0.001$. 
SUPPLEMENTARY FIGURE 5.2(B) Shannon index of α diversity between healthy control and T1D rats at week 5. T1D was induced in the T1D group at week 1. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$. *** $P < 0.001$. 
SUPPLEMENTARY FIGURE 5.3(A) Simpson index of $\alpha$ diversity between healthy control and T1D rats at weeks 0, 1, 2 and 4. T1D was induced in the T1D group at week 1. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$. ***$P < 0.001$. 
SUPPLEMENTARY FIGURE 5.3(B) Simpson index of α diversity between healthy control and T1D rats at week 5. T1D was induced in the T1D group at week 1. The nonparametric Mann-Whitney test was used to estimate the relationship between the groups. Statistical significance was accepted at $P < 0.05$. *** $P < 0.001$. 
SUPPLEMENTARY FIGURE 5.4 Rarefraction curves seen to be parallel, signifying that additional sequencing would yield a limited increase in species richness.
SUPPLEMENTARY FIGURE 5.5 Phylum level distributions of the microbial communities between weeks 0 and week 5 for the healthy control group. The nonparametric Kruskal-Wallis test was used to estimate the relations between the healthy control group over the 5 time points taken. Statistical significance was accepted at $P < 0.05$. * $P < 0.05$, **$P < 0.001$. 
SUPPLEMENTARY FIGURE 5.6 Family level distributions of the microbial communities between weeks 0 and week 5 for the healthy control group. The nonparametric Kruskal-Wallis test was used to estimate the relations between the healthy control group over the 5 time points taken. Statistical significance was accepted at $P < 0.05$. * $P < 0.05$, **$P < 0.001$
SUPPLEMENTARY FIGURE 5.7 Genus level distributions of the microbial communities between weeks 0 and week 5 for the healthy control group. The nonparametric Kruskal-Wallis test was used to estimate the relations between the healthy control group over the 5 time points taken. Statistical significance was accepted at $P < 0.05$. * $P < 0.05$, **$P < 0.05$, ***$P < 0.001$. 

![Graph showing the relative abundance of different genera over time and statistical significance levels.](image-url)
SUPPLEMENTARY FIGURE 5.8 Phylum level distributions of the microbial communities between weeks 0 and week 5 for the T1D group. The nonparametric Kruskal-Wallis test was used to estimate the relations between the healthy control group over the 5 time points taken. Statistical significance was accepted at $P < 0.05$. * $P < 0.05$, **$P < 0.05$, ***$P < 0.001$
SUPPLEMENTARY FIGURE 5.9 Family level distributions of the microbial communities between weeks 0 and week 5 for the T1D group. The nonparametric Kruskal-Wallis test was used to estimate the relations between the healthy control group over the 5 time points taken. Statistical significance was accepted at $P < 0.05$. * $P < 0.05$, **$P < 0.05$, ***$P < 0.001$
**SUPPLEMENTARY FIGURE 5.10** Genus level distributions of the microbial communities between weeks 0 and week 5 for the T1D group. The nonparametric Kruskal-Wallis test was used to estimate the relations between the healthy control group over the 5 time points taken. Statistical significance was accepted at $P < 0.05$. * $P < 0.05$, **$P < 0.05$, ***$P < 0.001$

Please note that Chapter 6 (pp.272-308) is unavailable due to a restriction requested by the author.
Chapter 7

General Discussion
High-throughput sequencing technologies have demonstrated that approximately 100 trillion microorganisms reside within the adult intestine (Qin et al., 2010). Over 90% of the bacterial species residing in the intestine belong to the phyla Firmicutes and Bacteroidetes (Sommer and Backhed, 2013). A plethora of evidence indicates links between perturbations of the intestinal microbiota with inflammatory and metabolic disorders, such as obesity (Ley et al., 2006; Zhang et al., 2009), malnutrition (Kau et al., 2011) and diabetes (de Goffau et al., 2013; Qin et al., 2012).

Diet impacts on the composition (De Filippo et al., 2010; de Wit et al., 2012; Devkota et al., 2012; Huang et al., 2013; Mujico et al., 2013) and balance of metabolic products produced by the intestinal microbiota (Scott et al., 2008). Alterations in the Firmicutes: Bacteroidetes ratio has been implicated in obesity whereby an “obese” microbiota exhibits an increased capacity to extract energy from the diet (Murphy et al., 2010), which impacts on weight gain. Previously, different dietary fat types have been associated with altering the intestinal microbiota and metabolic activity of the host (Devkota et al., 2012; Mujico et al., 2013). In Chapter 2, the impact of dietary fat quantity and quality on metabolic health status and intestinal microbiota composition was assessed in mice. The data presented highlight the effects of dietary fat quality on metabolic health and intestinal microbiota composition. For example, dietary palm oil (saturated fat (SFA)) had a negative impact on metabolic health by increasing hepatic triglyceride content, body weight gain and percentage body fat mass and was associated with a decreased relative abundance of Bacteroidetes ($P < 0.05$) and an increased Firmicutes: Bacteroidetes ratio, previously associated with obesity (Murphy et al., 2010). Dietary flaxseed/fish oil ($n$-3 polyunsaturated fatty acids (PUFA)) led to increased tissue concentrations of the beneficial long-chain $n$-3 PUFA,
eicosapentaenoic acid (EPA) \( (P < 0.05) \), docosapentaenoic acid \( (P < 0.05) \) and docosahexaenoic acid (DHA) \( (P < 0.05) \) and was associated with an increased intestinal abundance of *Bifidobacterium* \( (P < 0.05) \). Overall, the data indicated that as the degree of saturation of dietary fat decreased from SFA to \( n \)-3 PUFA, liver triglycerides \( (P < 0.05) \) and intestinal abundances of *Lachnospiraceae* decreased (51% following SFA feeding to 26% following \( n \)-3 PUFA feeding), while proportions of *Erysipelotrichaceae* increased (17% following SFA feeding to 42% following \( n \)-3 PUFA feeding). Potential mechanisms involved in such effects of dietary fat quality on intestinal microbiota composition may include dietary fat promoted changes in hepatic bile acid composition, altering conditions for intestinal microbial assemblage (Devkota et al., 2012) or through SFA triggered extension of fat absorption, impacting on lipid-metabolism gene expression in the distal parts of the small intestine (de Wit et al., 2012).

Intestinal microbiota derived PUFA metabolites such as conjugated linoleic acid (CLA), have been shown to exert health benefits based on *in vitro* and *in vivo* studies. It has previously been shown that dietary supplementation with linoleic acid in combination with CLA-producing *Bifidobacterium breve* NCIMB 702258 led to enhanced liver CLA status, in addition to higher concentrations of EPA and DHA in adipose tissue of mice and pigs (Wall et al., 2009). Furthermore, dietary supplementation with *B. breve* NCIMB 702258 resulted in higher EPA concentrations in the liver (Wall et al., 2010) and higher DHA concentrations in the brain of mice (Wall et al., 2012; Wall et al., 2010). The mechanism by which *B. breve* NCIMB 702258 altered tissue fatty acid profiles is unclear and may either involve its ability to modulate fat-absorption in the small intestine and/or affect host lipid metabolism including hepatic
desaturase and elongase enzyme activity involved in n-3 PUFA metabolism. In Chapter 3, the impact of dietary supplementation with *B. breve* NCIMB 702258 and *B. breve* DPC 6330 on mRNA gene expression levels of fatty acid transport and uptake enzymes in the distal small intestine and liver and hepatic desaturase/elongase enzymes were investigated. The data indicated that *B. breve* NCIMB 702258 and *B. breve* DPC 6330 reduced hepatic triglyceride levels \( (P < 0.05) \) and this was associated with reduced hepatic CD-36 and fatty acid binding protein (FABP)-1 mRNA gene expression, involved in the uptake of fatty acids to the liver (Garcia-Monzon et al., 2011), compared with unsupplemented controls. In addition, *B. breve* DPC 6330 exerted a strain-specific effect on the mRNA gene expression levels of enzymes involved in fatty acid uptake to the ileum \( (P < 0.05) \). The role of the upper and lower intestine in the absorption of gut microbiota derived PUFA metabolites has recently been demonstrated (Druart et al., 2014). Dietary supplementation with ALA increased liver, adipose tissue and brain concentrations of n-3 PUFA, ALA, EPA and docosapentaenoic acid \( (P < 0.05) \), compared with unsupplemented controls and this was associated with an increase in mRNA gene expression of Δ-6 desaturase \( (P < 0.05) \), regardless of microbial supplementation, for both *B. breve* strains used in this study. The data therefore indicate that the mechanisms by which *B. breve* impacts on tissue fat content and fatty acid composition include effects on fatty acid uptake enzymes in the intestine and liver. Future studies using a microarray may yield more detailed information on how *B. breve* supplementation may function in the proximal and distal gut.

Certain commensal bacteria possess the ability to produce exopolysaccharides (EPS) such as beta-glucan with known immuno-stimulatory, antitumoral and anticarcinogenic activity. It has been shown that dietary beta-glucan may modulate
satiety in the host and thus control appetite (Huang et al., 2011). The effect of microbial beta-glucan production on health status was assessed in Chapter 4 using recombinant Lactobacillus paracasei NFBC 338, genetically engineered to heterologously express the glycotransferase (gtf) gene responsible for microbial beta glucan production and compared with an isogenic control Lb. paracasei NFBC 338 strain (Stack et al., 2010). Specially equipped TSE Phenomaster cages measured the impact of feeding a beta-glucan producing probiotic on feeding patterns and energy metabolism in mice. The data indicated that an altered feeding pattern was obtained due to microbial beta-glucan production (P < 0.05), with mice consuming smaller meal sizes (P < 0.05) more frequently (P < 0.05) during the experiment, but overall dietary intake remained unchanged. Moreover, the group supplemented with beta-glucan producing Lb. paracasei NFBC 338 maintained a stable oxygen consumption (VO$_2$) and heat production during a 24hr food deprivation period, compared with unsupplemented controls (P < 0.05). The altered feeding pattern associated with ingesting the probiotic beta-glucan producer may be associated with increased viscosity and thus, increased colonic bulking capacity in this group, compared with isogenic controls, although this remains to be clarified. Overall, the study demonstrated that feeding a beta-glucan producing probiotic alters feeding patterns leading to ingestion of smaller meal portions and thus, may have future applications as dietary adjuncts, particularly for weight loss/management. The genetically engineered beta-glucan producing Lb. paracasei NFBC 338 used in this study is considered a genetically modified organism (GMO) and not Generally Recognised As Safe (GRAS). Future studies to screen for natural beta-glucan producing strains for use as probiotics may lead to new nutritional supplements for weight management.
Perturbations of the intestinal microbiota have been linked with diabetes (de Goffau et al., 2013; Qin et al., 2012). The relationship between type-2-diabetes and the intestinal microbiota have been extensively studied in recent literature (Karlsson et al., 2013; Qin et al., 2012; Zhang et al., 2013). Type-1-diabetes (T1D) is more complex, despite alterations in the microbiota observed in both animal (Roesch et al., 2009) and human models (Brown et al., 2011; de Goffau et al., 2013; Giongo et al., 2011; Murri et al., 2013). Whether the changes observed in the intestinal microbiota represent a cause or an effect of T1D development remain to be elucidated and this was the subject addressed in Chapter 5. Sprague-Dawley rats were induced with T1D through a single intraperitoneal injection of the pancreatic β-cell toxin, streptozotocin (STZ). With preferential toxicity towards insulin-producing pancreatic β-cells, T1D rapidly and irreversibly develops and injected animals display hyperglycemia (>200mg/dL blood glucose) as soon as one week following injection. In Chapter 5, intestinal microbial composition of rats that developed T1D was compared with healthy controls over 5 weeks. The data indicated that T1D development and progression had a dramatic impact on intestinal microbial composition and diversity. In agreement with previous studies, the data revealed that STZ induced T1D was associated with an altered Bacteroidetes: Firmicutes ratio, favouring the abundance of Bacteroidetes, with depletion of Firmicutes following disease onset (de Goffau et al., 2013; Giongo et al., 2011; Murri et al., 2013). Moreover, the abundances of lactic acid producing bacteria such as *Bifidobacterium* and *Lactobacillus* and caecal lactate levels were higher in T1D rats, compared with healthy controls (*P* < 0.05). Brown et al., (2011) reported that bacterial fermentation products, such as SCFA and lactic acid play a distinct role in the maintenance of intestinal integrity, often compromised in T1D (Brown et al., 2011).
Butyrate is an anti-inflammatory SCFA which induces mucin synthesis and is involved in maintaining intestinal integrity, often compromised in T1D (Brown et al., 2011) and was found to be significantly decreased in T1D rats in this study, compared with healthy controls. The data presented in Chapter 5 also indicate that T1D onset and progression immediately and irreversibly reduces intestinal microbial diversity. Future studies involving metagenomics would provide information on the functionality of the microbiota following T1D onset and its impact on different metabolic pathways. STZ treatment did not result in T1D development in all injected rats and therefore it would be interesting to compare the intestinal microbiota of such rats to investigate what impact the toxin had on the intestinal microbiota, without T1D development. Furthermore, as none of the STZ injected T1D rats received insulin therapy, future studies addressing the impact of how insulin treatment and maintenance of T1D progression would impact on intestinal microbial composition and diversity would be of interest.

Due to the increasing demand for health-promoting probiotics, continuous screening processes to discover bacteria with such properties as CLA, EPS, vitamin K, gamma-aminobutyric acid (GABA) and serotonin production, for example, are desirable. These screening processes are fundamental for discovering the bioactive potential of future potential probiotics. In Chapter 6, we assessed the impact of feeding a recently discovered conjugated fatty acid producing strain, isolated from a faecal swab taken from a 3 day old breast-fed infant and identified as Propionibacterium avidum DPC 6544 (Hennessy et al., unpublished data). In light of recent investigations which demonstrate the impact of probiotic bacteria on the microbiota-gut-brain axis, with implications on psychiatric and neurodevelopmental
health of the host (Bravo et al., 2011; Desbonnet et al., 2008), the effects of this strain on behaviour patterns was assessed in a feeding study in mice. Feeding the strain to mice did not alter fatty acid composition after 10 weeks, possibly due to the limited availability of substrate for conjugation in vivo. The data did however demonstrate a potential use of *P. avidum* DPC 6544 as a “psychobiotic”. Recently defined, a psychobiotic is a live organism that, when ingested in adequate amounts, produces a health benefit in patients suffering from psychiatric illness (Dinan et al., 2013). Following *P. avidum* DPC 6544 ingestion, mice exhibited reduced anxiety-like behaviour (*P* < 0.05), compared with unsupplemented controls. The phenomenon of using probiotic bacteria to decrease anxiety in the host has previously been demonstrated (Bravo et al., 2011; Hsiao et al., 2013). Furthermore, the essentiality of the gut microbiota for social interaction and development in the host have been described (Desbonnet et al., 2013). Hsiao et al., (2013) recently demonstrated that feeding the probiotic *Bacteroides fragilis* to animals symptomatic of autism spectrum disorder (ASD), provided some alleviation of the social abnormalities associated with the disease (Hsiao et al., 2013). In this study, supplementation with *P. avidum* DPC 6544 exhibited dietary dependent increases in social interaction (*P* < 0.05), compared with unsupplemented controls. Clinically, replacement of anxiolytic and antipsychotic drugs with a probiotic which induces a similar affect in the host provides an attractive novel approach for the treatment of psychiatric illness. Furthermore, prospective studies to determine whether the psychobiotic potential of *P. avidum* DPC 6544, as observed from this study, could alleviate symptoms of ASD, depression, or anxiety would be desirable. Metagenomic studies could uncover pathways unique to *P.*
avidum DPC 6544 responsible for its unusual fatty acid conjugating abilities and psychobiotic potential.

The data presented in this thesis reveals the impact of nutrition and disease (T1D) on intestinal microbiota composition while also highlighting how modulation of the intestinal microbiota through probiotic supplementation, capable of producing bioactive metabolites can positively shape health status. An unperturbed, stable intestinal microbial ecosystem is important for health and the recent advent of high-throughput sequencing technology has allowed an in-depth analysis of a once unexplored world of gastrointestinal microbiology. We are still at the very early stages of understanding how nutrition modulates intestinal bacteria and indeed how probiotic bacteria mechanistically alter metabolic processes as well as the intestinal microbial ecosystem to impact on health. Further in vivo studies are required to understand the role of the intestinal microbiota on health as well as effects on psychiatric and neurodevelopmental health and development.

REFERENCES


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