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Omega-3 fatty acids, gut microbiota and associated inflammatory outcomes

A thesis presented to the National University of Ireland, Cork for the degree of Doctor of Philosophy

By

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Declaration

This thesis has not been previously submitted 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 by the author with the following exceptions:

Chapter 1b

Mr. Ruairi Robertson and Ms. Clara Seira Oriach are joint first authors of this chapter and of the subsequent publication. Both researchers contributed equally to the preparation, review and editing of the manuscript.

Chapter 2

Mr. Ruairi Robertson and Ms. Clara Seira Oriach are joint first authors of this chapter and of the subsequent publication. Both researchers performed all aspects of study design, data collection, analysis and manuscript preparation for publication. Dr. Kiera Murphy performed part of the bioinformatics analysis of 16s sequencing data including QIIME processing and production of PCoA plots.

Chapter 3

Ms. Clara Seira Oriach assisted in conducting the animal work. Dr. Kiera Murphy performed part of the bioinformatics analysis of 16s sequencing data including QIIME processing and production of PCoA plots. LC-MS was performed by a commercial company.

Chapter 4

Dr. Conall Strain assisted with 16S sequencing library preparation. Dr. Kiera Murphy performed part of the bioinformatics analysis of 16s sequencing data including QIIME processing and production of PCoA plots.

Chapter 5

Dr. Freddy Guiheneuf and Mr. Matthias Schmid conducted collection, culture and characterization of the algae extracts. Dr. Bojlul Bahar assisted with the PCR array.

Chapter 6

Dr. Freddy Guiheneuf conducted culture and characterization of the microalgae extract. Dr. Michael O'Grady assisted with sensory analysis and fat/moisture analysis

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Ruairi Robertson

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Date

Publications

THESIS PUBLICATIONS

- **Robertson R. C.*,** Seira Oriach C.*, Murphy K., Moloney G. M., Cryan J. F., Dinan T. G., Stanton, C.. Omega-3 polyunsaturated fatty acids critically regulate behaviour and gut microbiota development in adolescence and adulthood. *Brain Behav Immun*. (2016).

- Seira Oriach, C.*, **Robertson, R. C.***, Cryan, J. F., Stanton, C., Dinan, T. G. Food for Thought: The role of nutrition in the microbiota-gut-brain axis. *Clin Nutr Exp* (6) 25-38 (2016)

- **Robertson, R. C.,** Guihéneuf, F., Bahar, B., Schmid, M., Stengel, D. B., Fitzgerald G. F., Ross, R. P., Stanton, C. The Anti-Inflammatory Effect of Algae-Derived Lipid Extracts on Lipopolysaccharide (LPS)-Stimulated Human THP-1 Macrophages. *Mar. Drugs* 13(8) 5402-5424 (2015)

- **Robertson, R. C.,** Gracia Mateo, M. R., O'Grady, M. N., Guihéneuf, F., Stengel, D. B., Ross, R. P., Fitzgerald, G. F., Kerry, J. P. & Stanton, C. 2016. An assessment of the techno-functional and sensory properties of yoghurt fortified with a lipid extract from the microalga *Pavlova lutheri*. *Innovative Food Sci. Emerging Technol*. (2016)

- **Robertson, R. C.,** Guihéneuf, F., Schmid, M., Stengel, D. B., Fitzgerald G. F., Ross, R. P., Stanton, C. Algae-derived Polyunsaturated fatty acids: Implications for Human Health. In A. Catala, *Polyunsaturated Fatty Acids: Sources, Antioxidant Properties and Health Benefits*. Nutrition and Diet Research Progress. USA: Nova Sciences Publishers, Inc.; (2013)

OTHER PHD PUBLICATIONS

- Pusceddu, M.M., Nolan, Y. M., Green, H. F., **Robertson, R. C.,** Stanton, C. Kelly, P., Cryan, J. F., Dinan, T. G. The Omega-3 Polyunsaturated Fatty acid Docosahexaenoic acid (DHA) reverses Corticosterone-induced Changes in Cortical neurons. *Int J Neuropsychopharmacol*. 1-10 (2015)

  Moroney, N.C., O'Grady, M.N., **Robertson, R. C.,** Stanton, C., O'Doherty, J. V., Kerry, J. P. Influence of level and duration of feeding polysaccharide (laminarin and fucoidan) extracts from brown seaweed (Laminaria digitata) on quality indices of fresh pork. *Meat Sci,* 99**,** 132-41. (2015)

Thesis Abstract

ABSTRACT

The overall aim of this thesis was to investigate the potential of omega-3 polyunsaturated fatty acids (n-3 PUFA), particularly from algal sources, as regulators of gut microbiota development, behaviour and associated inflammatory and metabolic outcomes.

In chapter 2, omega-3 supplemented (O3+), omega-3 deficient (O3-) and control diets were compared for their effect on behaviour and gut microbiota development in both adolescence and adulthood. O3- impaired communication, social and depression-related behaviours, whereas O3+ enhanced cognition. These behavioural changes were associated with alterations to gut microbiota composition and inflammatory outcomes such that O3- mice displayed an elevated Firmicutes:Bacteroidetes ratio and blunted systemic LPS responsiveness. Contrastingly, O3+ mice displayed greater fecal *Bifidobacterium* and *Lactobacillus* abundance and dampened hypothalamic-pituitary-adrenal axis activity. These results suggest that the beneficial effects of n-3 PUFA on neurobehavioural development are closely associated with comprehensive alterations in gut microbiota composition, HPA-axis activity and inflammation.

Chapter 3 further assessed the role of n-3 PUFA on the cecal microbiota and metabolome. n-3 PUFA status induced subtle changes to the cecal microbiota in mothers and their offspring and, in addition, O3+ induced increased production of energy metabolites. O3- reduced production of short chain fatty acids. These results indicate that n-3 PUFA modulate cecal microbiota composition and function, which may have implications for chronic disease risk.

In chapter 4, the transgenic *fat-1* mouse model was utilised to examine the effects of maternal n-3 PUFA on offspring obesity risk. Offspring of mothers with a lower n-6/n-3 tissue ratio gained significantly less weight on a high fat diet. This

reduced weight gain was associated with reduced gut permeability and changes to the gut microbiota and inflammatory outcomes. These results indicate that maternal n-3 PUFA status can significantly influence offspring adiposity through changes associated with gut microbiota.

Algae are potent sources of n-3 PUFA and pose potential as alternative sustainable sources of such fatty acids than fish. However, little research has examined the effect of algae-derived n-3 PUFA on health. The aim of Chapter 5 was to screen the anti-inflammatory bioactivity of n-3 PUFA-rich algae lipid extracts. Exposure of the algae lipid extracts to lipopolysaccharide-stimulated THP-1 macrophages significantly reduced production of the pro-inflammatory cytokines IL-6 and IL-8 and the expression of a number of pro-inflammatory genes associated with toll-like receptor and chemokine activity. These data reveal that such n-3 PUFA-rich algae lipid extracts pose potential as anti-inflammatory functional ingredients.

The aim of chapter 6 was to investigate the suitability of yoghurt as a food matrix in which to supplement an n-3 PUFA-rich lipid extract of *Pavlova lutheri*. Addition of the extract had little negative effect on the techno-functional properties of yoghurt. Furthermore, addition of the extract dose-dependently increased concentrations of n−3 PUFA. However, sensory analysis revealed that supplemented yoghurts were not well accepted. These results suggest that addition of this extract to yoghurt is a suitable method to increase n-3 PUFA concentration.

Finally, Chapter 7 discusses the major findings and general conclusions arising from the work presented in this thesis.

Chapter 1

Literature Reviews

Chapter 1.1

Algae-Derived Polyunsaturated Fatty Acids: Implications for Human Health

Published - Modified from book chapter in: A. Catala, *Polyunsaturated Fatty Acids: Sources, Antioxidant Properties and Health Benefits*. Nutrition and Diet Research Progress. USA: Nova Sciences Publishers, Inc.; (2013)

ABSTRACT

Polyunsaturated fatty acids (PUFA) have been shown to exert a multitude of beneficial health effects. Growing evidence suggests that dietary omega-3 (n-3) PUFA, which are abundant in foods of marine origin, play a major role in early development and in the prevention and treatment of chronic disease, notably inflammatory disease. Recent research has highlighted mechanisms by which the well-characterised fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and less common PUFA, such as docosapentaenoic acid (DPA) and stearidonic acid (SDA) ameliorate disease-causing physiological processes. Such PUFA are responsible for gene and cytokine expression, eicosanoid production, neurodevelopment and gut microbiota composition among other processes, all of which can significantly affect human health. The western diet, however, is deficient in n-3 PUFA and abundant in other lipids, notably n-6 PUFA and saturated fats. Hence, increasing n-3 PUFA consumption may contribute to improved public health outcomes linked to chronic disease. Algae pose potential as a vast and untapped resource of n-3 and n-6 PUFA, one that may be more resourceful and sustainable than fish. This chapter will discuss established and recent evidence for the role of dietary PUFA in chronic disease, notably their effects on inflammatory disorders and brain development and function. Furthermore, the bioactive properties of algae-derived PUFA and their potential as dietary lipids to prevent modern chronic disease epidemics will also be discussed.

1. INTRODUCTION

As the incidence of nutritionally-linked chronic disease increases worldwide (Laslett et al., 2012, Shaw et al., 2010, Kelly et al., 2008), nutrition-based prevention and treatment strategies are continuously being explored. Dietary n-3 polyunsaturated fatty acids (n-3 PUFA) have shown the potential to regulate pathways that lead to chronic diseases, and since the 1970s, when the low incidence of cardiovascular disease in Greenland Inuits was associated with a high fish intake (Bang et al., 1971, Bang et al., 1976, Dyerberg et al., 1975), investigations into the health benefits of long-chain omega-3 (n-3) PUFA have increased dramatically. Dietary long-chain n-3 PUFA are now known to exhibit cardioprotective (Adkins and Kelley, 2010), anti-inflammatory (Calder, 2006), immunomodulatory (Sijben and Calder, 2007), cognitive (Dangour et al., 2009), neurobehavioural (Boucher et al., 2011), anti-proliferative (Yang et al., 2012), anti-cancer (Chapkin et al., 2007) and anti-microbial (Huang and Ebersole, 2010) properties, amongst others. Hence, these functional fatty acids may assist in the treatment and prevention of chronic diseases such as obesity, cardiovascular disease (CVD), Alzheimer's disease (AD) and inflammatory bowel disease (IBD). Evidence associating these bioactive molecules with such multifactorial effects has led to an abundance of fortified foods and hence a rapid increase in PUFA health claims (Agostoni, 2010). The western diet, however, is characterised by an imbalance in the ratio of n-6/n-3 PUFA intake. It is estimated that humans evolved on a ratio of 1:1 however this ratio in the western diet may be as high as 10-40:1 (Simopoulos, 2002). Hence the proportions of dietary lipids are critical in the prevention and onset of chronic disease (Calder, 2006, Bazinet and Layé, 2014).

The most bioactive n-3 PUFA are the long-chain n-3 PUFA, which are derived from marine sources, notably oily fish. Microalgae, macroalgae (seaweeds) and certain microorganisms are capable of *de novo* PUFA synthesis and hence represent a potentially vast and untapped resource of these fatty acids, one that may be more resourceful and sustainable than fish. Macroalgae are known producers of marine bioactive compounds, and are largely exploited for their nutritional properties in Asian diets. Microalgae are a diverse group of unicellular organisms found in marine, freshwater and subaerial environments, comprised of thousands of different species, most of which have not been studied in detail, yet some are already exploited as sources of bioactive ingredients such as PUFA. PUFA profiles vary widely across algal species, and thus they may be resources of common as well as lesser-known unsaturated fatty acids. However, much has yet to be discovered about the compositional and functional properties of algal PUFA, and hence their potential as disease-preventing functional food ingredients.

2. THE STRUCTURE AND BASIC FUNCTION OF PUFA

2.1. PUFA Structure and Metabolism

PUFA are hydrocarbon chains, bound by 2 or more double bonds, and terminate in a methyl group at one end and a carboxyl group at the other. The reactive carboxyl group readily combines with alcohol groups to form various lipid-based molecules such as triglycerides and phospholipids. PUFA are characterised primarily by the number and position of their double bonds, which allows them to be classified into two distinct groups; n-3 and n-6 PUFA (or omega-3 and omega-6 PUFA). n-6

PUFA are synthesized from the essential fatty acid precursor linoleic acid (LA, 18:2), whilst n-3 PUFA are synthesized from the essential fatty acid α-linolenic acid (ALA, 18:3). LA and ALA are termed 'essential' as they cannot be produced *de novo* in mammals, due to a predisposed genetic lack of the Δ-15 desaturase enzyme. Thus LA and ALA must be obtained extrinsically from the diet. These two fatty acids are primarily found in plant sources such as soybean, canola, flaxseed and sunflower oils. Land plants such as these lack the enzymatic capacity to further metabolize LA and ALA into longer chain molecules and hence do not generally contain long-chain PUFA.

In mammalian tissues LA and ALA are subject to a series of elongation and desaturation reactions giving rise to longer chain PUFA (Fig. 1). The n-3 (ALA) and n-6 (LA) PUFA synthesis pathways are competitive, such that they occur adjacently, competing for the same enzymes. Thus, up-regulation of one pathway can lead to down-regulation of the other. These reactions rely on a combination of elongases, Δ-5 and Δ-6 desaturases (Sprecher et al., 1999). Arachidonic acid (AA, 20:4) is the major product of the n-6 pathway and eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) are the major products of the n-3 pathway, however, a number of intermediate and less common fatty acids are produced along these reaction chains. Hence humans can obtain long-chain PUFA from non-marine based plant sources via tissue conversion of LA and ALA to longer chain PUFA. However, the n-3 conversion process from ALA to EPA/DHA is inefficient (Burdge and Wootton, 2002, Burdge et al., 2003, Arterburn et al., 2006), thus it is necessary to obtain a majority of n-3 fatty acids directly from the diet. The inefficiency of this conversion process increases with age and in certain disease states (Gao et al.,

2012, Le et al., 2009), thus dietary intake of PUFA becomes even more essential in such cases. Dietary EPA and DHA are abundant in oily fish such as tuna, salmon and mackerel, and are also found in other food sources such as eggs and algae.

2.2. PUFA Functions

PUFA have a number of structural and functional roles in human tissues. Most notably, they form the core structure of the phospholipid bilayer in all cell membranes, regulating fluidity and rigidity, and cell signalling. Distribution of PUFA in the phospholipid bilayer of cell membranes varies according to dietary intake and cell type. For example, brain and retinal cells contain a higher proportion of DHA in comparison to most other cell types (Arterburn et al., 2006). DHA represents up to 60% of total fatty acids in the rod outer segment in the retina (Giusto et al., 2000). Cell PUFA composition is readily altered by diet and hence a high dietary intake of certain PUFA will be reflected by cell PUFA accumulation. Research has also shown that tissue DHA content following supplementation is dose-dependent (Arterburn et al., 2006). Although a certain amount of conversion and even retroconversion (Vidgren et al., 1997, Brenna et al., 2009) occurs between fatty acids *in vivo*, it is limited, thus it has been suggested that the most effective way to increase the tissue content of a certain fatty acid is dietary supplementation with that fatty acid (Arterburn et al., 2006).

Aside from structural properties, PUFA also regulate a number of functional and metabolic processes. PUFA possessing 20 carbons, notably AA and EPA, are capable of producing potent lipid mediators, termed eicosanoids. There are several classes of eicosanoids, including prostaglandins (PG), leukotrienes

(LT) and thromboxanes (TX), along with a number of subclasses, all of which play differing roles in inflammatory regulation (Calder, 2006). In recent times, DHA (22:6 n-3) and n-6 docosapentaenoic acid (n-6 DPA, 22:5 n-6) have been shown to produce other eicosanoid-like lipid mediators such as resolvins and protectins, which can also regulate inflammation (Dangi et al., 2009, Dangi et al., 2010, Serhan et al., 2002).

PUFA are known ligands for peroxisome proliferator-activated receptors (PPAR) which function as nuclear receptors and transcription factors by activating genes that regulate a number of activities including lipid metabolism and storage. PUFA are also capable of activating a number of other transcription factors and receptors such as nuclear factor kappa B (NF-κB) and toll-like receptor 4 (TLR-4) and thus are potent regulators of gene expression (Jump, 2002). Additionally, similar to other fatty acids, PUFA also provide a very good source of energy, as well as regulating plasma lipids and circulating cholesterol (Kelley et al., 2007).

2.3. The Health Effects of PUFA

As a consequence of their vital structural and metabolic functions, PUFA are responsible for a number of health effects as outlined below. By producing eicosanoids and regulating gene expression, PUFA play a significant role in inflammatory disorders. Inflammation is a complex biological process in which the body's immune responses are stimulated, and in some cases over-stimulated, in response to harm, such as the presence of pathogenic bacteria or physical damage to tissues. Low-grade chronic inflammation has recently been identified as the cornerstone of a number of chronic disorders including obesity (Wellen and Hotamisligil, 2003), type 2 diabetes mellitus (T2DM) (Pradhan et al., 2001),

rheumatoid arthritis (RA) (Choy and Panayi, 2001), AD (Akiyama et al., 2000) and IBD (Fiocchi, 1998). As potent regulators of inflammatory gene expression and precursors to eicosanoids, dietary PUFA play a major role in the onset, prevention and treatment of such inflammatory disorders. The role of PUFA and algal PUFA in inflammatory disorders will be discussed in the following sections.

AD, another inflammatory disorder, is characterized by the build-up of amyloid plaques in the brain, a symptom which occurs in tandem with neuroinflammatory processes. This plaque accumulation leads to brain cell degeneration and cognitive decline in the elderly (Leon et al., 2010, Janus et al., 2000). n-3 and certain n-6 PUFA have been shown to protect against age-related neuro-inflammation and cognitive impairment (Labrousse et al., 2012) and hence may play a role in the prevention of AD. This, in recent times, has led to PUFA-fortified dietary products specifically designed for brain health (www.nutricia.ie/souvenaid/) (Nutricia). PUFA have also been shown to be important for brain development. During the neonatal and early postnatal periods there is a significant dietary requirement for PUFA leading to a rapid uptake of DHA and AA in the infant brain (Schiefermeier and Yavin, 2002). Hence, the concentration of PUFA is high in human breastmilk. The role of PUFA and algal PUFA in brain development and decline will be discussed in the following sections.

One of the earliest known benefits of long-chain PUFA was in terms of cardiovascular health. As mentioned above, Greenland Inuits were noted for their lack of cardiovascular diseases in the 1970s and this was attributed to their high intake of PUFA from fish (Bang et al., 1971, Dyerberg et al., 1975). Dietary n-3 PUFA contribute to cardiovascular health by increasing the beneficial cholesterol, high density lipoprotein (HDL) cholesterol, and decreasing circulating

triglycerides (TAG) and low density lipoprotein (LDL) cholesterol (Kelley et al., 2007). n-3 PUFA are also potent inhibitors of platelet aggregation, hence may be beneficial in preventing the build-up of atherosclerotic lesions (Phang et al., 2009). Recent research has associated long-chain n-3 PUFA with anti-microbial effects (Huang and Ebersole, 2010), but there is a lack of *in vivo* data to validate this association. Similarly, PUFA have been associated with gut health by enhancing the adhesion capabilities of beneficial probiotics (Kankaanpää et al., 2001, Bomba et al., 2002, Bomba et al., 2003). In addition, n-3 PUFA appear to exert beneficial effects on the gut microbiota and the intestinal inflammatory environment (Robertson et al., 2016). n-3 PUFA have been also been associated with anti-viral effects (Morita et al., 2013) and anti-proliferative (Yang et al., 2012) effects which could have profound implications for the prevention of the progression of viral related diseases and certain cancers.

3. DIETARY PUFA IN HEALTH AND DISEASE

3.1. PUFA and Inflammatory Disease

3.1.1. Inflammation in Disease

The immune system is vital to combat injury and infection, however immune response can be harmful to the host if it is activated in an unregulated manner for a long period of time. Acute inflammatory response is beneficial to the host in cases of short-term infection or injury as it helps to fight infection and repair injured tissue. However, over-production of inflammatory mediators, immune activation by a non-pathogenic host antigen, or the lack of a mediator to terminate

inflammation, can cause immune molecules to attack and damage host tissues, which can lead to pathological disease progression.

Inflammation is a process of the body's innate immune system that occurs in response to infection or injury and manifests itself in the form of swelling, heat and redness. This process can also occur at a chronic subclinical level. The presence of a pathogen (such as pathogenic bacteria, viruses and parasites) in a host system activates the immune system in order to locate, identify and eliminate the invading pathogen. Immune system activation also occurs in response to other stimuli including physical injury, surgery or the presence of tumour cells, in an effort to heal and resolve. The presence of these stressors initiates increased blood flow and blood vessel permeability in order to allow immune cells to cross from the blood stream to the site of infection/injury. A number of biochemical modulators and cell types are involved in this process and they interact and communicate with each other in order to regulate immune response and resolve the infection/injury. The dominant immune cells are leukocytes such as monocytes, macrophages, lymphocytes and granulocytes. These cells adhere to endothelial cells supported by the increased expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1), and then transverse the permeable capillary membranes from the bloodstream into the affected tissue. Once at the affected site, leukocytes release potent biochemical molecules depending on the strength, stage and type of inflammatory stimulus. Lipopolysaccharide (LPS) is a common example of an inflammatory stimulus. LPS is an integral part of Gram-negative bacterial cell walls, and once identified by host immune cells, can stimulate the release of inflammatory mediators, which assist in the destruction of the pathogenic bacteria.

These mediators are wide ranging in structure and function and include cytokines, eicosanoids, reactive oxygen species (ROS) and a number of other biochemical molecules which all play differing roles in the inflammatory response. Some of these mediators can have anti-inflammatory or even both pro- and anti-inflammatory activities under different conditions, as well as having the ability to 'switch-off' inflammation, and hence, immune and inflammatory responses vary greatly under differing stress conditions.

When the inflammatory process becomes unregulated, it can supplement chronic inflammatory disease states. IBD (Fiocchi, 1998), RA (Choy and Panayi, 2001), AD (Wyss-Coray, 2006) and CVD (Ridker et al., 2000) are just a number of disorders that can progress as a result of unregulated chronic inflammation. In certain cases such as RA, these inflammatory disease states are alleviated through the use of non-steroidal anti-inflammatory drugs (NSAID). However, as inflammation is now recognised as a cornerstone of chronic disease states such as obesity and type-2 diabetes, nutritional rather than pharmacological treatments are being sought after as long-term remedies.

3.1.2 Processes by Which PUFA Regulate Inflammation

PUFA make up a significant proportion of the phospholipid membrane of immune cells such as monocytes and macrophages, and their incorporation into these cells is significantly altered by dietary PUFA intake (Endres et al., 1989, Gibney and Hunter, 1993). The n-6 PUFA AA constitutes a significant proportion of the fatty acids in immune cell phospholipids, whereas n-3 PUFA such as EPA and DHA are present at much lower levels. Numerous cellular, animal and human trials have shown that increased dietary intake of long-chain n-3 PUFA results in increased

incorporation of these fatty acids into immune cell membranes in place of n-6 fatty acids such as AA (Caughey et al., 1996, Lee et al., 1985). PUFA are directly and indirectly responsible for the production of potent inflammatory mediators and hence play a major role in inflammation through a number of immune cell-mediated processes.

Eicosanoid production

Eicosanoids are a group of potent inflammatory mediators produced by the 20-carbon PUFA EPA and AA, and hence play a key role in inflammation. As previously mentioned, AA makes up a significant proportion of the fatty acids in immune cell membranes, alongside lower proportions of other fatty acids such as EPA and DHA. The presence of an inflammatory stimulus, such as LPS, activates phospholipase A2 which in turn releases AA from the phospholipid membrane. The free AA is then oxidised by either cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 enzymes to produce immune-modulating eicosanoids (Figure 2). The COX pathway is a target for pain relieving drugs such as aspirin (Smith and Willis, 1971). Aspirin targets the COX pathway and prevents the production of pro-inflammatory eicosanoids from the prostanoid group.

Eicosanoids are classified in 2 distinct groups, the prostanoids (prostaglandins and thromboxanes) which are products of COX, and the leukotrienes which are products of LOX, and within these groups, there are a number of individual eicosanoids each with varying potencies and functions. Other groups of eicosanoids such as hydroxyeicosatetraenoic acid (HETE) and hydroxypero-xyeicosapentaenoic acid (HEPE) have also been identified and are produced via the action of P450 enzymes. Eicosanoids are primarily derivatives of

AA but can also be products of EPA oxidation following release from phospholipids.

In general, AA eicosanoids are considered proinflammatory and EPA eicosanoids are considered less inflammatory or anti-inflammatory (Wada et al., 2007). Hence, a high level of AA in the phospholipid membranes of cells results in a high level of substrate for inflammatory eicosanoids. Replacement of AA with EPA in membranes through the diet thereby increases the level of substrate for less inflammatory eicosanoids and hence reduces inflammatory disease risk. However, in recent times it has been shown that this is not always the case. Prostaglandin E_2 (PGE_2), an AA-derived eicosanoid, has been shown to display both pro- and anti-inflammatory effects (Levy et al., 2001), and additionally, it has been shown that both PGE_2 (an AA- derived eicosanoid) and PGE_3 (an EPA-derived eicosanoid) have equivalent potencies with regard to inhibition of the proinflammatory cytokines TNFα and IL-1β in human mononuclear cells (Miles et al., 2002). Hence, eicosanoid metabolism is highly complex and can vary greatly under different conditions.

PUFA have long been recognised as mediators of inflammation (Samuelsson, 1983, Tilley et al., 2001), and by acting as substrates for eicosanoids, dietary PUFA have since been recognised as mediators of inflammatory processes and immune function (Calder, 2002, James et al., 2000). Studies *in vitro* and intervention trials in humans and animals have repeatedly shown that dietary long-chain omega-3 PUFA, primarily EPA and DHA, reduce the production of inflammatory eicosanoids (Gibney and Hunter, 1993, Trebble et al., 2003). Much of this evidence has focused on dietary fish oil supplementation in healthy humans resulting in decreased production of the pro-inflammatory

eicosanoids PGE_2 (Endres et al., 1989, Lee et al., 1985) and the 4 series leukotrienes (Weaver et al., 2009). In recent times there has been increasing focus on the anti-inflammatory effects of the individual omega-3 PUFA, EPA and DHA, rather than the combination of PUFA in fish oil (Peterson et al., 1998, Weldon et al., 2007), and it has been reported that these PUFA exert differential effects on eicosanoid and other inflammatory mediator production (Corey et al., 1983, Kelley et al., 1999). A study in 2012 by Norris and Dennis provided the most in-depth analysis of differential eicosanoid production following *in vitro* supplementation with EPA, DHA or AA and subsequent inflammatory stimulation (Norris and Dennis, 2012). This research showed that DHA reduced AA-derived COX eicosanoids (primarily the 2 series prostaglandins) more effectively than EPA in mice macrophages. Furthermore, it was shown that a significant proportion of EPA was elongated to DPA in EPA-supplemented cells. Subsequent supplementation of cells with DPA showed that it was the only PUFA released from cells at a comparable level to AA following inflammatory stimulation, and hence competed for COX enzymes and reduced pro-inflammatory AA eicosanoid production. These results suggest a major role for n-3 DPA in eicosanoid metabolism and hence the inflammatory process. Indeed, it has been postulated that DPA n-3 may directly affect inflammation by producing its own eicosanoid-like molecules (Kaur et al., 2011b), but such compounds have yet to be identified.

Because of its ability to compete for the same enzymes as AA and influence eicosanoid production, EPA was initially considered as the sole anti-inflammatory n-3 PUFA. However, recent research has elucidated a novel group of eicosanoid-like molecules derived from DHA in addition to EPA. These molecules, termed resolvins and neuroprotectins, have shown equal and greater

anti-inflammatory activity than some EPA-derived eicosanoids in a number of immune cells (Serhan et al., 2008). Additionally, resolvins exhibit the novel ability to resolve or switch-off inflammation, rather than prevent its initiation. Resolvin synthesis following PUFA supplementation has been widely examined in cell and animal models, where fish oil, EPA and DHA supplementation have been shown to increase resolvin production in mice (Serhan et al., 2000). However, there have been few reported studies in humans that measured resolvin production following PUFA supplementation (Mas et al., 2012). Further clinical trials may identify the anti-inflammatory role of resolvins in humans. There is also growing interest in some of the DHA-derived neuroprotectins because of their potent activity in the brain and their ability to prevent neurodegeneration (Serhan et al., 2004). Further examination of these metabolites may have significant implications for identifying compounds to fight neurodegenerative diseases such as Alzheimer's disease (Lukiw et al., 2005). The growing interest into resolvins and protectins has also led to the discovery of n-6 DPA derived resolvins. These molecules have been identified and characterized and shown in mice models to have anti-inflammatory activity and alleviate experimental colitis (Dangi et al., 2009, Dangi et al., 2010, Chiu et al., 2012).

Gene Transcription and Gene Expression

In addition to their role in eicosanoid production, PUFA can also modulate inflammation through altered gene-expression. NF-κB is a transcription factor that plays a major role in inflammatory activation, by activating pro-inflammatory genes. Cytosolic NF-κB is inactive and is composed of 3 subunits, IκB, P65 and P50. An inflammatory trigger in the cell is detected by TLR4, this subsequently

causes phosphorylation and ubiquitination of the inhibitory IκB subunit and hence the active NF-κB translocates to the nucleus and activates pro-inflammatory genes (Figure 3). However, long-chain omega-3 PUFA such as EPA and DHA can prevent NF-κB activation and hence prevent the up-regulation of pro-inflammatory genes. This has been identified in human macrophages, where binding of NF-κB to DNA was significantly decreased following DHA supplementation (Weldon et al., 2007) and EPA supplementation (Zhao et al., 2004). The mechanisms by which omega-3 PUFA prevent NF-κB activation were recently reported. Omega-3 PUFA were found to activate G protein-coupled receptor 120 (GPR120) on the surface of macrophages, and GPR120 then prevents the signalling cascade caused by LPS activation of TLR4, and hence prevents the activation of NF-κB (Oh et al., 2010). Additionally, EPA and DHA act as ligands for PPARs, which act as nuclear receptors and transcription factors for genes associated with lipid metabolism and inflammation. When activated by PUFA, PPARs bind with retinoid X receptors (RXR) which then alter transcription and expression of particular genes. Much research has investigated the activation of PPARs, particularly PPARγ, by PUFA and the subsequent anti-inflammatory response (Li et al., 2005). Inflammatory gene expression changes have also been observed following EPA and DHA supplementation in human studies. Bouwens et al. (2009) reported a change in expression of 1040 genes from peripheral blood mononuclear cells (PBMC) isolated from healthy individuals following 26 weeks of EPA+DHA supplementation (Bouwens et al., 2009). The gene expression changes showed marked decreases in inflammatory and atherogenic pathways. EPA and DHA supplementation resulted in the reduced expression of a number of NF-κB target genes, interleukin receptors and enzymes involved in pro-

inflammatory eicosanoid synthesis. Hence, this study showed that long-term EPA and DHA supplementation can up regulate the anti-inflammatory gene expression profile in human PBMCs.

Cytokine Production

The up-regulation of pro-inflammatory genes stimulates the production of pro-inflammatory cytokines, which are potent forms of inflammatory mediators. Cytokines are potent, fast-acting and have a short half-life, and can affect cell proliferation, differentiation, apoptosis and acute phase response. These molecules are primarily produced by immune cells such as monocytes and macrophages, but other cells such as adipocytes have also been shown to have the ability to produce cytokines and hence elucidate a role of inflammation in obesity (Tilg and Moschen, 2006). Interleukin 6 (IL-6), IL-1β and Tumor Necrosis Factor-alpha (TNFα) are the classic pro-inflammatory cytokines, and show marked elevations in cases of sepsis (Cavaillon et al., 2003), fever (Netea et al., 2000), and IBD (Rogler and Andus, 1998). *In vitro* cell culture studies have shown the ability of EPA, DHA and fish oil to reduce the production of IL-1β, TNFα and IL-6 following inflammatory stimulation with LPS (Weldon et al., 2007, Zhao et al., 2004), most likely by preventing activation of NF-κB and inflammatory genes. The reduction of proinflammatory cytokines and increase in anti-inflammatory cytokines such as IL-10 and IL-4 has been shown in murine models of colitis and dermatitis following n-3 PUFA supplementation (Whiting et al., 2005, Renier et al., 1993, Sierra et al., 2006).

Human intervention studies have primarily involved dietary supplementation with fish oil, EPA or DHA followed by *ex vivo* examination of

PBMCs and cytokine production. These studies have shown consistent evidence that omega-3 PUFA supplements decrease inflammatory TNFα, IL-6 and IL-1β production (Endres et al., 1989, Caughey et al., 1996, Vedin et al., 2008) however it seems that this effect is dose-dependent and relies on large supplementary doses (>2g/day), and lower doses were reported to have no effect (Thies et al., 2001, Wallace et al., 2003). It has also been shown in a cross-sectional study of over 3,000 subjects that fish consumption of >300g/week results in significantly decreased levels of IL-6, TNFα and C-reactive protein (CRP) compared with non-fish consumers (Zampelas et al., 2005), most likely due to the high levels of omega-3 PUFA in fish.

Interaction with gut microbiota

In recent times, the gut microbiota has been identified as a critical regulator of immune and metabolic health (Patterson et al., 2016). Comprised of all the microbial species in the intestines, the gut microbiota plays key roles in inflammation, intestinal permeability, nutrient absorption, neuroendocrine signaling and a number of other physiological processes (Marchesi et al., 2016). A complex relationship exists between commensal gut bacteria and intestinal epithelial cells which uniquely modifies the host intestinal environment. This relationship is strongly influenced by diet through modulation of gut microbiota composition. Studies from germ-free animals have demonstrated that the composition of the gut microbiota independently regulates obesity and metabolic health (Ridaura et al., 2013).

The role that both n-3 and n-6 PUFA play in the gut-microbiota relationship has been explored in recent times (Kaliannan et al., 2015). Greater n-6

PUFA intake is reflected in the fatty acid composition of intestinal tissues, which contributes to a pro-inflammatory intestinal environment. Conversely, higher n-3 intake and subsequent incorporation into intestinal tissue can result in dampened intestinal inflammation (Kaliannan et al., 2015). In addition, n-3 PUFA and n-6 PUFA can directly interact with commensal gut microbiota by modulating the adhesion of certain species to intestinal epithelial cells (Bomba et al., 2003, Kankaanpää et al., 2001). n-3 PUFA have also been shown to stimulate the production of a number of antimicrobial peptides, including intestinal alkaline phosphatase (IAP), which scavenge LPS-producing bacteria such as *e. coli* (Kaliannan et al., 2015). Hence dietary PUFA play a critical role in the host intestinal environment by interacting with both host tissues and resident microbes.

Patterson et al demonstrated that microbiota composition is dependent upon type of dietary fat, with *Bifidobacteria* being more abundant following fish oil feeding (Patterson et al., 2014). Similarly, Caesar et al reported that a lard oil rich diet induced dysbiosis and elevated white adipose tissue inflammation in comparison to a diet rich in fish oil (Caesar et al., 2015). Wall et al showed a novel link between gut microbiota and host tissue fatty acid composition in both rodents and pigs (Wall et al., 2009, Wall et al., 2010, Wall et al., 2012). Oral administration of *Bifidobacterium breve* species significantly altered liver, adipose and brain fatty acid composition. Most interestingly, n-3 docosapentaenoic acid was significantly increased in the brain of the probiotic fed animals. Conversely, it has been shown that n-3 PUFA consumption in pharmacological excess (18% energy) can stimulate the overgrowth of opportunistic pathogens in the gut microbiota and impair the inflammatory response (Ghosh et al., 2013, Gibson et

al., 2015), however the doses used in these studies may not be biologically relevant.

The role of algae-derived PUFA on the gut microbiota has yet to be examined, however recent data suggests that EPA and DHA from microalgal oils elevate abundance of both *bifidobacteria* and *lactobacillus* in mice (Robertson et al., 2016). Further research must now address the role of dietary PUFA intake on both the developing and developed microbiota in humans to supplement the little data already available (Andersen et al., 2011). Clinical research such as this will provide novel evidence for the role of dietary fat type on the gut microbiota and associated outcomes and may help public health guidelines for PUFA intake.

Other Processes

A number of other mechanisms by which PUFA regulate inflammation have been reported. Dietary supplementation with EPA and DHA have been shown to reduce leukocyte chemotaxis to the site of inflammation in *ex vivo* studies of human leukocytes (Lee et al., 1985); reduce the expression of adhesion molecules such as ICAM-1 and VCAM-1 which allow stimulated macrophages to adhere to endothelial cells (Yamada et al., 2008); reduce the production of reactive oxygen species (ROS), when supplemented at high levels (Luostarinen and Saldeen, 1996); and disrupt membrane lipid raft assembly, which are rich in saturated fatty acids, play a role in T-cell response and cell-signalling (Calder, 2011) and alter gut microbiota composition (Caesar et al., 2015).

3.1.3 PUFA Supplementation and Inflammatory Disease Outcome

Obesity and metabolic disease

Obesity and its associated metabolic abnormalities are heavily influenced by an altered immune environment. Adipose tissue inflammation, characterized by macrophage accumulation and cytokine production, is a hallmark of adipogenesis leading to fat mass accumulation (Greenberg and Obin, 2006). Dietary lipids play a role in this process partially through the regulation of inflammation. n-3 PUFA and n-6 PUFA appear to play opposing roles in this process. Caesar et al showed that n-3 PUFA dampen white adipose inflammation through a mechanism involving toll-like receptor activity and modification of gut microbiota composition (Caesar et al., 2015). As well as regulating the adipose immune system, n-3 PUFA also upregulate UCP-1 expression in both brown and white adipose tissue thereby influencing thermogenesis and energy metabolism (Kim et al., 2015).

Evidence from clinical trials is conflicting regarding n-3 PUFA supplementation, weight loss and the resolution of inflammation. These discrepancies may be due to supplementation dose, length and indeed inter-individual differences in underlying tissue PUFA status. Itariu et al reported that eight-week supplementation with fish oil in non-diabetic obese patients significantly reduced inflammation in adipose tissue and circulation (Itariu et al., 2012). Fish oil supplementation in obese pregnant women has also been shown to dampen adipose and placental inflammation (Haghiac et al., 2015). The effect of n-3 PUFA supplementation on absolute weight loss appears to be inconclusive,

however a meta-analysis reported that fat mass is significantly reduced in obese subjects following fish oil supplementation (Du et al., 2015).

Rheumatoid Arthritis

RA is an autoimmune disorder that involves uncontrolled infiltration of B-lymphocytes, T-lymphocytes and macrophages to the synovium surrounding joints, and hence over-production of inflammatory cytokines TNFα, IL-1β, IL-6 and IL-8. There have been a number of intervention trials using a wide range of n-3 PUFA doses, from 1-7g per person daily (Kremer et al., 1985, Kremer et al., 1987, van der Tempel et al., 1990, Lau et al., 1993, Cleland et al., 1988), that have resulted in significant improvements in qualitative clinical outcomes such as duration of morning stiffness, joint swelling and grip strength. Perhaps the most replicated improved outcome following supplementation is reduced use of non-steroidal anti-inflammatory drugs (NSAID) during PUFA supplementation (Calder and Zurier, 2001). A small number of meta-analyses assessing alleviation of RA symptoms following n-3 PUFA supplementation have been carried out (Fortin et al., 1995, Goldberg and Katz, 2007, Lee et al., 2012, MacLean et al., 2004), the details of which are summarised in Table 1. Goldberg and Katz (2007) analyzed 17 randomized controlled trials of 3-4 month PUFA supplementation and found standard mean differences of -0.26 for patient reported joint pain intensity; -0.43 for minutes of morning stiffness; -0.29 for number of painful and/or tender joints; and -0.4 for NSAID consumption. The meta-analysis of Fortin et al. (1995) found comparable evidence for reduction in number of tender joints and morning stiffness, whereas Maclean et al. (2004) reported conflicting conclusions that may be accounted for by inappropriate study inclusion and clinical outcomes. The most

recent meta-analysis by Lee et al. (2012) reported a standard mean difference of NSAID use as -0.518. Trends towards reduction in other outcomes such as morning stiffness and tender joint count were found but did not reach statistical significance.

Thus, much of this evidence warrants the use of omega-3 PUFA supplements in RA cases, but more research needs to be undertaken to examine dose response, as low-dose supplementation has been reported as having little effect (Remans et al., 2004). Additionally, further research is needed in order to understand which fatty acid, or combinations of fatty acids are most effective in treating RA.

Inflammatory Bowel Disease

IBD is a pathological autoimmune disorder that has been shown to react to dietary PUFA supplementation in animal models and some human clinical trials (Whiting et al., 2005, Belluzzi et al., 1996). IBD is a term to describe two disorders, Crohn's Disease and Ulcerative Colitis, and involves intestinal inflammation and can range widely in severity. Ulcerative Colitis occurs in the colon and involves inflammation of the intestinal mucosa. Crohn's disease, however, can occur at any part of the digestive tract from mouth to anus, and similarly is characterized by mucosal inflammation. Animal models of colitis are suitable for studying IBD and have shown promising results in terms of omega-3 PUFA supplementation and reduction of colonic damage and inflammatory processes in IBD (Whiting et al., 2005).

However, results from human clinical trials have been conflicting. A small number of randomized controlled trials show benefits of PUFA supplementation in

human IBD with regard to histological scoring and maintenance of IBD remission (Belluzzi et al., 1996), however the available systematic reviews and meta-analyses fail to conclude that supplementary PUFA have a profound effect (Table 2). Turner et al. (2011) have established that there is insufficient evidence to associate dietary omega-3 PUFA with maintenance of remission of Ulcerative Colitis and Crohn's Disease in a meta-analysis of 9 randomised controlled trials (Turner et al., 2011). Maclean et al. (2005) and Cabré et al. (2012) both conclude in systematic reviews that current evidence shows little benefit of dietary omega-3 PUFA in human IBD clinical trials (MacLean et al., 2005, Cabré et al., 2012). Similar to RA, supplementary dose may play a role in IBD, and hence higher prescribed doses may be more effective. Indeed, further research into the role of the microbiota in IBD may elucidate mechanisms behind therapeutic effectiveness of n-3 PUFA supplementation.

Other Diseases

Asthma is an inflammatory disease that involves broncho-constriction as a result of overproduction of inflammatory eicosanoids, and thus holds promise to be modulated by dietary fatty acid supplementation. However, systematic reviews of the literature have all concluded that current evidence is insufficient to make any recommendations regarding PUFA supplementation and asthma (Reisman et al., 2006), and that large randomised clinical controlled trials are necessary in order to analyse any associations. Promising evidence has been generated for the prevention and treatment of other inflammatory disorders such as diabetes, non-alcoholic fatty liver disease (NAFLD) (Parker et al., 2012) and cardiovascular disease (CVD) (Kris-Etherton et al., 2002), following omega-3 PUFA

supplementation. The evidence for the use of omega-3 PUFA supplementation in the prevention and treatment of certain inflammatory diseases is robust. However the question that remains to be answered refers to the effective dose of supplementation required to provide a therapeutic effect. Hence, it remains to be confirmed if omega-3 PUFA supplementation in inflammatory disorders is a nutritional or pharmacological solution (Calder, 2013).

3.1.4 Algal PUFA and Inflammatory Disease

Much of the aforementioned research employed the use of fish oil or fish-derived PUFA, but there are a growing number of trials examining the anti-inflammatory effect of algal oils and algal-derived PUFA. Rats fed an algal oil from *Schizochytrium* sp. containing n-6 DPA (16% of total fatty acids) and DHA (40% of total fatty acids) exhibited significantly reduced inflammation in a rodent paw edema model compared with control and compared with an oil from *crypthecodinium cohnii* which contained DHA (42% of total fatty acids) but no DPA n-6 (Nauroth et al., 2010). This result identified for the first time the anti-inflammatory potential of DPA n-6, but also the potential of a microalgal oil as an anti-inflammatory dietary supplement. Anti-inflammatory effects have also been observed in studies examining macroalgal PUFA. Khan et al. (2007) isolated EPA and stearidonic acid (SDA) from the seaweed *Undaria pinnatifida* and reported that these PUFA reduced phorbol myristate acetate (PMA) induced inflammation in mice when applied topically (Khan et al., 2007). The brown seaweed *Ishige okamurae* was shown to alleviate allergic inflammation by reducing histamine release and modulating inflammatory cytokine production in human basophilic cells (Vo et al., 2011) and the efficacious component was confirmed to be the

PUFA. Indeed maternal and lifelong n-3 deficiency or supplementation with an algal oil appeared to modulate the immune response in mice, an effect that appeared to be associated with changes to the gut microbiota (Robertson et al., 2016). A meta-analysis of eleven clinical trials found that algae-derived n-3 PUFA significantly reduced circulating triglycerides and increased both high density (HDL) and low density (LDL) lipoprotein cholesterol (Bernstein et al., 2012). Cholesterol metabolism is intrinsically linked with the immune system and plays an important role in chronic disease, notably inflammatory disease.

3.2. PUFA and Brain Health

3.2.1. Modulating Brain FA Composition

Perhaps one of the most important roles of dietary PUFA is in brain health, from brain and retinal development, to brain maintenance, to prevention of brain degeneration. Lipid content can reach up to 40% in the brain's grey matter, over 60% in white matter and up to 80% myelin (O'Brien and Sampson, 1965b), whereas the essential fatty acids i.e. LA, ALA and their longer chain derivatives such as AA and DHA, can make up to 20% of brain dry weight (Muskiet et al., 2006). The accumulation, distribution and interconversion of the various fatty acids in the brain varies over the lifespan (O'Brien and Sampson, 1965a) and hence specific PUFA play differing roles in brain development, maintenance and function.

3.2.2. PUFA and Brain Development

Rapid accretion of the fatty acids DHA and AA occurs in the foetal brain during the last trimester *in utero* and these fatty acids are vital in neurodevelopment (Clandinin et al., 1980). It has been shown that DHA and AA are preferentially transported across the placenta from mother to foetus in place of other fatty acids (Campbell et al., 1996, Haggarty et al., 1997). During growth and development DHA subsequently remains as the most abundant PUFA in the brain, making up approximately 8% of brain dry weight, with AA constituting up to 6% of dry weight (Muskiet et al., 2006).

Foetal fatty acid accumulation however, is dependent on the fatty acids available, and hence foetal brain fatty acid accretion is highly dependent on maternal diet. Elias et al. (2001) have shown that concentrations of fatty acids in maternal phospholipids at 35 weeks of gestation were significantly related to fatty acids present in infant plasma lipids at birth (Elias and Innis, 2001). Additionally, animal models of n-3 PUFA deficiency have identified the important role of these fatty acids in development and physiological homeostasis. Maternal DHA deficiency altered neurogenesis in embryonic rat brains, measured by thickness of the cortical plate (Coti Bertrand et al., 2006). Similarly, deprivation of n-3 PUFA in the maternal and infant diets of rhesus monkeys resulted in delayed retinal and neuronal development as measured by visual acuity (Neuringer et al., 1986).

PUFA deficiency in infants was found as a result of un-supplemented formula feeding compared with breastfed infants. Post-mortem fatty acid analysis was undertaken on infants' brains who were breastfed or formula fed (Farquharson et al., 1992) and results showed that DHA content of brains of breastfed infants was significantly higher than formula-fed infants. Additionally, pre-term and term

infants fed a long-chain omega-3 PUFA source or displaying higher erythrocyte DHA levels show better visual acuity development compared with those with lower DHA levels (Birch et al., 1992, Makrides et al., 1993). A meta-analysis has also associated breast-feeding with improved cognitive development when compared to formula fed infants (Anderson et al., 1999), an effect that is likely to be attributed in part to dietary DHA and other PUFA present in human milk. Hence, recommendations have followed that all infant formula be supplemented with long-chain fatty acids, specifically AA and DHA (Koletzko et al., 2005).

There is however conflicting evidence for the association between PUFA supplemented formula and cognitive development. A recent meta-analysis showed no improvement in cognitive development after 1 year in infants fed PUFA supplemented formula compared with unsupplemented formula (Qawasmi et al., 2012). These data matched results from previous meta-analyses (Simmer et al., 2008, Smithers et al., 2008) regarding PUFA supplementation of infant formula and cognitive development. Thus, further studies are required to determine optimum dose response for neurodevelopment, and the effects at different stages of development from foetus to infant.

Supplemented formulas primarily obtain PUFA from microalgal sources, as discussed below. This is partly due to the ability of microalgae to produce a specific fatty acid rather than a mixture, as is found in fish oil. Additionally, fish oil poses the risk of containing harmful levels of pollutants and contaminants such as mercury that can be harmful to infant health, hence algae provide a safer and more economical PUFA resource.

3.2.3. PUFA and Brain Maintenance

The maintenance of ordinary brain function is vital to prevent the onset of brain degenerative disorders during ageing. Depression and stress are both brain related disorders that have clinical and biochemical as well as psychological components (McEwen, 2011, Jacobs et al., 2000). These disorders have significant implications in the progression of neurodegeneration in later life, and hence by preventing depression and stress-related pathologies via dietary intervention, it is possible to slow or prevent brain degeneration (Jacobs et al., 2000).

Chronic and acute stress activates the hypothalamus-pituitary-adrenal (HPA) axis in the brain which stimulates the production of glucocorticoid hormones cortisol in humans and corticosterone in animals (Lupien et al., 2009). Regulation and modulation of this pathway can dampen the harmful response of stress on the brain and neuronal cells, and this can be achieved via a nutritional approach. Chronic stress can alter triglyceride and phosphatidylethanolamine omega-6/omega-3 ratio as shown in monkeys (Laugero et al., 2011), and hence suggests a relationship between fatty acid status and stress. In a recent study, omega-3 PUFA supplemented rats exhibited significantly lower HPA activation, corticosterone peak and stress-induced weight loss compared to control or omega-3 PUFA deficient rats following long-term induced stress by restraint (Hennebelle et al., 2012). Similarly, DHA deficiency at the pre-weaning period increased HPA activation through elevated serum corticosterone levels and increased anxiety-like behaviour following short-term induced stress in comparison to a DHA adequate diet, and in comparison to DHA deficiency at post-weaning (Chen and Su, 2013). These results suggest that dietary DHA adequacy is essential in alleviating short-term stress but also shows that DHA deficiency during brain development in early

life could lead to stress and anxiety in later life. Stress also includes an inflammatory component and this was shown in mice injected with the pro-inflammatory cytokine IL-1β, who subsequently displayed anxiety and stress-like behaviour (Song et al., 2008). Ethyl-EPA supplementation diminished stress-induced inflammatory factors such as PGE_2 and increased the anti-inflammatory cytokine IL-10. This was coupled with a reduced rise in corticosterone and reduced anxiety-like behaviour in comparison to animals supplemented with AA.

In humans, the effect of omega-3 fatty acid status and supplementation has been examined in relation to stress-associated inflammation. Stimulated production of TNFα and IFN-γ following psychological stress, an academic examination, was significantly lower in students with higher serum omega-3 PUFA levels, compared with those with low serum omega-3 PUFA levels (Maes et al., 2000). Similarly students supplemented with omega-3 PUFA displayed reduced production of LPS stimulated IL-6 production and reduced anxiety-like symptoms during a psychological stress, the day prior to an exam (Kiecolt-Glaser et al., 2011). Observational studies have also associated omega-3 PUFA status with psychological distress in Inuit women (Lucas et al., 2009).

Stress and anxiety are linked closely to depressive disorders and hence suggest a role for PUFA in treating depression. Depression is majorly underestimated in terms of its impact globally as a disease. According to the World Health Organisation, depression is the fourth leading cause of disease burden globally, and is expected to rise to second place by the year 2020, only behind heart disease (Murray and Lopez, 1996). Depressive symptoms can be alleviated through endocrine and inflammatory regulation in the brain among other

pathways and hence depression pathology is a complex process that is not full understood (Logan, 2003).

The consumption of fish, which contain high PUFA levels, is strongly inversely associated with rates of depression globally (Hibbeln, 1998), such that countries which consume the highest levels of fish, display the lowest rates of major depression. In randomised controlled trials, omega-3 PUFA supplementation has been shown to improve depressive symptoms, but similar to other disease pathologies, optimum PUFA supplementary dose is still to be realised. Peet and Horrobin (2002) undertook a dose response clinical trial of ethyl-EPA supplementation on depressed patients and found that 1g/day ethyl-EPA supplementation improved depression in those who were unresponsive to standard therapy, and this dose was more effective than 2g/day or 4g/day (Peet and Horrobin, 2002). However, clinical trials have displayed conflicting results (Bourre, 2005), for example depressed patients who received 2g/day DHA supplement for 6 weeks showed no improvement in depressive symptoms (Marangell et al., 2003). It has been suggested that EPA is more effective in treating depressive symptoms than DHA, but larger clinical trials are warranted to understand effective dose and length of supplementation (Logan, 2003).

The neuro-inflammatory component of depression is being realised and has been examined in cell culture (Lu et al., 2010) and observational human studies (Dinan et al., 2009). Dinan et al. (2009) demonstrated the association between PUFA status, inflammatory mediators and major depression by examining the fatty acid and cytokine profiles of plasma from depressed patients and controls (Dinan et al., 2009). It was shown that plasma AA and IL-6 levels were significantly higher in depressed patients compared with controls. In addition

EPA/AA ratio was significantly lower in depressed patients who did not respond to previous antidepressant treatment compared to controls. The authors concluded that major depression is associated with a proinflammatory phenotype and an elevated omega-6/omega-3 PUFA ratio. A meta-analysis of 10 double-blind placebo-controlled trials found a significant effect of omega-3 PUFA supplementation in treating clearly defined depression (effect size 0.69), however the authors cite a number of limiting factors such as publication bias and 'unblinding' due to fishy taste of supplements (Lin and Su, 2007). This meta-analysis also discusses the issue of dose and length of supplementation, and the need for larger randomised controlled trials.

3.2.4. PUFA and Neurodegeneration

Alzheimers disease (AD) is a form of dementia that involves degeneration of the brain in ageing, and currently has no cure. The psychological degeneration associated with AD is characterized by short-term memory loss and cognitive decline. Biological degeneration of the brain in AD is characterized by the formation of neural amyloid plaques, the accumulation of Tau protein, death of neuronal cells, neuro-inflammation, and hence alteration of synaptic plasticity. The impact and importance of this disease is increasing rapidly due to the increasing age of the population and it is expected that the number of people living with dementia will increase from 35.6 million in 2010, to 115.4 million in 2050 (Alzheimer's Disease International, 2009). As in early life and brain development, the intake of long-chain omega-3 PUFA in later life is also extremely important, in particular, PUFA have been shown to prevent the neurodegenerative processes that lead to AD, and hence may act as a nutritional or pharmaceutical prevention

and treatment. There are many mechanisms by which PUFA exert their effects on the ageing brain, one of which is through the regulation of inflammation. Inflammation is one of the primary components in the pathogensis of AD (Akiyama et al., 2000) and supplementary PUFA have been shown to dampen the inflammatory effect of AD-associated inflammation in cell, animal and human models (Orr et al., 2012). Much of this research has focused on DHA and EPA but a study by Kelly et al. (2009) showed that dietary n-3 DPA in aged rats downregulated microglial activation and activation of cell stress molecules such as caspase-3, suggesting that n-3 DPA has a role to play in the prevention of brain degeneration (Kelly et al., 2011). Additionally, the discovery of neuroprotectins, the DHA derived anti-inflammatory eicosanoids, suggest a role for these molecules in protection against AD (Lukiw et al., 2005). Aside from neuro-inflammation, PUFA can also regulate AD-related processes such as regulation of cholesterol and apolipoprotein E (ApoE), regulation of cell signalling and alteration of lipid raft assembly, as discussed by Boudrault et al. (2009) (Boudrault et al., 2009).

Data from human trials suggest an association between long term PUFA status and risk of Alzheimer's disease, but further research is needed to identify a conclusive role for supplementary PUFA in the prevention and treatment of AD. Participants in the highest quartile for DHA status in the Framingham Heart Study had a 47% reduced risk of dementia in a 9-year prospective follow-up study (Schaefer et al., 2006) compared with those in lower quartiles of DHA status. Similarly, higher fish consumption was associated with reduced cognitive decline over a 5-year period in individuals aged 70-89 (van Gelder et al., 2007). However, randomised controlled trials have thus far failed to present an effect of

supplementary PUFA in the treatment or prevention of AD and cognitive decline. No effect was observed on cognitive performance in patients with mild to moderate AD receiving 1.7g DHA and 0.6g EPA for 6 months, but a small significant improvement was observed in a subgroup of patients with very mild AD (Freund-Levi et al., 2006). Similarly, no significant effect of 2g algal DHA supplement for 18 months was observed in Alzheimer's patients with regards to improvements in cognitive performance (Quinn et al., 2010). Other studies have also reported no observable effect (Chiu et al., 2008). Meta-analyses have identified associations between PUFA status, fish consumption and the slowing of cognitive decline as well as improvement in mild cognitive impairment, but have failed to identify an association between PUFA intake and the treatment of AD (Fotuhi et al., 2009, Mazereeuw et al., 2012). These results suggest that the role of PUFA in AD progression may lie in n-3 PUFA adequacy or deficiency, but further large-scale clinical trials are required in order to find clearer evidence relating to PUFA dose, length of supplementation and stage of disease in AD.

3.2.5. Algal PUFA and Brain Health

Algal extracts have displayed therapeutic potential to reduce inflammation associated with brain degeneration. *Ecklonia cava*, a brown seaweed, showed anti-inflammatory and anti-oxidant effects in LPS-stimulated microglial cells by reducing NF-kB activation and inhibiting PGE_2, nitric oxide (NO) and COX-2 (Jung et al., 2009). Similarly, neuro anti-inflammatory effects have been observed in murine hippocampal and microglial cells following exposure to extracts from the seaweeds *Ulva conglobata* and *Neorhodomela aculeate* (Jin et al., 2006, Lim et al., 2006). Although it is unclear what component of these algae are causing the

anti-inflammatory effect, it is likely that PUFA play a role, and hence suggest that algal PUFA can help in the prevention of brain degeneration. Indeed recent studies have suggested that microalgal oil supplementation can prevent behavioural deficits in animals related to depression (Weiser et al., 2015). Furthermore, maternal and lifelong omega-3 supplementation with an algal oil has been shown to benefit early-life behavioural development related to communicative behaviour and cognition in mice (Robertson et al., 2016). These behavioural outcomes were also associated with dampeneing of the corticosterone response to stress, suggesting algal n-3 PUFA can dampen activity within the HPA-axis. A number of PUFA supplements marketed for brain health use algae as a source of EPA and DHA, such as Life'sDHA™ produced by DSM (www. lifesdha.com).

A recent study has shown the benefit of algae-derived DHA on memory function and learning in patients with age-related cognitive decline (Yurko-Mauro et al., 2010). A large trial involving 485 subjects examined the impact of dietary supplementation with 900mg/day of DHA derived from *Schizochytrium* sp. for 24 weeks. Patients taking the supplement showed a two-fold reduction in errors in visuospatial learning and memory tests compared with those taking placebo. Plasma DHA levels also significantly correlated with cognitive improvements, and hence showed a significant role for algal PUFA supplementation in the improvement of age-related cognitive decline.

4. THE EMERGING USE OF ALGAL PUFA

As PUFA are considered essential for early infant development (Fleith and Clandinin, 2005) and important for the prevention of chronic disease in later life, many national and international health organisations now provide daily intake

recommendations for PUFA in parallel with existing recommended daily intakes for essential nutrients. The World Health Organisation (WHO) recommends the intake of 2 portions of oily fish per week in order to obtain sufficient dietary EPA and DHA (200-500 mg) (FAO/WHO, 2003), and similar recommendations are provided by a number of health organisations throughout the world (Kris-Etherton et al., 2009).

There are a number of barriers to fish consumption, notably a rapid global decline in fish stocks (Branch et al., 2011, FAO Fisheries and Aquaculture Department, 2010), and similarly barriers to fish oil consumption such as undesirable taste and smell. Furthermore, increasing levels of toxic metals such as mercury have been detected in fish which if consumed over a long period of time can be detrimental to human health (Kris-Etherton et al., 2002). The realisation of the beneficial effects of PUFA in early infant development and disease prevention has led to a significantly increased demand for these lipids, primarily in the industrial production of infant formula. Thus there is a need for alternative sources of long-chain PUFA in order to meet commercial demands and health-related requirements and hence prevent the onset of chronic disease.

Macroalgae and microalgae present a vast and relatively untapped resource of marine fatty acids, thus providing an alternative source to declining fish stocks and a solution to increasing PUFA demand. Unlike fish, the thousands of different species of marine algae and microbes are capable of *de novo* production of long-chain PUFA (Ratledge, 2004). *De novo* production of PUFA occurs through a unique system as a result of specific enzymatic and genetic properties in certain species of microalgae (Metz et al., 2001). PUFA are subsequently obtained by fish followed by humans, via bioaccumulation through the food chain.

The potential of microalgae as direct sources of fatty acids is now being realised and is leading to the large-scale production of PUFA supplements (Ward and Singh, 2005) and research into their exploitation for biofuels (Hu et al., 2008). Production of lipids from microalgae and other microorganisms, known as single cell oil (SCO) production, was only proposed in recent times and is now being exploited at industrial levels (Ratledge, 2004). Algal PUFA production is more economical than biofuel production (Ratledge and Cohen, 2008) and hence a number of large-scale producers are now focusing on nutritional PUFA production as opposed to biofuels. *Crypthecodinium cohnii* is just one example of microalgal species currently cultivated and exploited at an industrial scale for the production of PUFA, primarily for the supplementation of infant formula (de Swaaf et al., 2003, Sijtsma and de Swaaf, 2004). A number of *Schizochytrium* species have been assessed for their PUFA production potential (Sijtsma and de Swaaf, 2004), and are also of increasing interest due to their ability to produce relatively high concentrations of other fatty acids such as n-6 and n-3 DPA (Hauvermale et al., 2006). There is also growing interest in algal production of PUFA other than DHA and AA. For example, microalgal production of EPA has significant importance because of its association with cardiovascular health, however until recent times EPA production from purified algal oil could not compete economically with that of fish oil (Belarbi et al., 2000). *P. tricornutum* (Yongmanltchal and Ward, 1992), *Nannochloropsis* sp. (Zittelli et al., 1999), and a number of other species can produce relatively high concentrations of EPA under specific conditions but must overcome issues of low specific growth rate and low cell density in order to reach industrial production potential. The realisation that heterotrophic microalgae are also potent producers of long-chain fatty acids expands the scope for industrial

PUFA production using different algal species without the dependence on light as an energy source (Perez-Garcia et al., 2011, Wen and Chen, 2003), as is the case with phototrophic species. In 2011, a group from the University of Göttingen determined the fatty acid profiles of over 2000 microalgae species and presented considerable variation in lipids between species (Lang et al., 2011). This data suggests that there is still much to be learned about the potential of the significant diversity of microalgae that exist for industrial PUFA production.

In macroalgae, lipid content is typically quite low (~2-5% of dry weight), but the proportion of these lipids that occur as PUFA can be relatively high (up to 70%) (Dawczynski, 2007). The ratio of omega-6 to omega-3 PUFA is also at an optimum dietary level in a number of macroalgal species (< 4:1) (van Ginneken et al., 2011, Pereira et al., 2012). Due to the low lipid content, cultivation of macroalgae for PUFA at an industrial level offers less potential than microalgae, however due to their broad content of functional compounds, macroalgae represent an emerging and beneficial product as whole foods in the functional foods market. Furthermore, there is an increasing interest in the genetic manipulation of land plants in order to produce long-chain n-3 PUFA (Napier and Sayanova, 2005, Qi et al., 2004). Further research into this area will require the close study and use of a number of macroalgae and microalgae species in order to identify specific genes and enzymes involved in PUFA synthetic pathways. With such a diverse range and large number of species yet to be examined, marine macroalgae, microalgae and certain microorganisms possess considerable potential for PUFA production and the prevention and treatment of chronic disease.

5. CONCLUSION

Dietary long-chain n-3 PUFA have significant beneficial effects on a number of chronic disorders associated with inflammation, and in addition are essential for neurodevelopment and the prevention of cognitive decline. However recommendations of PUFA intakes vary widely between countries, most likely due to the lack of clarity in PUFA dose and their therapeutic effect in a number of diseases. Conflicting evidence for the impact of n-3 PUFA supplementation in clinical trials may be due to ineffective doses or length of supplementation, or inter-individual differences in underlying PUFA tissue status. Hence, future research must consider underlying tissue PUFA composition in addition to the n-6/n-3 ratio as an important biomarker of PUFA status. Further research into the role of dietary PUFA on the gut microbiota may also elucidate a novel mechanism by which dietary lipids regulate the inflammatory response.

The role of algae as sources of PUFA must be investigated and applied further. The potential of algae as sources of PUFA and other bioactives is globally under-recognised, and further research and exploitation of these organisms holds promise for the benefit of human nutrition and pharmacology. In order to cope with projected increases in population and decreases in food availability, it will be vital to examine novel food sources such as algae. Hence, the incorporation of algae as functional foods in the western diet poses great potential to reduce chronic disease progression due in part to the biological potency of long-chain n-3 PUFA.

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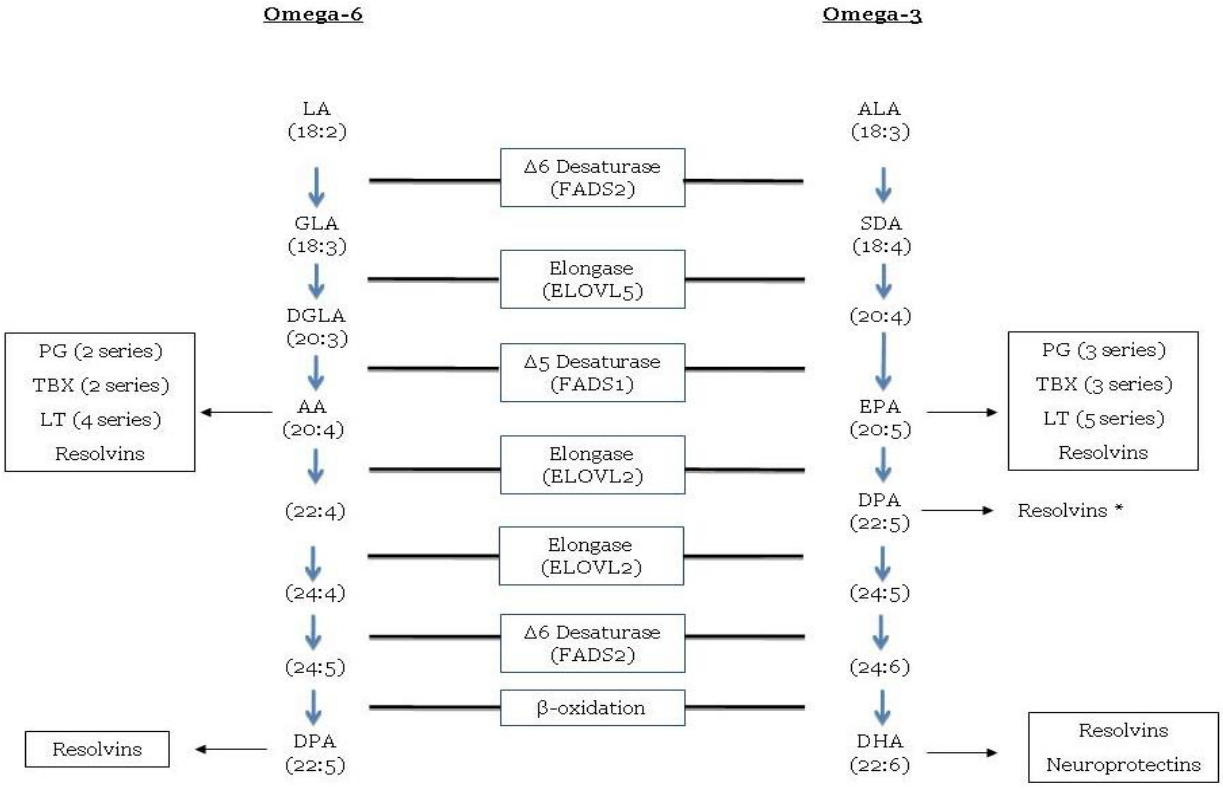
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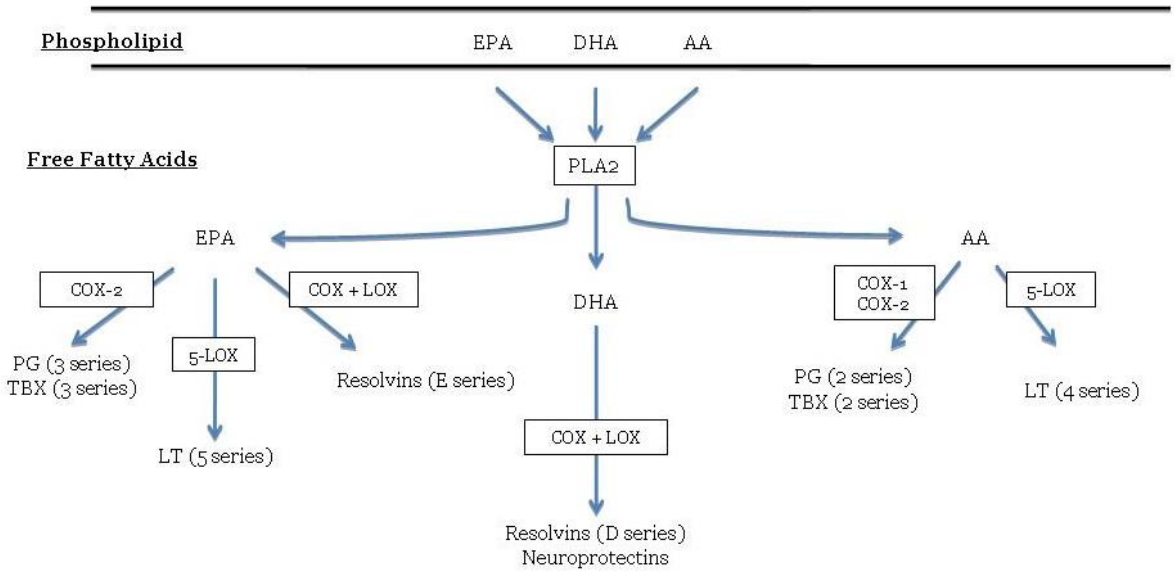
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FIGURES



Elaborated from (Patterson et al., 2012).
*It has been hypothesized that n-3 DPA may produce resolvin-like molecules (Kaur et al., 2011a).
Abbreviations: LA, Linoleic acid; GLA, Gamma linolenic acid; DGLA, Dihomo gamma linolenic acid; AA, Arachidonic acid; DPA, Docosapentaenoic acid, ALA, Alpha linolenic acid; SDA, Stearidonic acid; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; PG, Prostaglandins; TBX, Thromboxanes; LT, Leukotriene.

Figure 1. Metabolism of omega-3 and omega-6 PUFA in humans. Omega-3 and omega-6 PUFA metabolism involves a number of elongase and desaturase enzymes which are competitively inhibited by the substrates in each pathway.



Abbreviations: EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; AA, Arachidonic acid; PLA2, Phospholipase A2; COX, Cyclooxygenase; LOX, Lipoxygenase; PG, Prostaglandin; TBX, Thromboxane; LT, Leukotrienes.

Figure 2. Cyclooxygenase and lipoxygenase metabolism of AA, EPA and DHA.

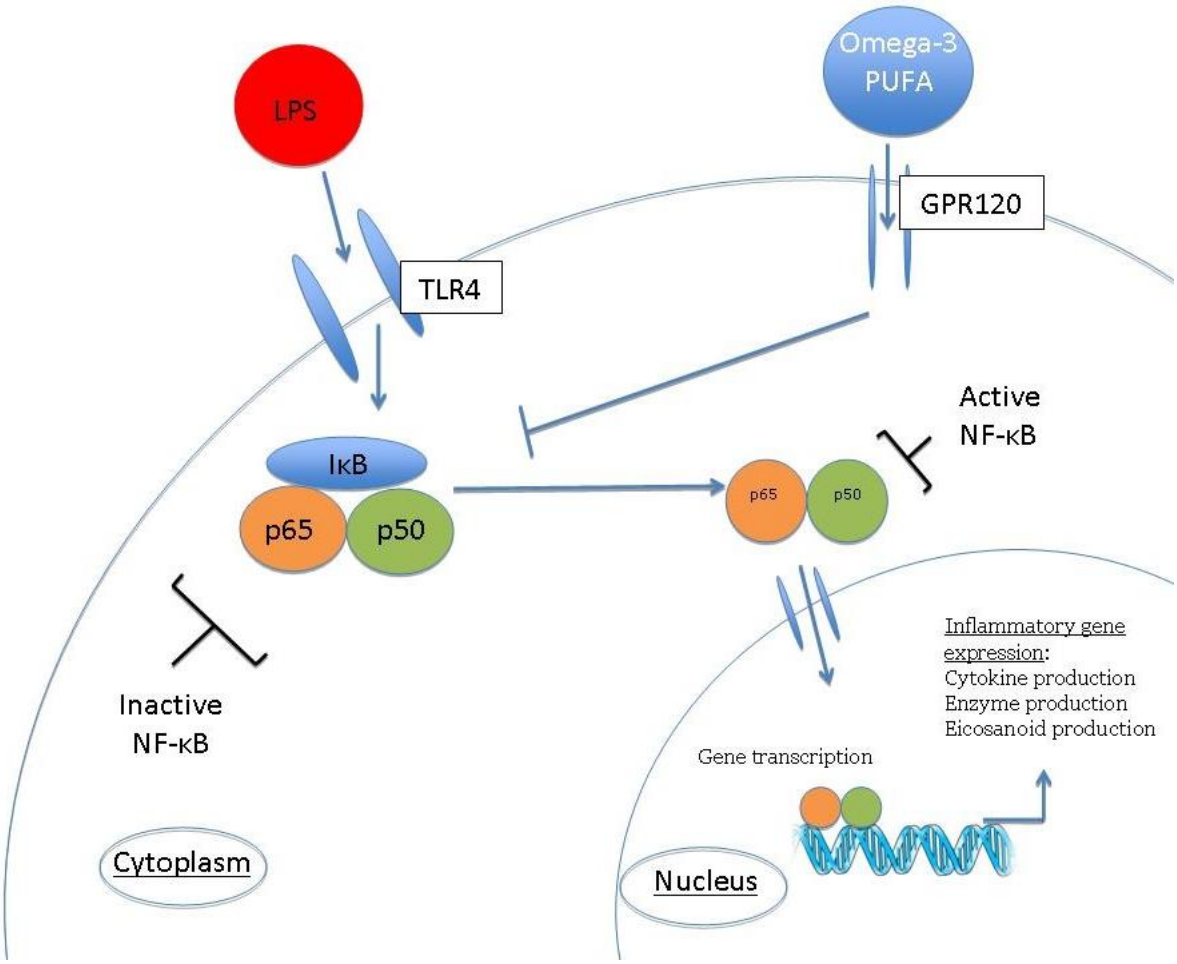


Figure 3. Process by which dietary PUFA regulate inflammatory response through activation of GPR120 and preventing the activation of NF-κB and subsequent gene transcription. The presence of LPS, a bacterial endotoxin, is detected by TLR4, which subsequently results in activation of the inactive cytosolic NF-κB, through phosphorylation and ubiquitination of the inhibitory IκB subunit. The active NF-κB then translocates to the nucleus where it acts as a transcription factor for a number of inflammatory genes. Inflammatory gene transcription leads to the up-regulation of genes involved in the expression of pro-inflammatory cytokines, eicosanoids and enzymes. The presence of omega-3 PUFA, such as EPA and DHA, in the cell prevents this process. Omega-3 PUFA activate the GPR120 receptor which prevents the activation of NF-κB and hence prevents the transcription and up-regulation of inflammatory genes.

TABLES

Table 1. Meta-analyses investigating the role of n-3 PUFA supplementation in rheumatoid arthritis

Meta-analysis	No. of studies involved	Primary outcomes	Conclusions/Comments
Fortin et al. (1995) (Fortin et al., 1995)	10	Significant reduction in: - Tender joint count (RD = -2.9) - Minutes morning stiffness (RD = -25.9) Non significant change in: - Swollen joint count - Grip strength - Patient/Physician global assessment - Visual analogue scale	'There was a modest, statistically significant improvement in tender joint count and morning stiffness in patients with RA treated with dietary fish oil supplementation for 3 months as compared with placebo control'
Goldberg and Katz (2007) (Goldberg and Katz, 2007)	17	Significant reduction in: - Joint pain intensity (SMD = -0.26) - Minutes morning stiffness (SMD = -0.43) - Painful/tender joint count (SMD = -0.29) - NSAID use (SMD = -0.4) Non significant change in: - Physician assessed pain - Ritchie articular index	'n-3 PUFAs are an attractive adjunctive treatment for joint pain associated with rheumatoid arthritis, inflammatory bowel disease, and dysmenorrhea'
Lee et al. (2012) (Lee et al., 2012)	10	Significant reduction in: - NSAID use (SMD = -0.518) Non significant reduction in: - Tender joint count - Swollen joint count - Minutes morning stiffness - Physical functioning - Pain - Physician/patient global assessment	'Use of omega-3 PUFAs at dosages of >2.7 g/day for >3 months may be effective at reducing NSAID use in RA patients. Additional studies are needed to explore clinical effects including the NSAIDs sparing effect of omega-3 PUFAs in RA' - Despite not reaching statistical significance, there was a trend for improved tender joint count, swollen joint count, morning stiffness and physical functioning.
MacLean et al. (2004) (MacLean et al., 2004)	9	Non significant reduction in: - Pain - Swollen joint count - Patient global assessment - Erythrocyte sedimentation rate A qualitative assessment found that 6 out of 7 studies reported a reduced requirement for anti-inflammatory drugs and corticosteroids during omega-3 PUFA supplementation	'The available evidence suggests that omega-3 fatty acids reduce tender joint counts and may reduce requirements for corticosteroids, but does not support an effect of omega-3 fatty acids on other clinical outcomes' The studies examined in this meta-analysis included a study of ALA supplementation and one which involved transdermal rather than dietary administration of PUFA. Inappropriate study inclusion and conclusions may affect the results and conclusions of this meta-analysis.

Table 1. Meta-analyses investigating the role of n-3 PUFA supplementation in inflammatory bowel disease

Meta-analysis/systematic review	No. of studies involved	Primary outcomes	Conclusions/Comments
Turner et al. (2011) Meta-analysis (Turner et al., 2011)	9	Maintenance of remission of IBD was significant for Crohns Disease patients during PUFA supplementation (RR = 0.77) however studies were heterogeneous. Exclusion of a small paediatric trial from the meta-analysis resulted in the RR no longer being significant. For Ulcerative Colitis there was no significant reduction in relapse rate following PUFA supplementation (RR = 1.02)	'The existing data do not support routine treatment with n-3 fatty acids for maintaining remission in IBD' This meta-analysis purely focussed on maintenance of IBD remission rather than improvement of clinical outcomes during disease state
MacLean et al. (2005) (MacLean et al., 2005) Systematic review	13	PUFA supplementation had a small significant improved effect on IBD clinical score in 3 out of 5 studies examined. Endoscopic score significantly improved in 3 studies following supplementation, but discrepancy in results of one study suggest a Type 1 or Type 2 error. Histological score did not change in 2 out of 3 studies following supplementation IBD remission and relapse were not significantly changed following supplementation Reduction in the use of immunosuppressive drugs varied widely across studies, but some showed reduced requirement for the drugs during PUFA supplementation	'The available data are insufficient to draw conclusions about the effects of n-3 fatty acids on clinical, endoscopic, or histologic scores or induced remission or relapse rates'
Cabré et al. (2012) (Cabré et al., 2012) Systematic review	19	Active ulcerative colitis appeared to improve following PUFA supplementation in 7/7 trials examining histological, endoscopic, steroid use and remission data. Inactive ulcerative colitis was not significantly changed following supplementation as measured by relapse rate in 4 trials. Two trials examining active Crohns disease identified no significant effect on remission following enteral supplementation, however one of these studies used solely ALA as the supplementary PUFA Six trials examined inactive Crohns disease by assessment of remission, where four of these trials showed no difference in remission rates between PUFA supplemented groups and placebo	'The available data do not allow to support the use of omega-3 PUFA supplementation for the treatment of both active and inactive inflammatory bowel disease' The authors note the potential effect of study heterogeneity on the inconclusive results alongside the limitations of a small number of randomized controlled trials and inappropriate study designs such as choice of placebo.

Chapter 1.2

Food for thought: The role of nutrition in the microbiota-gut- brain axis

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ABSTRACT

Recent research has provided strong evidence for the role of the commensal gut microbiota in brain function and behaviour. Many potential pathways are involved in this bidirectional communication between the gut microbiota and the brain such as immune mechanisms, the vagus nerve and microbial neurometabolite production. Dysbiosis of gut microbial function has been associated with behavioural and neurophysical deficits, therefore research focused on developing novel therapeutic strategies to treat psychiatric disorders by targeting the gut microbiota is rapidly growing. Numerous factors can influence the gut microbiota composition such as health status, mode of birth delivery and genetics, but diet is considered among the most crucial factors impacting on the human gut microbiota from infancy to old age. Thus, dietary interventions may have the potential to modulate psychiatric symptoms associated with gut-brain axis dysfunction. Further clinical and *in vivo* studies are needed to better understand the mechanisms underlying the link between nutrition, gut microbiota and control of behaviour and mental health.

1. INTRODUCTION

The microbial population residing in the small and large intestine represents the largest microbial population of the human microbiota. Estimates suggest that bacterial cells within the gut microbiota outnumber human eukaryotic cells by ten to one (Hamady and Knight, 2009). Moreover, the genes encoded by the gut microbiota, the gut microbiome, outnumber the human genome by one hundred to one (Qin et al., 2010). This complex ecosystem is formed mainly by bacteria, but also viruses, archae, protozoa and fungi. Due to the advances in genomic technologies it has been possible to unravel around 75% of the adult gut microbiota bacterial composition, which is predominantly composed of the Firmicutes and Bacteroidetes phyla (Collado et al., 2012). Furthermore, the gut microbiota plays a major role in host health by shaping the development of the immune system, metabolizing dietary nutrients (such as fatty acids, glucose and bile acids) and drugs, digesting complex indigestible polysaccharides and synthesizing vitamins and bioactive molecules (Lankelma et al., 2015).

Throughout different life stages, various changes occur in the microbial diversity of humans. Early studies suggested that the fetus first came in contact with microbes during birth. However, it has been posited that as early as the prenatal period, an initial inoculum of microbes may be translocated via the bloodstream and placenta from the mother to the fetus, thus contradicting the "sterile womb" hypothesis (Borre et al., 2014). After birth, the first colonizers of the gut are facultative anaerobes such as *Streptococcus*, *Enterobacteriaceae* and *Staphylococcus*. These colonizers consume oxygen, creating an anaerobic environment leading to an increase of *Clostridium*, *Bacteroides* and *Bifidobacteria*, which are strict anaerobes. During this early post-natal period, diet (breast milk/formula feeding) plays a key role in shaping the gut microbiota composition (Thum et al., 2012). This unstable infant gut microbiota with low diversity goes through a number of compositional changes during the first two years of life. From

the second year of life onward, the microbial composition undergoes an important shift toward the stable gut microbiota profile of the adult, which is composed mainly of *Bacteriodetes* and *Firmicutes*. During healthy adulthood the gut microbiota remains relatively stable until ageing, when considerable changes occur (Claesson et al., 2012).

The intestinal microbes are markedly affected by numerous factors such as host genetics, mode of delivery, lifestyle (urbanization and global mobility), medical interventions (use of antibiotics, vaccinations and hygiene) and health status (Burokas et al., 2015). Furthermore, diet has repeatedly shown to be one of the most important factors affecting gut microbiota establishment and composition throughout the lifespan (Lankelma et al., 2015). Indeed, more than 50% of the variation of gut microbiota has been related to dietary changes (Zhang et al., 2010) and major changes in diet during adulthood can modify the microbiota in a matter of days (David et al., 2014). Furthermore, an alteration of gut microbiota and metabolism, through dietary or other environmental influences, can cause a state of dysbiosis, which is characterized by an overgrowth of potentially pathogenic organisms (pathobionts) (Kamada et al., 2013). This change in the balance of symbionts/pathobionts can induce reduced intestinal barrier function (leaky gut) and subsequent chronic inflammation. Such dysbiosis may be associated with some metabolic and inflammatory disorders, visceral pain and even alterations to central nervous system functioning (DuPont and DuPont, 2011, Sekirov et al., 2010). Hence the relationship between the gut microbiota, chronic inflammation and the central nervous system (CNS) suggests that microbial dysbiosis could alter brain function and hence contribute to behavioural and cognitive abnormalities (Cryan and Dinan, 2012). A wealth of preclinical research is now showing potential for the treatment of dysbiosis, through dietary measures, to improve cognitive and behavioural outcomes.

Given such evidence, there is a growing appreciation for the importance of the gut microbiota in health and disease, including mental health. Bearing in mind that diet is one of the most crucial factors in the development of the human gut microbiota from infancy to old age, this review focuses on the role of the gut-brain axis in brain function and behaviour and the potential nutritional interventions to target this axis as psychiatric disease therapies.

2. How does diet influence human microbiota?

The human gut harbours over ten thousand species of microorganisms (Eckburg et al., 2005), hence such taxonomic diversity requires a wide array of nutrients and energy sources for normal microbial growth and function. Narrowing of host dietary diversity and reduced intake of essential nutrients can therefore reduce availability of substrates for specific microbial growth and contribute to intestinal dysbiosis. Over recent decades, modern dietary patterns have undergone major compositional changes, with increased intakes of red meat, high fat foods, and refined sugars. This 'Westernization' of diets together with sedentary lifestyles results in modifications to the gut microbiota, which may partially contribute to the higher incidences of chronic inflammatory disorders, such as cardiovascular disease, obesity, depression, allergies, diabetes and autoimmune disorders (Maslowski and Mackay, 2011). It is therefore clear that in order to improve the nutritional value of food and thus, human health, it is essential to understand the biological interactions between the diet and microbiota.

Many human studies have assessed dietary impact on the gut microbiota. However, as is the case with many human studies, they are limited by the difficulties to control potential confounding variables such as habitual diets and lifestyle behaviours. Moreover, it is worth noting that, typically, sequencing of the human gut microbiota is carried out on faecal samples, which may not accurately reflect the microbiota

composition of the different intestinal segments. Despite these limitations, much of this human data is useful to assess the role of varying dietary patterns on microbiota composition and function.

Rural vs Western diet

Many studies comparing rural and western communities have revealed specific gut microbiota adaptations to their respective environments. The adaptations to westernization have resulted in an important loss of several bacterial species, and hence subsequent reduction in microbial diversity and stability. Recent studies have clearly showed this reduction in microbiota diversity such as the one comparing an Italian urban control group compared to a hunter–gatherer community (Schnorr et al., 2014). Moreover, recent investigations have reported the impact of diet on the microbial biodiversity within different human populations (De Filippo et al., 2010). African children, who consume a low-fat and high-fibre diet, presented less potentially pathogenic bacteria and greater degree of diversity and microbial richness than European children consuming a high-fat diet (Western diet). African children had a depletion in *Firmicutes* and a greater abundance of the phylum *Bacteroidetes* (*Xylanibacter* and *Prevotella*), while European children showed a significant increase of *Firmicutes* (*Feacalibacterium* and *Acetitiomaculum*) and *Enterobacteriaceae* (*Shigella* and *Escherichia*) (De Filippo et al., 2010). Similar findings were observed in terms of an increase of *Prevotella* genus in rural African populations compared to US Americans (Ou et al., 2013).

Mediterranean diet

The Mediterranean diet is characterised by an abundance of fruits, vegetable, grains and monounsaturated or n-3 polyunsaturated fats. Hence it is regarded as the

gold-standard for optimum health. A recent study showed the ability of a Mediterranean-inspired anti-inflammatory diet to reduce inflammation in Crohn's disease. The results demonstrated a small reduction of the acute phase protein C-reactive protein (CRP), an increase in *Bacteriodetes* and *Clostridium* clusters and a decrease in *Proteobacteria* and *Bacillaceae* population (Marlow et al., 2013). Similarly De Filippis et al recently observed that Italian subjects with a high adherence to a Mediterranean diet had greater abundance of *Prevotella* and short chain fatty acids. Conversely, those with low adherence had higher urinary trimethylamine oxide (TMAO), which has associations with gut dysfunction, cardiovascular disease and colorectal cancer (De Filippis et al., 2015).

Vegetarian/vegan diets

Vegetarian diets have also gained recognition as a healthy and therapeutic dietary pattern for a number of chronic diseases, while vegan diets may confer protective benefits beyond that of vegetarian diets (Glick-Bauer and Yeh, 2014). Vegan diets may have protective effects against metabolic and inflammatory diseases. Moreover, they appear to lead to a unique gut microbiota profile characterized by a reduction of pathobionts (Glick-Bauer and Yeh, 2014). Some studies have shown that vegetarian and vegan diets significantly decrease microbial counts of *Bacteroides fragilis* compared to an omnivore diet (Ferrocino et al., 2015). Another study comparing vegetarian to omnivore diet observed a higher ratio (%) of *Bacteroides–Prevotella*, *Bacteroides thetaiotaomicron*, *Clostridium clostridioforme* and *Faecalibacterium prausnitzii* but a lower ratio (%) of the *Clostridium cluster XIVa* in vegetarian diet (Matijašić et al., 2014).

High-fibre diets

Numerous studies support the idea that diets rich in plant fibres may promote the diversification of the microbiota by promoting hydrolytic bacteria and stimulating the production of short chain fatty acids (De Filippo et al., 2010). High-fibre diets have been positively associated with *Actinobacteria* and *Bacteriodetes* abundance (Wu et al., 2011). One study showed that three diets with different fibre-rich whole grains (barley, brown rice or combination of both) increased microbial diversity, the *Firmicutes*/*Bacteroidetes* ratio, and the abundance of the genus *Blautia* in fecal samples (Martínez et al., 2013). Furthermore, the administration of whole grain barley induced an increase in *Bifidobacteria* which is considered a positive indicator of prebiotic activity (Carvalho-Wells et al., 2010). A recent study found an elevation of *Bifidobacteria* and a reduction of *Bacteroides spp*. and *Clostridium histolitycum* group in a cohort of overweight adults after administration of prebiotics (GOS) (Vulevic et al., 2013). Davis et al showed as well that an administration of GOS increased abundance of *Bifidobacteriaceace* and decreased *Bacteroidaceae* (Davis et al., 2011).

High-fat diets

Over the last few decades, the increase in the consumption of high-fat diets has been associated with the obesity epidemic (Torres-Fuentes et al., 2015). Many studies have shown that high-fat diets lead to a decrease in *Bacteroidetes* and an increase in *Firmicutes* (Murphy et al., 2015). These effects may be associated with increased gut permeability, a higher capacity for energy harvest and storage, and inflammation (Murphy et al., 2015). Several studies have focused on dietary supplementation as a possible way to attenuate the gut microbiota dysbiosis and metabolic impairments produced by high-fat diets. For example, polyphenols, conjugated linoleic acid and short chain fatty acids supplementation during high-fat diet consumption, have

displayed an improvement of gut microbiota dysbiosis (den Besten et al., 2015, Roopchand et al., 2015, Chaplin et al., 2015).

High-protein diets

The western diet has experienced a considerable increase in protein content in recent times. This has led to much research examining variations in macronutrients intake in order to manage body weight (Johnstone, 2012). Dietary proteins undergo luminal proteolysis and subsequent metabolism by the large intestine microbiota triggering the production of numerous amino acid-derived metabolites such as phenols, indoles, amines, sulphide, ammonia and monocarboxylic acids (Nyangale et al., 2012).

Dietary protein intake in humans has been positively associated with the *Bacteroides* enterotype (Wu et al., 2011). An animal-based diet in humans showed an increase in the abundance of bile-tolerant microorganisms (*Alistipes*, *Bilophila* and *Bacteroides*) and a decrease in the levels of *Firmicutes* that metabolize dietary plant polysaccharides (*Roseburia*, *Eubacterium rectale* and *Ruminococcus bromii*) (David et al., 2014). Interestingly, Clarke et al. showed the importance of exercise in the relationship between the microbiota, host immunity and host metabolism, and the important role played by the diet. They compared male elite professional rugby players to healthy male controls, finding a positive correlation between protein consumption with microbial diversity (Clarke et al., 2014).

3. Gut-Microbiota-Brain Communication

As previously discussed, diet significantly modifies host gut microbiota composition and function. Simultaneously, however, gut microbiota determine what the

host is capable of extracting from its diet, from nutrients to bioactive signalling molecules such as neurometabolites, vitamins and short-chain fatty acids (SCFA) (Tan and O'Toole, 2015). Many of these molecules such as serotonin and gamma-aminobutyric acid (GABA), have neuro-active functions due to their capacity to modulate neural signalling within the enteric nervous system and consequently influence brain function and host behaviour (Wall et al., 2014). This gut-brain axis, the bidirectional communication system between the gastrointestinal system and the CNS, plays an important role in homeostasis between neural (both enteric and central nervous systems), hormonal and immunological signalling (Cryan and Dinan, 2012). Through this complex network the gut can influence the brain via visceral messages, and conversely, the brain is able to influence gastrointestinal functions (like motility, secretion and mucin production) and immune functions, such as the modulation of cytokine production by cells of the mucosal immune system (Tracey, 2009).

Both luminal nutrients and gut microbiota metabolites stimulate enteroendrocrine cells (EECs) located throughout the gastrointestinal (GI) tract, which represents the largest endocrine organ in the human body (Rehfeld, 2003). These EECs contain most of the nutrient receptors such as those for amino acids, peptones, SCFAs, long-chain fatty acids (LCFAs) and oleoylethanolamide (OEA) (Figure 1). Molecular sensing by these EEC's are crucial in the control of multiple functions during digestion, the initiation of neural and hormonal responses or changes in mucosal ion transport which controls appetite, insulin secretion and motility (Furness et al., 2013). Moreover, as the nervous and endocrine signalling between the gut and the brain is essential for the modulation of many GI functions, the sensing receptors of the gut that control the release of many hormones play a key role (Figure 1). Several interacting factors such as diet and microbiota composition modulate the activation of different sensory receptors in the gut, and consequently stimulate up or down-regulation of hormonal release,

which can induce a number of functional GI changes. Interestingly, increasing evidence indicates that animals fed on a high-fat diet present numerous changes in gastrointestinal function, particularly in the secretion and signalling of gastrointestinal hormones, which may predispose to an increase in energy intake, and consequently, to weight gain and obesity (Little et al., 2007).

In addition to its role as a sensory organ, the gut forms part of the enteric nervous system, which makes up a comprehensive division of the autonomic nervous system, containing between 200 and 600 million neurons (Furness et al., 2013). The vagus nerve (the major nerve of the parasympathetic division of the autonomic nervous system) is crucially involved in bidirectional signalling between the gastrointestinal and nervous systems (Figure 2). A landmark study by Bravo et al (Bravo et al., 2011) found that probiotic modulation of the gut microbiota induced behavioural and neurochemical changes in mice. However, this was not apparent in mice that had undergone vagotomy suggesting a crucial role for the vagus nerve in the gut-brain axis.

The human intestine also acts as an endocrine organ through direct and indirect production of microbial metabolites and neurometabolites such as short chain fatty acids (SCFAs), vitamins and neurotransmitters, which have also been shown to influence gut-brain interactions (Burokas et al., 2015). GABA and serotonin are neurotransmitters that can influence host behaviour and are produced directly or indirectly by certain commensal microbes (Barrett et al., 2012, Reigstad et al., 2015). SCFAs including butyrate, propionate and acetate can be produced by species such as *Roseburia spp* and *Faecalibacterium* following fermentation of indigestible polysaccharides (Brestoff and Artis, 2013). Butyrate and propionate can modulate brain functioning, in particular appetite regulation and energy homeostasis (Byrne et al., 2015) through regulation of neuropeptide production.

The role of the gut microbiota in immune activation also has strong associations with neurological functioning. The gut microbiota regulate intestinal epithelial barrier integrity and hence control the translocation of viable bacteria or bacterial endotoxins into the bloodstream (Bischoff et al., 2014). Increased intestinal permeability can lead to increased lipopolysaccharide (LPS) in the bloodstream, which increases inflammatory status. Diet and obesity significantly alter gut microbiota composition and hence have been shown to affect inflammatory status. Cani et al showed that prebiotic supplementation dampened inflammatory status and improved gut barrier function in genetically obese mice (Cani et al., 2009). Many supporting studies have demonstrated the potential for high fat and other obesogenic diets to promote inflammation and microbiota-targeted interventions, such as prebiotics and probiotics to reverse inflammatory status (Everard et al., 2014). Chronic inflammation has been linked to a number of neurological disorders including depression and dementia (Yong, 2010) and hence microbiota-associated chronic inflammation may influence risk of such disorders.

Due to the fact that many of these gastrointestinal pathways significantly influence neurological function, there is potential for dietary interventions that increase bacterial metabolism and promote growth of beneficial bacteria, to positively modulate the gut-brain axis and improve symptoms of psychiatric illness. Moreover, bearing in mind the potential link between the gut microbiota and anxiety-related behaviour (Burokas et al., 2015), research has recently focused on the health benefits of probiotic administration on psychiatric illnesses (Dinan et al., 2013b).

4. Microbiota-targeted dietary interventions and behavioural outcomes

The gut microbiota have been implicated in a number of clinical neuropsychiatric disorders (Cryan and Dinan, 2012). Also, the role of nutrition in the

aetiology and treatment of psychiatric disorders has come to light in recent times (Sarris et al., 2015). The development of next generation sequencing technologies has also allowed for increased understanding of human gut microbial composition in healthy and disease states and how environmental factors such as diet influence this composition.

Probiotic interventions

A number of studies have reported certain probiotic strains, primarily *Lactobacillus* and *Bifidobacteria,* to enhance brain function in both rodents and humans. Hence there is potential for 'psychobiotics' (live organisms that, when ingested, confer a benefit to host psychiatric health) to modulate the gut microbiota and act as therapies for psychiatric disorders (Dinan et al., 2013a). Table 1 summarizes a non-comprehensive list of human and preclinical studies investigating the role of probiotics in behaviours associated with psychiatric illness. Despite the promising evidence in animals, these results have yet to be fully translated into humans. However with larger randomised controlled trials, there is potential for psychobiotics to be effective psychiatric therapeutics.

Dietary interventions

Many studies have shown a clear association between the gut microbiota and behavioural alterations, and given that gut microbiota is affected by diet, the composition of the diet may be a crucial factor contributing to these behavioural changes, as summarized in table 2.

Large macronutrient alterations as reflected in western style diets have been shown to induce microbial dysbiosis, which has been linked to impaired cognition. Magnusson et al examined microbiota compositional changes following high fat, high sucrose or standard chow diets and assessed associations with cognitive capabilities in

mice (Magnusson et al., 2015b). *Lactobacillus* was significantly increased in the high sucrose group whereas *Erysipelotrichales* was significantly increased in the high fat group. High fat and high sucrose both had increased *Coriobacteriales* and reduced *Bacteroides*. Both high-energy treatments induced impaired cognition in the Morris water-maze and step-down latency tasks. In addition these behavioural changes displayed significant correlations with the alterations in *Lactobacillus*, *Erysipelotrichales*, *Coriobacteriales* and *Bacteroides*. These results suggest that the cognitive changes induced by the western-style diets are mediated through alterations to the gut microbiota. Jørgensen et al performed a similar study with the same treatment groups and found similar correlations between microbiota alteration and memory which was also associated with inflammatory status (Pyndt Jørgensen et al., 2014).

Li et al also reported diet-induced changes to microbial diversity to improve cognition and working memory in mice. In this study, a meat-containing diet led to greater gut microbiota diversity than found in the control diet group. Moreover, the meat-containing diet group had improved working and reference memory on the hole-board open field test and less anxiety-like behaviour, assessed during the novel encounter in the hole-board open field (Li et al., 2009a).

Mental decline is increased by obesity, which may be in part regulated by gut microbiota dysbiosis. A recent study showed that an obese-type microbiota, induced by high-fat feeding, induced cognitive disruptions when transplanted into healthy rodents (Bruce-Keller et al., 2015b). The high-fat diet microbiota led to a significant increase of anxiety-like behaviour in the elevated plus maze, open field, and marble burying test. Moreover, this transfer of the high-fat diet microbiota decreased the cued fear memory in comparison to mice that received microbiota from chow-fed mice. Furthermore, inflammatory markers in the medial prefrontal cortex and intestinal permeability were increased in the mice who received the high-fat diet microbiota, suggesting that immune

signalling pathways may be key mediators of microbiota-brain communication. These interesting results revealed that even in the absence of obesity, an obese-type microbiota profile could induce behaviour deficits similar to those seen in obesity and hence suggesting the potential for microbiota-based dietary interventions to treat obese-associated psychiatric disorders. In addition, Ohland et al reported a western-style diet to induce anxiety-like behaviour in mice, as assessed in the Barnes maze (Ohland et al., 2013b). Moreover, western-style diet feeding increased the *Firmicutes*/*Bacteriodetes* ratio, and the abundance of *Proteobacteria* and *Spirochaetes* as well as reducing total SCFA contents. This increased anxiety-like behaviour was not apparent in mice who had been fed a western-style diet supplemented with the probiotic *Lactobacillus helveticus*. Another study found that mice on a high-fat diet presented major shifts in the gut microbiota (increase of *Firmicutes* and decrease of *Bacteroidetes* and *Tenericutes*) and a robust anxiety phenotype (Kang et al., 2014). A diet deficient in Mg, increased depressive-like behaviour and altered the gut microbiota, which suggested that magnesium deficiency could be a mediator of the behavioural effects through an altered gut microbiota (Winther et al., 2015). Interestingly, a significant correlation was found between the gut microbiota of the diet deficient in Mg and a decrease in hippocampal IL-6 levels, suggesting that this immune-modulation could be the mechanism by which diet induced changes in the gut microbiota composition alter behaviour (Winther et al., 2015). A similar study found that a diet deficient in Mg decreased bacterial diversity and altered anxiety-like behaviour (Pyndt Jørgensen et al., 2015).

Certain prebiotics have the potential to influence central nervous system functioning through stimulation of specific microbial growth and production of SCFAs. Tarr et al reported that a social disruption stressor significantly altered gut microbiota composition in mice, which resulted in anxiety-like behaviour and a reduction in the growth of neurons in the denate gyrus region of the hippocampus (Tarr et al., 2015).

Interestingly, supplementation of the human milk oligosaccharides 3′Sialyllactose or 6′Sialyllactose, which have anti-inflammatory properties and stimulate bifidobacterial growth, prevented the stressor-induced alterations to the gut microbiota, in addition to preventing the behavioural, microbial and neurophysical defects (Tarr et al., 2015).

A number of small clinical controlled trials have assessed the efficacy of certain prebiotics on psychological outcomes with promising results. Schmidt et al demonstrated that 3-week supplementation with a GOS prebiotic, which has been shown to stimulate *bifidobacteria* growth, in healthy volunteers significantly reduced waking cortisol response, a stress hormone strongly linked to anxiety and depression (Schmidt et al., 2015). Moreover, a Bimuno®-galactooligosaccharides cohort demonstrated altered behavioural outcomes through a decrease in attentional vigilance to negative versus positive information in a dot-probe task compared to placebo. It is interesting to note, however, that fructooligosaccharide (FOS) supplementation had no effect. These results suggest that shaping of microbiota composition through prebiotic intake could influence behavioural outcomes (Schmidt et al., 2015). In humans, prebiotic supplementation with *trans*-galactooligosaccharide (trans-GOS) not only enhanced bifidobacterial growth and improved bloating symptoms, but in addition significantly reduced anxiety scores in IBS sufferers (Silk et al., 2009).

Conclusions and future implications

It is evident that there are a number of major metabolic, endocrine and neural pathways connecting the gut and the brain. Indeed, the trillions of microbes and microbial by-products within the gut contribute to the plasticity of these pathways. Despite the rapid growth of this area of research, it is still in its infancy. Relatively little is known about the extent to which bacterial metabolites can influence brain function, something which could be addressed with further advances in metabolomic

technologies. In addition, the complexity of the pathways involved in the gut-brain axis contributes to the difficulty of identifying true mechanisms of action. Moreover, the role of individual nutrients to affect signalling within these pathways requires further examination. Sequencing technologies have grown extensively in recent times allowing deeper insight into gut microbial composition and associations between altered microbiota and psychiatric illnesses. Further research in this field should address mechanistic evidence for gut microbiota to alter brain and behaviour. The majority of the data available is preclinical and few of these promising studies have been translated into humans, which warrants the need for more clinical trials in the area. There are very little data reporting clinical interventions targeting the microbiota in psychiatric illness. Indeed, diet has a significant impact on the microbiota and hence dietary interventions can beneficially modulate microbial diversity and function. Caution must be taken on assigning the term 'probiotic' to a specific strain of bacteria until its health effects can be replicated in both humans and animals. Indeed, commercial availability of true 'psychobiotics' (a live bacteria that may benefit mental health) will only become apparent after rigorous human trials. Prebiotics and other larger dietary interventions, including dietary fats and polyphenols also pose potential to alter the gut-brain axis and hence neuropsychiatric disorders, and may be feasible as long-term interventions for mental health.

In conclusion, diet-induced gut microbiota modifications may be associated with brain dysfunction, behavioural and metabolic deficiencies. The emerging evidence of a microbiota-gut-brain axis dysregulation in certain neuropsychiatric disorders warrants further clinical and *in vivo* studies to investigate gut microbiota-targeted interventions as novel therapeutic strategies. Indeed, dietary interventions to treat dysfunction of the gut-brain axis may pose potential as therapeutic strategies for psychiatric disorders.

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Table 1. Probiotic interventions and behavioural outcomes

Intervention	Species	Health status	Microbiota changes	Behavioural/neurochemical outcomes	References
Lactobacillus casei	Humans	Healthy	——	↑ mood (self reported) ↓ memory	(Benton et al., 2007)
Bifidobacteria longum	Mice	Healthy anxious strain (BALB/c)	——	↑ memory and cognitive performance (novel object recognition, barnes maze, fear conditioning)	(Savignac et al., 2015)
VSL#3	Rats	Aged	↑Bacteroidetes	↓ deficit in age-related LTP ↓ microglial activation ↑BDNF and synapsin	(Distrutti et al., 2014)
Lactobacillus helveticus	Mice	Healthy or fed western-diet	Normalized the increase in proteobacteria following "western diet" feeding	↑ memory (Barnes maze) ↓ anxiety-like behaviour (Barnes maze)	(Ohland et al., 2013a)
Bacteroides fragilis	Mice	MIA treated	Restored relative abundance of lachnospiraceae following MIA treatment	↓ anxiety-like behaviour (Open field) ↑ communication (ultrasonic vocalization) ↓stereotyped behaviour (marble burying)	(Hsiao et al., 2013)
Lactobacillus casei	Humans	Chronic fatigue syndrome	↑Bifidobacteria ↑Lactobacillus	↓anxiety	(Rao et al., 2009)
Lactobacillus helveticus and *bifidobacterium longum*	Rats	Healthy	——	↓anxiety (conditioned defensive burying)	(Messaoudi et al., 2011)
	Humans	Healthy	——	↓anxiety	
Bifidobacteria infantis	Rats	Healthy	——	↓proinflammatory immune response ↑ tryptophan	(Desbonnet et al., 2008)
Lactobacillus rhamnosus and *bifidobacterium animalis*	Humans	Schizophrenic	Microbiota data not reported however probiotic group significantly less likely to experience severe bowel	No observed differences	(Dickerson et al., 2014)

			difficulty		
Bifidobacterium infantis	Rats	Healthy normosensitive (Sprague-Dawley) and healthy hypersensitive (Wistar-Kyoto)	——	↓visceral pain (colorectal distension)	(McKernan et al., 2010)
Lactobacillus rhamnosus	Mice	Healthy anxious strain (BALB/C)	——	↓corticosterone, anxiety behaviour, depressive behaviour. Altered GABA receptor expression	(Bravo et al., 2011)

Abbreviations: MIA, maternal immune activation; LTP, long-term potentiation; GABA, gamma-aminobutyric acid

Table 2. Microbiota-targeted dietary interventions and behavioural outcomes

Diet	Species	Intervention length	Microbiota changes	Behavioural outcomes	Biochemical outcomes (possible mechanisms)	Reference
High-fat diet	Mice	13weeks	↑Firmicutes (mainly Ruminococcaceae and Lachnospiraceae) ↓ Bacteroidetes (S24-7)	↓ Burrowing (Burrowing Test) ↓ Memory (Morris water maze test)	No difference between diet groups was observed for sucrose preferences, LPS, cholesterol, HbA1c, BDNF and the cytokines IL-1α, IL-1β, IL-6, IL-10, IL-12(p70), IL-17 and TNF-α. Low-grade levels of the systemic inflammatory mediators IL-6, IL-12p70 and IL-17A correlated to memory, anxiety, anhedonia and species-typical behaviour	(Jørgensen et al., 2014)
High-fat diet	Mice	2-5weeks	↑Clostridiales and Erysipelotrichales ↓ Bacteroides	No significant differences from the control mice, except for remaining focused on the old platform position during the reversal probe trial	——	(Magnusson et al., 2015a)
High-sucrose diet	Mice	2-5weeks	↑Clostridiales, Lactobacillus (Enterococcus, Lactococcus and Lactobacillus) and Lactococcus ↓ Bacteroides	↓ Learning (Morris water maze test) Cognitive deficits (in spatial short-term memory) Impairments in early development of a spatial bias for long-term memory, short-term memory and reversal training, compared to mice on normal diet.	——	(Magnusson et al., 2015a)

Diet deficient in Magnesium (MgD)	Mice	6weeks	Principal Component Analysis plots illustrating differences in GM composition. The GM profile of MgD mice differed significantly from mice fed a standard control diet	↑ FST- Increased immobility (depressive-like phenotype)	Strong tendency towards decreased mRNA IL-6 levels in the MgD mice. The GM of MgD mice correlated significantly to hippocampal IL-6 levels	(Winther et al., 2015)
Diet deficient in Magnesium (MgD)	Mice	6weeks	↓ Bacterial diversity of the gut Principal component analysis plots illustrating differences in GM composition between mice fed a control diet or an MgD diet	Altered anxiety-like behaviour (↓ Latency to enter the light compartment in the Light Dark Box test)	——	(Pyndt Jørgensen et al., 2015)
Meat-containing diets	Mice	3months	↑ Bacterial diversity	↑ Working and reference (temporary and long-term) memory. ↓ Anxiety-like behaviour	——	(Li et al., 2009b)
Western-style diet high in fat	Mice	21 days	↑ Firmicutes/Bacteriodetes ratio ↑ Abundance of Proteobacteria and Spirochaetes.	Altered anxiety-like behaviour	↓ total levels of SCFAs in cecal contents ↓ levels of acetic, propionic and butyric acids ↑ levels of caproic acid)	(Ohland et al., 2013a)
High-fat diet (fecal transplantation from donors on high-fat diet)	Mice	10weeks	Sequencing-based phylogenetic analysis confirmed the presence of distinct core microbiota between groups, with alterations in α- and β-diversity, modulation in taxonomic distribution, and	Disrupted exploratory, cognitive, and stereotypical behaviour	Disrupted markers of intestinal barrier function ↑ circulating endotoxin ↑ lymphocyte expression of ionized calcium-binding adapter molecule 1 ↑ toll-like receptor 2 ↑ toll-like receptor 4	(Bruce-Keller et al., 2015a)

			statistically significant alterations to metabolically active taxa			
High-fat diet	Mice	8weeks	↑ Firmicutes ↓ Bacteroidetes and Tenericutes	Robust anxiety phenotype	——	(Kang et al., 2014)
Diet supplemented with prebiotics (trans-GOS)	Human	8weeks	Enhanced bididobacteria	Improved anxiety (HAD escale)	——	(Silk et al., 2009)

Abbrevitations: HAD, Hospital Anxiety and Depression scale; SCFAs, short-chain fatty acids; GM, gut microbiota; LPS, Lipopolysaccharide ; HbA1c, hemoglobin A1c ; BDNF, brain-derived neurotrophic factor

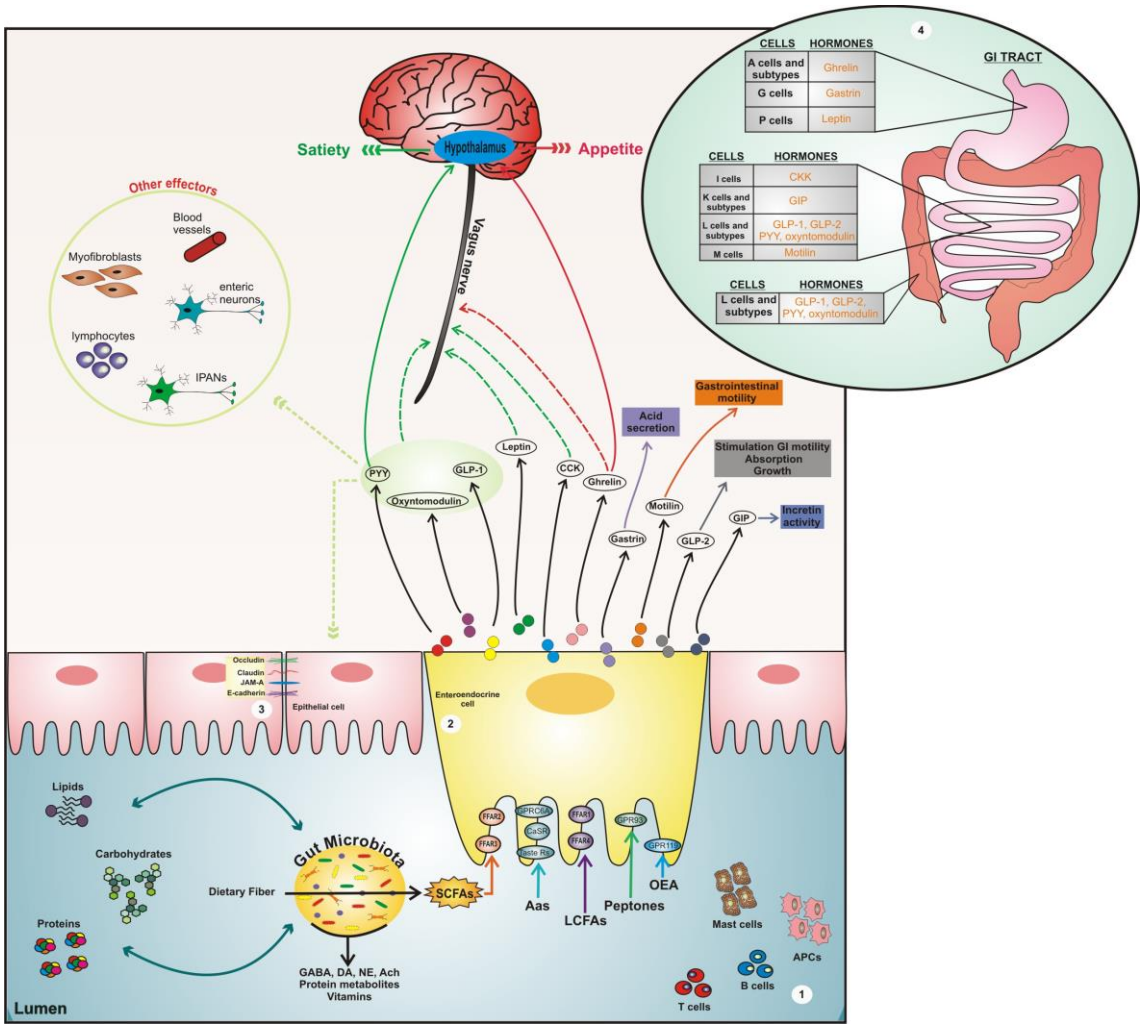


Figure 1. Interaction between luminal nutrients, gut microbiota metabolites and gut sensory receptors: the key communication pathways between endocrine, neuronal and immune systems.

Dietary composition determines the type of nutrients that reach the luminal gastrointestinal tract. Different dietary patterns can alter the composition of the gut microbiota and consequently the production of their metabolites, which can influence: epithelial permeability by acting on the cells from the immune system (1), activity of enteroendocrine cells (2) or tight junction protein function (3). The gut luminal content is continuously monitored by the intestine to optimize nutrient assimilation and protect against hazards which can affect its integrity. Therefore, the intestine is conferred with a range of sensory receptors which interact with major effector systems such as the endocrine system, the nervous system, the gut immune system, and the nonimmune defence systems of the gut. Hormone release triggered by the activation of nutrient-specific receptors found on the enteroendrocrine cells, occurs along the gastrointestinal tract from the stomach to the large intestine. There are several types of enteroendocrine cells such as L cells or I cells with sensory receptors that stimulate the release of different types of hormones (4), which have a wide range of effects such as satiety through the hypothalamus, gastrointestinal motility and acid secretion. Abbreviations: Aas, aminoacids; Ach, acetylcholine; APCs, antigen-presenting cells; CCK, cholecystokinin; DA, dopamine; GABA, gamma-aminobutyric acid; GIP, gastric inhibitory peptide; GLP, glucagon-like peptide; IPANs, intrinsic primary afferent neuron; LCFAs, long-chain fatty acids; NE, norepinepherine; OEA, oleoylethanolamide; PYY, peptide YY; SCFAs, short-chain fatty acids;

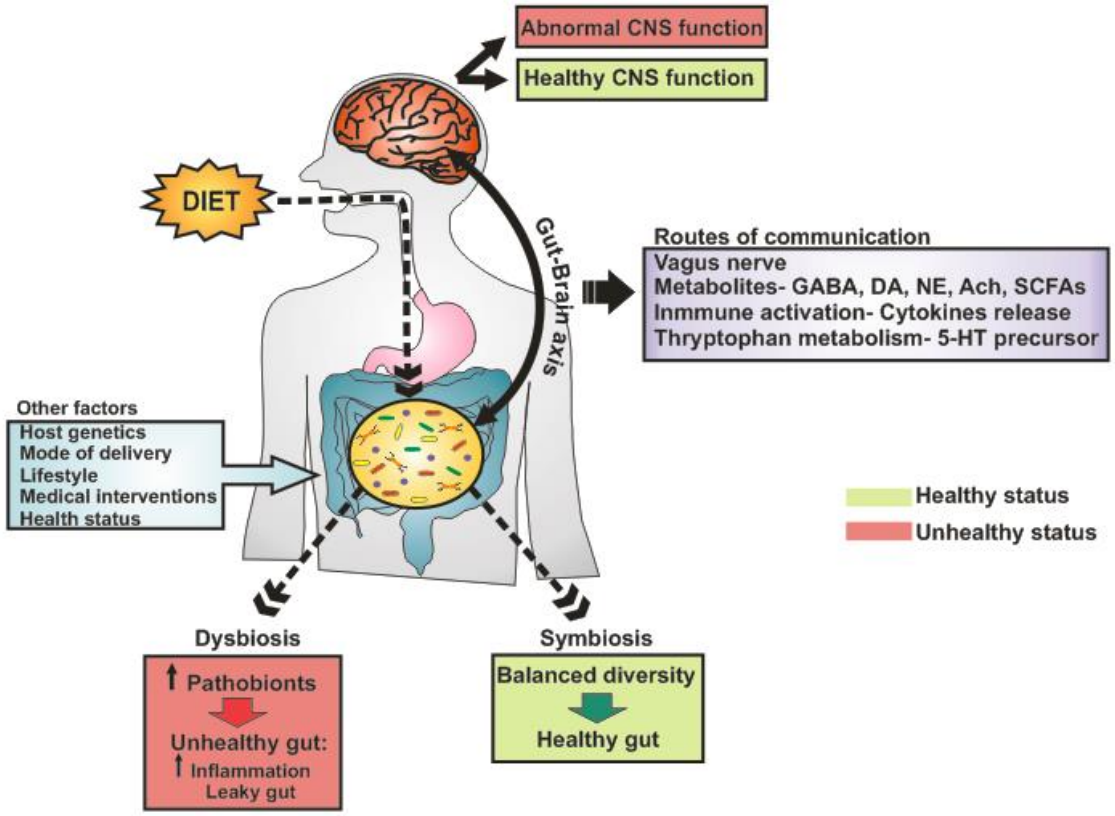


Figure 2. Impact of diet on the gut microbiota and routes of communication involved in the gut-brain axis.

Diet is one of the most crucial factors in the development of the human gut microbiota. Different dietary patterns can change the gut microbiota composition by keeping a balanced diversity of the gut microbiota (symbiosis) or causing a state of dysbiosis which is characterized by an overgrowth of potentially pathological organisms (pathobionts). A state of dysbiosis leads to an increased inflammation and leaky gut. Many mechanisms have shown to be involved in this bidirectional pathway between the gut microbiota and brain including vagus nerve signalling, immune activation, tryptophan metabolism and production of microbial metabolites and neurometabolites. Many of these bacterial metabolites significantly impact neurological function, therefore there is potential for dietary interventions that increase bacterial metabolism and promote growth of beneficial bacteria, to beneficially modulate the gut-brain axis and modulate CNS function. Abbreviations: GABA, gamma-aminobutyric acid; DA,dopamine ; NE, norepinepherine; Ach, acetylcholine; SCFAs, short-chain fatty acids; 5-HT,serotonine; CNS, central nervous system.

THESIS HYPOTHESIS

Based on the literature discussed here in chapter 1.1 and 1.2, there are a number of questions to be addressed in this thesis:

1. How does omega-3 sufficiency or deficiency interact with the microbiota composition and function, especially in early life?
2. How does this microbiota-lipid interaction impact metabolic and behavioural health outcomes?
3. Can algae act as novel functional foods to induce such health effects associated with long-chain omega-3 fatty acids?

Hence, the overarching hypothesis that underlies this thesis is that long-chain omega-3 fatty acids enchance the gut microbiota environment in early life thereby supporting metabolic health and behavioural development. In addition, algae, as novel sources of long-chain omega-3 fats, can act as novel functional foods to exert such health effects.

Chapter 2

Omega-3 polyunsaturated fatty acids critically regulate behaviour and gut microbiota development in adolescence and adulthood

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ABSTRACT

Background: Neurodevelopment is strongly influenced by maternal and early-postnatal diet. Omega-3 polyunsaturated fatty acids (n-3 PUFA) are vital structural and functional components of the developing brain. The gut microbiota is also influenced by n-3 PUFA status, however, little is known about the role of maternal and early-life n-3 PUFA intake on offspring gut microbiota development and subsequent interactions with central nervous system functioning and behavioural outcomes.

Methods: Pregnant female C57BL/6 mice and their male offspring were fed a control (CON), omega-3 deficient (O3-) or omega-3 supplemented (O3+) diet. Cognitive, depressive and social behaviours were assessed through a battery of behaviour tests in the male offspring at both adolescence (week 4-5) and adulthood (week 11-13). Hypothalamic-pituitary-adrenal axis (HPA) activation was assessed by analysis of stress-induced corticosterone production. Fecal microbiota composition was analysed by 16S sequencing at both adolescent and adulthood. In addition, stimulated spleen cytokine levels were assessed.

Results: n-3 PUFA interventions induced subtle changes in offspring early-life and adolescent behaviours, which were further evident in adulthood, such that O3- animals displayed impaired communication, social and depression-related behaviours and O3+ animals displayed enhanced cognition. O3- mice displayed an elevated Firmicutes:Bacteroidetes ratio and blunted systemic LPS responsiveness. Contrastingly, O3+ mice displayed greater fecal *Bifidobacterium* and *Lactobacillus* abundance and dampened HPA-axis activity.

Conclusions: Neurobehavioural development related to cognitive, anxiety and social behaviours, is highly dependent upon *in utero* and lifelong n-3 PUFA availability. In addition, neurobehavioural changes induced by altering n-3 PUFA status are closely associated with comprehensive alterations in gut microbiota composition, HPA-axis activity and inflammation.

1. INTRODUCTION

Neurodevelopment is characterised by rapid structural, neurochemical and molecular changes in the central nervous system (CNS) during both the prenatal and early postnatal periods. These critical physiological changes however, are uniquely sensitive to environmental stressors such as maternal health status and socioeconomic class (Reynolds et al., 2014, Talge et al., 2007, Hackman et al., 2010). Nutrition, in particular, plays a fundamental role in the growth and development of the infant brain, with particular importance being placed on the first '1000 days', referring to the nine months *in utero* and the first two years of life (Goyal et al., 2015, Wopereis et al., 2014). Due to the high metabolic demands of the brain during such a vulnerable developmental period, inadequate nutrient intake can inhibit normal neurodevelopment and may cause neurofunctional, structural and behavioural deficits that persist into later life (Seckl and Meaney, 2004, Bale et al., 2010). Indeed malnutrition, referring to the underrepresentation or overrepresentation of particular nutrients, has recently been recognised as a key contributor to mental illnesses such as major depressive disorders and cognitive impairment in adulthood (Sarris et al., 2015). However, particular aspects of neurodevelopment, such as synaptic pruning, continue late into the second or third decade of life (Crews et al., 2007) suggesting that the impact of nutrition on brain function and mental health is critical during the adolescent period. Indeed several psychiatric disorders including schizophrenia, substance abuse and mood disorders begin to appear during this maturation period of the brain, in adolescence (Davidson et al., 1999, Paus et al., 2008, Kessler et al., 2001, Spear, 2013, O'Connor and Cryan, 2014, McVey Neufeld et al., 2016), suggesting that this may be a critical timeframe in which inadequate nutrition may impose its effects on the brain and related behavioural outcomes.

One of these nutritional inadequacies associated with impaired mental health is omega-3 PUFA intake (Edwards et al., 1998, Innis, 2008). The CNS is rich in both long-chain omega-6 (n-6) and omega-3 (n-3) PUFA, particularly arachidonic acid (AA; n-6) and docosahexaenoic acid (DHA; n-3), which are crucial for infant brain development, comprising roughly 20% of brain dry weight (Muskiet et al., 2006). n-3 and n-6 PUFA cannot be synthesized de novo in mammalian tissue and hence are considered 'essential' fatty acids, that must be obtained from the diet. For infants, PUFA uptake occurs via the placenta during pregnancy and through breast milk during lactation, suggesting that maternal PUFA intake is critical to meet sufficiency (Innis, 2005). Long-chain n-3 PUFA, in particular, play vital roles in synaptic plasticity, dopaminergic and serotonergic neurotransmission (Chalon, 2006), hypothalamic-pituitary-adrenal axis (HPA-axis) activity (Chen and Su, 2013), hippocampal neurogenesis (Calderon and Kim, 2004) and a number of other neurological functions as shown in many animal studies (Bazinet and Layé, 2014). Through these mechanisms, it has been demonstrated that long-chain n-3 PUFA supplementation can protect against corticosterone-induced neuronal stress *in vitro* (Pusceddu et al., 2015c) and improve anxiety, depressive and cognitive behaviours in rodents (Pusceddu et al., 2015b, Labrousse et al., 2012).

However, in recent times the westernisation of modern diets has led to drastic decline in n-3 PUFA intake in place of n-6 PUFA. Humans evolved on a n-6/n-3 PUFA dietary ratio of around 1-2:1 however the modern ratio has increased dramatically to as high is 20-30:1 in the western diet (Simopoulos, 1999). This deficiency in n-3 PUFA intake in early life is associated with impaired psychomotor development, attention, cognition, visual acuity (Burgess et al., 2000, Innis and Friesen, 2008, SanGiovanni et al., 2000, Helland et al., 2003) and in addition has been implicated in neuronal and psychological abnormalities such as schizophrenia (Wood

et al., 2015), dementia (Cunnane et al., 2012) and major depressive disorders in later life (Peet et al., 1998). Hence, it has been suggested that an imbalanced n-6/n-3 ratio and relative n-3 deficiency can contribute to adolescence-sensitive mental disorders in animals, even across consecutive generations (Bondi et al., 2014). In Europe, for example, depression prevalence appears to be higher in Northern Europe than in the Mediterranean region where fish consumption, a potent source of n-3 PUFA, is much greater (Ayuso-Mateos et al., 2001).

The development of the brain during the early postnatal period occurs adjacently to the development of the complex microbiome that resides in the human intestine. Increasing evidence has revealed the interdependence of the brain's development on microbiota development (Borre et al., 2014, Sampson and Mazmanian, 2015). The microbiota–gut–brain axis, referring to the bidirectional network of communication between the intestinal gut microbiota, the gut, and the brain, interacts through inflammatory, nervous and endocrine systems to modulate immunological, gastrointestinal (GI) and CNS functions (Mayer et al., 2014). The mechanisms by which commensal microorganisms influence brain function are unclear but are slowly being resolved (Cryan and Dinan, 2012). Some evidence suggests that gut microbiota influence permeability at the gut epithelial barrier and hence control the passage of pro-inflammatory bacterial endotoxins such as LPS into circulation, which is elevated in certain neuropsychiatric disease states (Kelly et al., 2015). In addition certain species within the microbiota can influence mammalian behaviour through vagus nerve signalling and production of neurometabolites such as neurotransmitters and short chain fatty acids (SCFA) (Bravo et al., 2011, Cryan and Dinan, 2012). Moreover, studies from germ-free mice have shown that blood-brain barrier development and permeability are highly influenced by gut microbes (Braniste et al., 2014) as are prefrontal cortical myelination (Hoban et al., 2016), hippocampal

neurogenesis (Ogbonnaya et al., 2015) and microglia activation (Erny et al., 2015). Hence, alterations to gut microbiota composition in early life through environmental influences such as birth mode, antibiotics and diet can induce perturbations of the developing gut microbiota (Bäckhed et al., 2015) which may impact neurodevelopment and potentially lead to brain dysfunction, behavioural impairments and metabolic deficiencies later in life. Due to the strong interaction between diet and the gut microbiota, it has been suggested that the role of dietary changes in influencing brain biochemistry and behaviour may be mediated through changes in gut microbiota composition and function (Seira Oriach et al., 2016). In addition to improving neurological outcomes, n-3 PUFA have demonstrated a beneficial impact on the gut microbiota in mice, by increasing *bifidobacteria* growth, reducing *enterobacteria* growth and subsequently dampening inflammatory responses associated with metabolic endotoxemia (Kaliannan et al., 2015). Furthermore it has been shown that long-chain n-3 PUFA supplementation can correct the perturbations in the mouse gut microbiota induced by early life stress (Pusceddu et al., 2015a). In light of recent evidence of a gut-microbiota-brain axis, we hypothesize that the behavioural outcomes related to altered early-life n-3 PUFA status may be correlated with changes in the gut microbiota.

Here we examine the effects of *in utero* and early life n-3 deficiency or supplementation on depressive, cognitive and social behaviours during adolescence and adulthood in C57BL/6 mice, and examine correlations with inflammatory outcomes, HPA-axis activity and alterations to gut microbiota development.

2. MATERIALS AND METHODS

2.1. Animals

C57BL/6J female and male mice, aged 8 week-old on arrival were purchased from Harlan Laboratories, UK. Food and water was available *ad libitum* and animals were maintained on a 12:12-h dark–light cycle with temperature at 20 ± 1°C. After a week of habituation, females were mated with males and fed with either control standard chow, n-3 PUFA supplemented diet or n-3 PUFA deficient diet from gestational day 0. Male offspring were group-housed 3-4 per cage and weaned on to the same mothers corresponding diets (n=10 per dietary group) after lactation, at week 4. Moreover, 4 week-old C57BL/6J male mice, purchased from Harlan Laboratories, UK, were used as the conspecifics on the three chamber test.

Body weight was recorded weekly during the 13 weeks of dietary treatment. All experiments were conducted in accordance with the Directive 2010/63/EU and were approved by the Animal Experimentation Ethics Committee of University College Cork #2012/036.

2.2. Diets

Diets consisted of 1) control standard chow (CON); 2) Omega 3 polyunsaturated fatty acid supplemented diet (O3+) (~1g Eicosapentaenoic acid (EPA) + Docosahexaenoic acid (DHA) /100g diet); or 3) Omega 3 polyunsaturated fatty acid deficient diet (O3-). The O3+ diet was prepared with microalgal oil rich in EPA/DHA (*life'sOmega^{TM}45* – DSM, Netherlands). All three diets, which were matched for macronutrient content and differed only in fatty acid profile, were formulated by Harlan Laboratories, Inc. (Madison, WI, USA). The detailed macronutrient and fatty acid compositions of the three diets are shown in Tables S1 and S2. To prevent oxidation of long-chain n-3

PUFA in the O3+ diet, diets were kept in air-sealed bags, in the dark, at 4ºC. Diets were changed and weighed twice per week.

2.3. Experimental design

The experimental design is illustrated in Figure 1. C57BL/6J mothers were fed for 6 weeks, during pregnancy and lactation. Male offspring were subsequently weaned onto the same diets as their mothers. At both adolescence (4-5 weeks) and adulthood (11-13 weeks), male offspring underwent a battery of behavioural tests over a period of 2 weeks. Behavioural tests were conducted in sequence from the least to the most stressful test. In addition isolation-induced ultrasonic vocalization (USV) and nesting behaviour were performed at postnatal day (PND) 9 and week 8 respectively in male mice. At 13 weeks old, animals were sacrificed by cervical dislocation and tissue samples collected were rapidly harvested.

2.4. Behavioural testing

2.4.1. Three chamber test

The three-chamber test was performed to assess sociability and social novelty as described previously (Desbonnet et al., 2014). Test mice and conspecific mice (novel or familiar mice) were habituated to the testing room 30min prior to testing. The test was conducted in a rectangular box split into three connecting chambers. The test consisted of 3 sequential 10 min phases: (1) Habituation: the test mouse was allowed to explore the three empty chambers; (2) Sociability: an unfamiliar mouse was placed in an inner mesh wire cage in one chamber, an object was placed in the opposite chamber and the central chamber was empty; (3) Social novelty, the object was replaced by a novel mouse, allowing the conspecific mouse to become the familiar

mouse. During the three phases, the test mouse was placed in the middle chamber. All mice, test mice & conspecifics, were sex and age matched. The chamber was cleaned and the bedding was replaced between trials. All three phases were recorded and the time actively interacting with the object, the familiar mouse and the novel mouse were measured by an experimenter blind to the experimental groups.

2.4.2. Forced swim test

The forced swim test (FST) was carried out to determine depression-like behaviour as previously described (Cryan et al., 2001, Mombereau et al., 2004, Thakker et al., 2005). Mice were individually placed in a Pyrex cylinder (internal diameter of 24 cm × 21 cm), containing 15 cm-depth water at 23-25ºC, for 6min. Every mouse was recorded for 6 min and the immobility time was scored for the last 4 min by an experimenter blinded to the experimental groups. A mouse was recognised as immobile when it was floating and making only small movements necessary to keep the head and body above the surface of the water. Water was changed between each mouse to remove odors and the researcher remained in the testing room during the test.

2.4.4. Novel object recognition

Novel object recognition (NOR) test, used to assess memory and learning, was conducted as described previously (Burokas et al., 2014, Pusceddu et al., 2015b) with minor modifications. Mice were habituated to the room for 30min. prior to the test. The test consisted of three 10 min phases where mice were individually placed in a box (30 × 30 × 20 cm) and allowed to explore. On Day 1, the habituation phase, no objects were placed in the box. On Day 2, after 24h, two identical objects were placed

on adjacent corners of the box, approximately 5 cm from each wall. On Day 3, after 48h, one of the objects was replaced by a novel object. After each phase animals were returned to their home cages. Object exploration is defined as the time when the animal's nose comes within a 2cm radius of the object. Memory was defined by the discrimination index for the novel object (DI) as the difference of time mice spent investigating between the novel and the familiar object divided by the total time exploring both objects. [Discrimination Index, DI= (Novel Object Exploration time - Familiar Object Exploration time)/(Novel Object Exploration time + Familiar Object Exploration time)]. The box and objects were cleaned between trials.

2.4.5. Isolation-induced Ultrasonic Vocalization (USV)

Isolation-induced ultrasonic vocalization (USV) were assessed to determine the intensity of an aversive affective state and the communicative behaviour between pup and mother (Scattoni et al., 2009). Parameters for this isolation protocol were established based on previous published USV parameters (Hofer et al., 2002). The animals were habituated to the room for 30 min. Pups were individually isolated from their mother and littermates at PND9, and placed in a clean plastic container into a sound-attenuating chamber. Pup's vocalizations were recorded with an ultrasound sensitive microphone (US Mini-2 bat detector, Summit, Birmingham, USA) placed above the isolated pup and tuned in the range of 60-80 kHz, for 3 min. Once completed the mice were returned to their home cage.

2.4.6 Fear conditioning

Fear behaviour was determined by the freezing behaviour of mice, which is defined as immobility of the body. This fear conditioning protocol (Bravo et al., 2011, Brinks et

al., 2009) differentiates between context and context/cue related behavioural responses within the same procedure.

Day 1 (Training day) involved 3 min of baseline recording, followed by 6 light/tone conditioned stimulus (CS) and shock [unconditioned stimulus (US)] pairings with an interval of 1 min. Pairings consisted of the cue [e.g., a combined light (260 lx) and tone exposure (70 dB)] for 20 s and an electric footshock (0.4 mA) during the last 2 s of the cue.

Days 2-3, at 24 and 48 h after conditioning, the same experimental procedure as Day 1 was repeated in absence of shocks to test for memory and extinction of the conditioned fear response. The procedure lasted 12 min per mouse per day. After each procedure mice were returned to their home cage and the FC chambers were cleaned between subjects.

2.5. Stress-induced Corticosterone production

Blood samples were taken to assess the HPA axis in response to an acute stress (FST), through the analysis of corticosterone levels in plasma at different time points. First, a blood sample was taken before the FST as a corticosterone baseline level. After the acute stress every mouse was single-housed for 2 hours of recovery while blood samples at 5 different time points were taken: (1) 15min, (2) 45min, (3) 90min, and (4) 120min. Bleeding was performed in a separate room to the FST test room. Blood samples (50-70 ul) were taken from the tail and were placed in heparin-coated capillary tubes. The blood samples were centrifuged at 9198g for 15min. and plasma was collected in to new tubes and stored at -20ºC. Plasma was diluted 1:40 and total corticosterone was measured by ELISA (Enzo Lide Sciences, Farmingdale, NY, USA).

2.6. Tissue Harvesting

Trunk blood was collected in potassium EDTA (Ethylene Diamine Tetra Acetic Acid) tubes and centrifuged for 15min at 7774g. Plasma was then isolated and stored at -80ºC for future cytokine analysis. Brains were gross dissected upon the culling days and snap frozen on dry ice. Right hemisphere regions were utilized for brain fatty acid composition analysis and left hemisphere regions were used for gene expression. Weights from livers, adrenals, and fats pads (epididymal, subcutaneous, mesenteric) were measured and tissues were snap frozen for further analysis, such as long chain fatty acid (LCFA) composition analysis. Moreover, spleens were collected and kept in media until the preparation of splenocytes.

2.7. RNA isolation and synthesis cDNA

Total RNA was isolated from the hippocampus and amygdala using the *mir*Vana™ miRNA Isolation Kit as per manufacturer's instructions (Thermo Fisher Scientific). Briefly, tissue was homogenised using a denaturing lysis solution. The lysate was then extracted using phenol–chloroform and ethanol was then added to the solution before being filtered over a glass–fibre filter. The filter was then washed and finally the RNA was eluted with 100µl of a low-ionic strength solution. Isolated RNA was stored at −80 °C until further processing. RNA concentration was quantified using the ND-1000 spectrophotometer (NanoDrop®) and RNA quality was assessed using the Agilent 2100 Bioanalyzer.

After the RNA extraction, equal amounts of RNA were reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Carlsbad, CA). All cDNA was stored at − 20 °C until time of assay.

2.8. Real-time RT-PCR

Probed cDNA amplification was performed in a 20 μL reaction consisting of 10 μL KiCqStart qPCR Ready Mix with ROX (Sigma), 0.1 μL of primer (final concentration 250 nM), 1 μL cDNA template, and 8.8 μL RNase-free water run in duplicate in a 96-well plate (Applied Biosystems) and captured in real time using the StepOne Plus System (Applied Biosystems). β - actin was used as the housekeeping gene. Relative gene expression was adjusted to a housekeeping gene and quantified using the $2^{-\Delta\Delta CT}$ method. Primer sequences for PCRs are listed in Table S3.

2.9. Spleen Cytokine Assay

Spleens were collected immediately following sacrifice and cultured. To culture spleen cells, first the spleens where homogenised in media [RPMI (with L-glutamine and sodium bicarbonate, R8758 Sigma) + FBS (F7524, Sigma) + Pen/Strep]. The homogenate was then filtered over a 70um strainer, centrifuged at 200g for 5min and resuspended in media. Cells were counted and seeded (4,000,000/mL media). After 2.5h of adaptation, cells were stimulated with lipopolysaccharide (LPS-2 μg/ml) or concanavalin A (ConA-2.5 μg/ml) for 24h. Following stimulation, the supernatants were harvested to assess the cytokine release using Proinflammatory Panel 1 (mouse) V-PLEX Kit (Meso Scale Discovery, Maryland, USA) for TNFα, IL-10, IL-1β and IL-6. The analyses were performed using MESO QuickPlex SQ 120, SECTOR Imager 2400, SECTOR Imager 6000, SECTOR S 600.

2.10. Tissue fatty acid analysis

Fatty acid analysis on peripheral tissues and brain regions was performed as follows. Lipids were extracted from tissues using chloroform:methanol (2:1 v/v) as previously described (Folch et al., 1957). Fatty acid methyl esters (FAME) of the extracts were

prepared using 10 ml 0.5 N NaOH in methanol and heating at 90 °C for 10 min, followed by the addition of 10 ml 14% BF3 in methanol for 10 min at 90 °C (Park and Goins, 1994). FAME were recovered with hexane. To limit degradation of FAME, samples were dried over anhydrous sodium sulphate (0.5 g) for 1 h, concentrated under nitrogen and stored at −20 °C until analysis by gas chromatography (GC). FAME separation was performed via GC (Agilent 7890B, Agilent Technologies, Santa Clara, CA, USA) with flame ionisation detection (Chrompack CP Sil 88 column, Chrompack, JVA Analytical, 100 m × 0.25 mm internal diameter, 0.25 μm film thickness) and helium as the carrier gas. The GC column oven cycle was programmed initially at 80 °C for 8 min followed by an increase by 8.5 °C/min to a final column temperature of 200 °C. Sample injection (0.5 μl) was automated (CTC Autosampler, Model 80) on an Aglient Multimode Inlet on-column temperature programmable injector. Peak integration was performed using the Agilent OpenLAB Chemstation software (Version A.01.06.111). Identification of individual FAME was achieved by comparison of retention times with pure FAME standards (Nu-Chek Prep, Elysian, MN, USA) and quantification against an internal standard (C17:0). Results are expressed as g/100 g total FAME.

2.11. DNA extraction and 16S sequencing

Total bacterial metagenomic DNA was extracted from fecal pellets using a commercial DNA extraction kit (QIAmp DNA Stool Mini Kit, Qiagen, UK) following an initial repeated bead beating step to increase final DNA yield (Fouhy et al., 2015). Quantification of DNA was carried out using the Nanodrop 1000 (Thermo Scientific, Ireland) and stored at -20°C until analysis.

V3-V4 amplicons for Illumina sequencing were generated according to the 16S metagenomic sequencing library protocol (Illumina). Briefly, an initial PCR reaction utilised primers specific for amplification of the V3-V4 region of the 16S rRNA gene, (Forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). PCR product clean-up and purification was achieved using the Agencourt AMPure XP system (Labplan, Dublin, Ireland). A second PCR reaction incorporated a unique indexing primer pair each sample (Illumina Nextera XT indexing primers, Illumina, Sweden). The products were again purified using the Agencourt AMPure XP system. Samples were quantified using the Qubit broad range DNA quantification assay kit (Bio-Sciences, Dublin, Ireland). Following quantification, samples were pooled in equimolar amounts 4nM) and sequenced at Eurofins Genomics, Germany, using Illumina MiSeq 2x300 bp paired end sequencing.

2.12. Bioinformatics

300 bp paired-end reads were assembled using FLASH with parameters of a minimum overlap of 20 bp and a maximum overlap of 120 bp (Magoč and Salzberg, 2011). The QIIME suite of tools, v1.8.0, was used for further processing of paired-end reads, including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds (Caporaso et al., 2010). Denoising, chimera detection and operational taxonomic unit (OTU) grouping were performed in QIIME using USEARCH v7 (Edgar, 2010). Taxonomic ranks were assigned by alignment of OTUs using PyNAST to the SILVA SSURef database release 111 (Quast et al., 2013). Alpha and beta diversities were generated in QIIME

and calculated based on weighted and unweighted Unifrac distance matrices (Lozupone et al., 2011). Principal coordinate analysis (PCoA) plots were visualised using EMPeror v0.9.3-dev (Vázquez-Baeza et al., 2013).

2.13. Statistical Analysis

Statistical analyses were carried out using SPSS version 19 (Armonk, NY, USA). Results are presented as mean ± standard error of the mean (SEM). Differences between treatment groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison *post-hoc* test. Non-parametric data from 16S sequencing analyses were subject to Kruskal-Wallis tests followed by Mann-Whitney tests. False discovery rate (FDR) correction was assessed at $p<0.05$. The study was powered to detect differences at $p<0.05$.

3. RESULTS

3.1. Body composition and food intake

Offspring weights were recorded weekly at the beginning of weaning (postnatal week 4) until sacrifice at week 13 (Figure 2 A). Results of two-way ANOVA revealed a significant effect of time [$F_{(9,269)}$=228.4, p<0.0001)] and a significant effect of diet [$F_{(2,269)}$=12.15, p<0.0001)] but no significant interaction between the factors. Tukey HSD post-hoc testing revealed no significant differences in body weight between groups from week 4 to week 9, however, body weight was significantly reduced in the O3- group at week 10 and week 11 compared with the O3+ group (p<0.05) (Figure 2 A). These differences in body weight coincided with the second battery of behavioural testing.

Both subcutaneous fat [$F_{(2,27)}$=5.88, p=0.0076)] and epididymal fat [$F_{(2,27)}$=5.79, p=0.0081)] mass was significantly lower in the O3- group compared with O3+ (p < 0.01) (Figure 2 B and C), however, there were no significant differences compared with CON. Interestingly, adrenal gland weight was significantly lower [$F_{(2,27)}$=4.029, p=0.0294)] in the O3+ group compared with CON (Figure 2 D).

3.2 Fatty acid analysis

Feeding of CON, O3+ and O3- diets elicited significantly different fatty acid compositions between all groups in maternal milk and eight different brain regions as shown in Table 1 and Table S4. Maternal milk was significantly higher in total n-3 PUFA content and significantly lower in n-6 AA content in the O3+ group compared with both CON and O3-. EPA and DHA were not detected in O3- maternal milk.

Similar trends were observed in each brain region examined (prefrontal cortex, amygdala, hippocampus, frontal cortex, striatum, hypothalamus, cerebellum, brain stem). DHA was the most abundant PUFA in each brain region of the CON and O3+ groups, ranging from 7-16% of total fatty acids. There were no significant differences in DHA content between CON and O3+ in the prefrontal cortex, frontal cortex, hippocampus or hypothalamus however DHA was significantly higher in the amygdala, striatum, cerebellum and brain stem of the O3+ animals. Conversely, relative concentrations of DHA and total n-3 PUFA in the O3- group were less than half that of the CON and O3+ groups for each brain region examined, ranging from 3-7% of total fatty acids. In contrast to the other two groups, the most abundant PUFA within all brain regions of the O3- groups were n-6 AA and n-6 DPA (22:5 n-6), ranging from 5-9% and 2-8% respectively. n-6 DPA was undetectable or present in very low concentrations (0.1%) in all brain regions of the CON and O3+ groups.

3.3. Behavioural tests

3.3.1. Ultrasonic vocalization

As revealed by Mann Whitney test there was a significant effect of diet on the number of calls at PND9 after isolation from their mother and littermates (Figure 3A). The number of calls from the O3- group was significantly lower compared to the CON ($U=60.5$, $p=0.052$) and the Omega 3 O3+ group ($U=47$, $p=0.011$).

3.3.2. Forced Swim Test

Depression-like behaviour is represented as a function of immobility time. One-way ANOVA showed that there was not an overall effect of diet during adolescence

[$F_{(2,29)}$=0.325, p=0.726]**,** but there was a diet effect on the time spent immobile in adulthood [$F_{(2,29)}$=3.312, p=0.05] (Figure 3B). Further, post-hoc analysis showed that there was a statistical difference on the immobility time between the controls and the omega-3 deficient diet group (p=0.042). Altogether, these data show that omega-3 PUFA deficient mice presented a depressive-like phenotype displayed by a significant greater immobility during the forced swim test compared to the control group.

3.3.3. Novel Object Recognition

One-way ANOVA showed an overall dietary effect in adolescence [$F_{(2,29)}$=3.297,p=0.05] and adulthood [$F_{(2,29)=}$7.189, p=0.003)] (Figure 3C). Post-hoc analysis revealed that O3+ showed enhanced cognition compared with O3- at adolescence (p=0.04). Furthermore, at adulthood, O3+ improved cognitive performance, shown by enhanced memory, compared to both CON (p=0.019) and O3- (p=0.004).

3.3.4 Three Chamber Test

During adolescence, one-way ANOVA did not show an overall effect of diet on the time spent actively interacting with the object [$F_{(2,29)}$=0.483, p=0.622] and the mouse [$F_{(2,29)}$=0.939, p=0.403] (Figure 4). Furthermore, there was no significant effect of diet on the time spent interacting with the familiar mouse [$F_{(2,29)}$=0.453, p=0.641] and the novel mouse [$F_{(2,29)}$=0.042, p=0.959].

During adulthood, one-way ANOVA revealed a significant effect of diet on time spent interacting with the object [$F_{(2,29)}$=10.540, p<0.001] and the mouse [$F_{(2,28)}$=14.582, p<0.001]**.** Post-hoc analysis further showed that the omega-3 deficient

group spent significantly more time interacting with the object compared to the control group ($p<0.001$) and the omega-3 supplemented group ($p=0.05$). Moreover, the omega-3 deficient group spent significantly less time interacting with the mouse compared to the control group ($p<0.001$) and the omega-3 supplemented group ($p<0.001$).

Furthermore, one-way ANOVA showed no significant effect of diet on the time spent interacting with the familiar mouse [$F_{(2,29)}=1.819$, $p=0.182$] and the novel mouse [$F_{(2,29)}=1.500$, $p=0.241$].

3.3.5. Fear Conditioning

Cue-induced and contextual fear conditioning are shown in Figure 5.

Adolescence-context component: During acquisition, two-way ANOVA repeated measures revealed a significant effect of time [$F(7,175)=56.988$, ($p<0.001$)], no interaction between time vs diet [$F(14,175)=1.561$, ($p=0.095$)], and no significant effect of diet with respect to time [$F(2,25)=1.909$, $p=0.169$]. During memory/extinction at 24h, two-way ANOVA repeated measures revealed a significant effect of time [$F(7,175)=7.373$, ($p=<0.001$], no interaction between time vs diet [$F(14,175)=1.297$, ($p=0.393$)], and a significant effect of diet respect to time [$F(2,25)=5.070$, $p=0.014$]. Furthermore, Tukey's HSD posthoc showed significant differences between O3+ vs O3- at Cxt 2 ($p=0.002$), Cxt3 ($p=0.045$), Cxt6 ($p=0.048$), Cxt7 ($p=0.015$) and Cxt8 ($p=0.042$). Moreover, there was a significant difference between CON vs O3- at Cxt8 ($p=0.051$). During memory/extinction at 48h, two-way ANOVA repeated measures revealed a significant effect of time [$F(7,175)=5.849$, $p<0.001$], no interaction between time vs diet [$F(14, 175)=0.813$, $p=0.655$], and no

significant effect of diet with respect to time [F(2,25)=2.946, p=0.071]. Post-hoc analysis further showed significant differences between O3+ and O3- at Cxt1 (p=0.050), Cxt4 (p=0.021) and Cxt7 (p=0.025)

Adolescence-cue component: During acquisition, two-way ANOVA repeated measures revealed a significant effect of time [F(5,125)=62.139, p<0.001)], no interaction between time vs diet [F(10,125)=0.855, p=0.577)], and no significant effect of diet with respect to time [F(2,25)=1.628, p=0.216]. Post-hoc analysis further showed significant differences between O3+ and O3- only at Cue6 (p=0.037). During memory/extinction at 24h, two-way ANOVA repeated measures revealed no significant effect of time [F(5,125)=1.329, p=0.256], no interaction between time vs diet [F(10,125)=0.737, p=0.688], and no significant effect of diet respect to time [F(2,25)=3.235, p=0.056]. Tukey HSD post hoc test further revealed a significant difference between O3+ and O3- at Cue4 (p=0.05). During memory/extinction at 48h, two-way ANOVA repeated measures revealed significant effect of time [F (5,125)=4.831, p<0.001], no interaction between time vs diet [F(10,125)=0.364, p=0.960], and no significant effect of diet respect to time [F(2,25)=5.325, p=0.012]. Tukey HSD post hoc test showed significant differences between O3+ and O3- at Cue4 (p=0.009) and at Cue6 (p=0.027).

Adulthood-context component: During acquisition, two-way ANOVA repeated measures revealed a significant effect of time [F(7,189)=27.347, p<0.001], no interaction between time vs diet [F(14,189)=1.558, p=0.094], and a significant effect of diet with respect to time [F(2,27)=4.452, p=0.021]. Tukey HSD test further revealed significant difference between O3+ and O3- at Cxt3 (p=0.009), Cxt4 (p=0.008) and at Cxt6 (p=0.024). Moreover, there were significant differences between CON and O3+ at Cxt2 (p=0.052) and at Cxt4 (p=0.012). During

memory/extinction at 24h, two-way ANOVA repeated measures revealed a significant effect of time [F(7,189)=9.703, p<0.001], no interaction between time vs diet [F(14,189)=1.557, p=0.095], and a significant effect of diet with respect to time [F(2,27)=5.112, p=0.013]. Tukey HSD post hoc test further showed significant differences between O3+ and O3- at Cxt3 (p=0.004) and at Cxt4 (p=0.017), and a significant difference between CON and O3+ at Cxt2 (p=0.024). During memory/extinction at 48h, two-way ANOVA repeated measures revealed significant effect of time [F(7,189)=12.273, p<0.001], no interaction between time vs diet [F(14,189)=0.741, p=0.731], and no significant effect of diet respect to time 48h [F(2,27)=1.353, p=0.275]. Post-hoc analysis further showed no significant differences.

Adulthood-cue component: During acquisition, two-way ANOVA repeated measures revealed a significant effect of time [F (5,135)=21.070, p<0.001], no interaction between time vs diet [F(10,135)=1.765, p=0.073], and no significant effect of diet on respect to time during Acq [F(2,27)=1.679, p=0.205]. Tukey HSD showed significant differences at Cue4 between O3+ and O- (p=0.006) and between CON vs O3+ (p=0.014). During memory/extinction at 24h, two-way ANOVA repeated measures revealed significant effect of time [F(5,135)=2.296, p=0.049], no interaction between time vs diet [F(10,135)=1.036, p=0.417], and no significant effect of diet with respect to time [F(2,27)=2.377, p=0.112]. Post-hoc analysis further showed significant differences between O3+ and O3- at Cue4 (p=0.012). During memory/extinction at 48h, two-way ANOVA repeated measures revealed a significant effect of time [F (5,135)=1.942, p=0.091], no interaction between time vs diet [F(10,135)=0.353, p=0.964], and no significant effect of diet with respect to time [F(2,27)=0.015, p=0.985]. Post-hoc analysis further showed no significant differences.

3.4. HPA axis response to acute stress

Two-way ANOVA repeated measures revealed a significant effect of time [$F_{(4,88)}$=251.028, p<0.001)], significant interaction between time and diet [$F_{(8,88)}$=3.001, p=0.005)] within groups and a trend for a significant effect of diet with respect to time between the groups [$F_{(2,22)}$=3.059, p=0.067)] during adolescence (Figure 6A). At 45min post stress Tukey HSD post-hoc showed that O3+ was significantly lower compared with both CON (p=0.004) and O3- (p=0.011). O3+ corticosterone was also significantly lower at 90 min compared with CON (p=0.005) and O3-(p=0.035).

Two-way ANOVA repeated measures showed a significant effect of time [F(4,96)=425.104, (p<0.001)], no significant interaction between time and diet [F(8,96)=0.887, (p=0.531)] within groups, and no significant effect of diet with respect to time between the groups [$F_{(2,22)}$=3.059, p=0.067)] on the corticosterone recovery in response to acute stress (forced swim test) during adulthood (Figure 6B).

One-way ANOVA revealed a significant effect of diet on delta corticosterone after an acute stress during adolescence [$F_{(2,24)}$=9.825, p=0.001] (Figure 6C). Further Tukey HSD post-hoc showed decreased levels of delta corticosterone in the O3+ in comparison to CON (p=0.001). During adulthood, one-way ANOVA showed no effect of diet on delta corticosterone [$F_{(2,26)}$=0.522, p=0.600] (Figure 6D).

3.5. Spleen cytokine production after stimulation with ConA and LPS

Omega-3 dietary intervention showed no significant effect on TNFα [F (2,26)=2.739, p=0.085] and IL-10 [F (2,26)=2.474, p=0.105] levels in unstimulated splenocytes.

One-way ANOVA revealed a significant effect of diet on the splenic production of both tumor necrosis factor (TNFα) [F (2,27)=4.765, p=0.018] and Interleukin 10 (IL-10) [F(2,27)=6.135, p=0.007] after stimulation with Concanavalin A (ConA) (Figure 7A). Furthermore, Tukey HSD post-hoc test showed that O3- significantly decreased ConA-induced TNFα release in comparison to the control (p=0.023). Moreover, O3- presented a significant reduced production of IL-10 in comparison to both CON (p=0.027) and O3+ (p=0.008).

After a splenic stimulation with LPS, one-way ANOVA revealed a significant effect of diet on the release of TNFα [F(2,28)=3.742, p=0.037]; while there was no significant effect of diet on IL-10 release [F(2,27)=2.590, p=0.09] (Figure 7B). Tukey HSD post-hoc showed significant decreased levels of TNFα in O3- (p=0.030) compared with O3+.

3.6. Microglial gene mRNA expression levels in hippocampus and amygdala

One-way ANOVA revealed a significant effect of diet on CD206 mRNA expression in hippocampus [$F_{(2,23)}$=4.574, p=0.022] (Figure 8A), but only a trend toward a significant effect in the amygdala [$F_{(2,22)=}$3.219, p=0.061] (Figure 8B) during adulthood. Further Tukey HSD post-hoc test showed a significant lower mRNA expression of CD206 in the O3- group compared to the O3+ group (p=0.027) in the hippocampus. No other differences were observed between treatment groups for other genes associated with microglial activation (Figure S3).

3.7. Compositional Analysis of Fecal Microbiota

MiSeq sequencing of the faecal microbiota resulted in a total of 3.4 million sequenced reads, corresponding to an average of 56,509 reads per sample. To remove any bias from variation in sample read numbers, samples were rarefied to 25,000 reads.

In both adolescence and adulthood samples, distinct clustering and separation of the O3- samples from the CON and O3+ samples was observed (Figure 9A). Phylogenetic diversity was assessed using the Chao1, Simpson, Shannon and number of observed species indices. During adolescence, microbial diversity was significantly greater in the O3- group compared with both CON and O3+ as assessed by the Chao1 index and number of observed species. There were no significant differences between groups in microbiota diversity in adulthood. Interestingly however, results trended towards increasing diversity in both CON and O3+ from adolescence to adulthood, but decreased diversity in O3-. Principle coordinate analysis (PCoA) of the unweighted UniFrac showed distinct clustering of samples based on dietary group (Figure 9B).

Analysis of relative abundances at phylum and genus levels suggested that fecal microbiota composition differed significantly between dietary groups at both adolescence and adulthood (Figure 9C-G and Table S5 and S6). Bacteroidetes and Firmicutes were the most abundant phyla in each group at both time-points, and relative proportions of both taxa differed between treatment groups at adolescence. Bacteroidetes made up 51.09% of relative abundance at phylum level in O3+ animals, whereas O3- displayed 45.45%, which was significantly lower than CON (55.18%). In contrast, Firmicutes were present in significantly higher proportions in O3- animals (42.49%) compared with O3+ (30.88%). These major differences in the two most abundant OTUs led to a significant increase in the Firmicutes:Bacteroidetes ratio in

O3- animals at adolescence compared with both other groups. None of these differences were observed in adulthood, however differences were observed for other taxa. Actinobacteria, the phylum to which the genus *Bifidobacterium* belong, was significantly greater in abundance in O3+ animals during adolescence compared with both other groups. Conversely, Tenericutes was significantly less abundant in O3+ animals compared with both CON and O3-. Candidate division TM7 was significantly less abundant in O3- animals.

At genus level, O3+ mice had significantly greater proportions of *Bifidobacterium* and *Lactobacillus* during adulthood compared with both other groups, which were not seen during adolescence. Moreover the ratio of *bifidobacteria*/*enterobacteria* was significantly greater in the O3+ groups at both adolescence and adulthood. O3- animals exhibited significantly less abundance of taxa within the *Bacteroidales* family including *Bacteroidales S24-7* uncultured and *Bacteroides* at adolescence and *Parabacteroides* at adulthood. Conversely *Bilophila*, *Gelria* and *Lachnospiraceae uncultured* were present in significantly greater abundance in O3- animals. *Anaeroplasma*, *Clostridium* and *Peptostreptococcaceae* were significantly decreased in O3+ animals.

4. DISCUSSION

There is a growing appreciation of the role of nutrition in maintaining brain health and behaviour. In these studies we have demonstrated that adolescent neurobehavioural development is highly sensitive to *in utero* and early life dietary n-3 fatty acid exposure, in which n-3 deficiency impairs communicative behaviour. Moreover, these behavioural outcomes observed in adolescence are further evident in adulthood with continued supplementation or deficiency of n-3 fatty acids, such that n-3 deficiency increases depressive behaviour, impairs social behaviour and fear-induced freezing behaviour, and n-3 supplementation enhances cognition. Finally, these neurobehavioural changes are closely associated with wide-ranging alterations in gut microbiota composition, HPA-axis activity and inflammation. Hence, these results suggest that neuropsychiatric disorders, including those emerging during adolescence, such as stress-related disorders, mood disorders and social disorders may be influenced by maternal and early life omega-3 PUFA intake and subsequent changes in gut microbiota and inflammation.

Evidence has shown that nutritional challenges during early-life developmental periods can have a detrimental impact on development and can induce functional and structural physiological impairments that persist throughout the lifespan (Barker et al., 1993, Skinner et al., 2010, Seckl and Meaney, 2004). The results presented here further support this hypothesis and provide evidence of the essentiality of n-3 PUFA for the prevention of behavioural deficiencies in later life. These data are highly relevant to modern public health due to the rapid decline of n-3 PUFA in the Western diet, which are being replaced by other fat sources, rich in n-6 PUFA.

In states of n-3 deficiency, enzymatic elongation and desaturation of PUFA occur primarily in the n-6 pathway resulting in increased production of long-chain n-6 PUFA, notably AA and n-6 DPA (Coti Bertrand et al., 2006). Interestingly, this was observed here in the O3- mother's breast milk, and was further reflected in all eight brain regions of O3- offspring, such that n-6 DPA and AA were significantly higher, and DHA was significantly lower compared with CON and O3+ groups. It has previously been shown that n-6 22:4, n-6 DPA and AA partially replace DHA in rat cortices during early development following n-3 deficiency (Greiner et al., 2003). This n-6/n-3 imbalance has been associated with deficits in spatial task performance (Lim et al., 2005), suggesting that DHA deficiency during brain development may lead to irreversible damage to certain brain functions. Here, it is likely that such drastic conversion of n-3 to n-6 fatty acids in the developing CNS contributed to the behavioural deficits observed in later life.

However, it is unclear at what stage during prenatal or postnatal neurodevelopment, n-3 PUFA deficiency may induce its negative effects on the brain. Nutrient sufficiency in the postnatal diet may not correct all neurodevelopmental deficits induced by prenatal nutritional inadequacies. Moreover, neurodevelopment continues past childhood. Adolescence is a critical period at which diet may influence neurodevelopment (O'Connor and Cryan, 2014). Stress, impulsivity and emotional instability are all elevated in adolescence (McVey Neufeld et al., 2016) and hence dietary insufficiencies during development may exacerbate such behaviours. Our data reflected certain dietary-induced changes in early-life behaviours. Firstly, through analysis of isolation-induced ultrasonic vocalization (USV), our results demonstrated that social and communicative behaviour in omega-3 deficient animals was impaired as early as PND9, suggesting that prenatal and maternal fatty acid status can strongly influence neonatal communicative behaviours. USV impairment has been associated

with animal models of neurodevelopmental disorders such as autism spectrum disorder (Hsiao et al., 2013). Furthermore it has been previously shown that prenatal ethanol exposure, which can induce brain DHA deficiency, results in deficits in USV in offspring which are normalized following postnatal DHA supplementation (Wellmann et al., 2015). These findings have strong clinical relevance. Social and communicative deficits identified so early in the postnatal development give indications that maternal diet, particularly omega-3 deficiency, may influence progression of ASD-related deficits in communication. Importantly however, the previously published work by Wellmann and colleagues suggests that it may be possible to reverse these communicative and social deficits through adequate nutrition in the postnatal period.

Interestingly there were no differences observed between dietary groups in social behaviour or depression-like behaviour at adolescence. Nesting behaviour and anxiety behaviours were also unaffected by diet at either time-point (Figures S6 and S7). However, adolescent offspring from the O3+ group exhibited significantly enhanced cognition compared with O3- animals. Dietary n-3 deficiency has previously been associated with disrupted memory and learning in rodents (Catalan et al., 2002, Moriguchi et al., 2000, Yamamoto et al., 1987, Bondi et al., 2014) whereas n-3 supplementation has demonstrated positive effects (Carrié et al., 2000, Lafourcade et al., 2011, Labrousse et al., 2012, Ferraz et al., 2011) which have been associated with enhanced hippocampal neurogenesis (Cao et al., 2009, He et al., 2009, Dyall et al., 2010). Hippocampal BDNF mRNA was assessed as a marker of neurogenesis however no differences were observed between groups (Figure S4). In humans, the role of maternal n-3 supplementation in child cognitive development is unclear (Gould et al., 2013). The discrepancies in these data may be due to differences in age i.e. childhood vs adolescence. DHA concentrations in the frontal cortex, a region

mediating cognitive processes, continue to increase past childhood into adolescence reaching around 15% of total fatty acids (Carver et al., 2001) as observed here in mice. These lipid compositional changes in the frontal cortex during adolescence coincide with vital structural changes such as synaptic pruning and white matter maturation (Glantz et al., 2007, Paus et al., 1999), suggesting that the adolescent period may be a critical timeframe in which omega-3 PUFA exert their effects on cognitive processes.

We subsequently examined these behavioural phenotypes in adulthood and found that the behavioural outcomes assessed in adolescence were more evident in adulthood with continued long-term omega-3 supplementation or deficiency. Despite no differences at adolescence, sociability was significantly impaired in O3- animals at adulthood, which has previously been demonstrated following maternal feeding with a diet rich in n-6 PUFAs (Jones et al., 2013). In addition, the enhanced cognitive effects seen in O3+ animals at adolescence compared with O3- were further apparent at adulthood, in which O3+ showed enhanced cognition compared with both O3- and CON. This has been demonstrated previously by Janssen et al (Janssen et al., 2015) who showed that early life DHA adequacy enhanced cognitive performance in male mice in adulthood compared with DHA deficiency. Finally, depression-like behaviour, which was not influenced by diet at adolescence, was significantly increased in O3- animals at adulthood, measured by immobility time in the forced swim test. Importantly this difference in immobility time was independent of locomotor activity, which was not different between groups as observed in the open field (Figure S5), suggesting the observed differences were behavioural rather than activity related.

Fear-conditioning can be utilized in order to examine fear-induced memory and the cognitive aspects of anxiety and stress-related behaviours (Brinks et al., 2009, Bravo et al., 2011, Takeuchi et al., 2003). Importantly, different neurobiological substrates have been shown to underlie contextual and cue-induced fear learning (Kim et al., 1991, Zimmerman and Maren, 2010, Phillips and LeDoux, 1992). Here, O3- animals displayed significantly greater freezing behaviour compared with O3+ animals during both contextual and cue fear-conditioning conditions in adolescence which endured into adulthood. These data are in line with previous studies which have shown that n-3 deficiency increases and n-3 supplementation decreases freezing behaviour in mice during fear-conditioning (Takeuchi et al., 2003, Yamada et al., 2014). Together the data presented here supports the concept that n-3 PUFA significantly alter plasticity in both hippocampal and cortico-amygdalar circuits that underlie contextual and cue-induced fear-induced freezing behaviour.

It has been previously shown that n-3 deficiency increases and n-3 supplementation decreases immobility in the forced swim test (Huang et al., 2008, Park et al., 2012, Weiser et al., 2015, DeMar et al., 2006, Chen and Su, 2013, Lafourcade et al., 2011). This behavioural phenotype has been associated with alterations in corticosterone production and HPA-axis activity (Chen and Su, 2013) as was also observed here. The design of this study did not allow us to distinguish between the effect of n-3 interventions prenatally compared with posnatally. However, Chen and Su previously demonstrated that pre-weaning n-3 deficiency caused depression-like behaviour in adulthood while a post-weaning n-3 deficiency did not have any effect (Chen and Su, 2013).

Importantly, the observed differences between adolescent and adulthood behaviour is of note due to the varying periods of development at which symptomatic

onset occurs for different psychiatric disorders (Paus et al., 2008). These data suggest that reduced n-3 intake in early life can induce subtle psychiatric abnormalities during adolescence associated with communicative behaviour and fear-induced memory. Continued n-3 deficiency into later life can induce further psychiatric deficits and worsen behavioural outcomes associated with depressive behaviour and social behaviour in adulthood. Recent studies have begun to compare and exhibit the differences between adolescent and adult behavioural changes induced by differing n-3 PUFA status in rodents (Weiser et al., 2015, Bondi et al., 2014). Therefore, it is possible that due to the complex structural rearrangement that occurs in the brain at adolescence, differences in underlying nutritional status on behavioural outcomes may not become overly apparent until brain development is complete in adulthood.

We further examined biological outcomes induced by altering n-3 status that may explain the observed behavioural changes. O3+ adolescent animals displayed enhanced corticosterone recovery following a forced stress compared with both other groups. Interestingly, these differences disappeared in adulthood, contrasting to the trends seen in behavioural analysis. In addition to dampened corticosterone response, adrenal gland weight was significantly lower in O3+ animals. Increased adrenal gland weight has previously been associated with lower social rank in mice and primates (Shively and Kaplan, 1984, Davis and Christian, 1957) which has implications for stress-reactivity. The HPA axis is an important neuroendocrine system that is subjected to programming by early life events. Pubertal corticosterone and stress reactivity are greater than at adulthood (Goldman et al., 1973, Romeo et al., 2004, McVey Neufeld et al., 2016), which may account for the differences between the two time points assessed here. This suggests that nutritional inadequacies such as omega-3 deficiency that alter HPA-axis activity may exacerbate the already altered stress response observed in adolescence. The data presented here suggest that long-chain n-

3 PUFA supplementation can dampen HPA-axis activity in early life, however it is unclear whether these changes in corticosterone stress response are correlated with the altered depressive phenotypes observed in adulthood. n-3 PUFA therefore may enhance negative feedback within the HPA axis. However, it has been recently shown that n-3 PUFA deficiency does not impair negative feedback within the HPA axis and instead the effects on emotional deficits are mediated through inactivation of glucocorticoid receptor signalling in the prefrontal cortex (Larrieu et al., 2016). It is possible that this also occurs through GABA-receptor mediated pathways (Chen and Su, 2013) or reduced cortical neuronal atrophy (Larrieu et al., 2014) as has been suggested previously.

The close link between HPA axis activity and immune system activity suggest that the effects of n-3 PUFA supplementation or deficiency on corticosterone production and subsequent behaviours may be regulated by inflammatory status. Interestingly our results found that omega-3 deficiency led to a blunted systemic LPS and ConA responsiveness, dampening anti-inflammatory IL-10 in ConA-stimulated macrophages and proinflammatory TNFa production in ConA and LPS-stimulated splenocytes, as has been shown previously through maternal dietary interventions (Myles et al., 2013). Moreover, as PUFAs have the capacity to cause dramatic changes in TLR4 (Norris and Dennis, 2012, Shi et al., 2006), this altered response to LPS found in n-3 deficient group may be due to modifications in the sensitivity of toll-like receptor 4 (TLR4), which is the receptor for LPS and plays a critical role in innate immunity (Poltorak et al., 1998, Kawai and Akira, 2010). Immune-neural interactions have strong implications for behavioural outcomes (McCusker and Kelley, 2013). Impaired social behaviour has been associated with disrupted spleen cytokine production in mice, although these results are strain dependent (Schwartzer et al., 2013). Furthermore, depressive behaviours are also strongly associated with

altered immune response and low-grade chronic inflammation as shown in animals (You et al., 2011) and in humans which may be associated with increased bacterial translocation into the bloodstream (Kéri et al., 2014).

The altered inflammatory status was also reflected in the brain. mRNA expression of CD206, an anti-inflammatory M2 microglial marker, was significantly up-regulated in the hippocampus of O3+ mice compared with O3- mice, but not compared with controls. However, no significant differences were observed between groups for other markers of microglial activation (Figure S3). Microglial activation has been implicated in depression, schizophenia and bipolar disorder (Réus et al., 2015). DHA has previously been shown to upregulate the anti-inflammatory microglial marker CD206 positive cells following traumatic brain injury in rats (Harvey et al., 2015). It is possible therefore, that CD206 expression may play an intermediary role in the protective effects of n-3 PUFA on brain function and behavioural deficiencies associated with excess inflammation. Here however, as no down-regulation of CD206 was observed in O3- animals, further evidence is warranted to establish whether n-3 PUFA play a true role in CD206 expression.

Furthermore, our data suggested that the behavioural and inflammation associated changes may be regulated by gut microbiota. Recently it has been suggested that n-3 and n-6 PUFA differentially contribute to risk of systemic low-grade chronic inflammation by altering gut microbiota composition, gut permeability and hence systemic circulation of LPS (Kaliannan et al., 2015). Interestingly, our data showed that the neurobehavioural changes induced by the O3+ and O3- diets were closely associated with wide ranging compositional changes in the developing microbiota. *Bifidobacteria* have been shown to enhance intestinal epithelial integrity, dampen inflammation (Cani et al., 2007, Cani et al., 2009) and improve anxiety-

related behavioural deficits in rodents (Savignac et al., 2015). Here, there was a trend towards increased *Bifidobacteria* in O3+ animals at adolescence, which became significant compared with both other groups at adulthood. Similarly the ratio of *bifidobacteria* to the LPS-producing *enterobacteriaceae* was significantly greater in O3+ animals at adolescence, an effect that strengthened in adulthood. A similar trend was observed for significantly increased *Lactobacillus species* in the O3+ animals at adulthood but not at adolescence. Interestingly, diets high in fat and sugar have been linked with decreased *Lactobacillales* and a higher ratio of Firmicutes:Bacteroidetes as observed here, which have been associated with brain dysfunction and behavioural changes (Bruce-Keller et al., 2015, Pyndt Jørgensen et al., 2014, Magnusson et al., 2015). Interestingly, these results are associated somewhat with the behavioural data. It appeared that differences between groups in abundance of certain species including *Bifidobacteria* and *Lactobacillus* were not apparent at adolescence but became significantly evident in adulthood following continuation of each respective diets. In addition, a number of significant correlations were observed between microbiota and delta corticosterone, including a positive correlation between corticosterone area under the curve and *Firmicutes* abundance (Figure S1). Due to the evidence of a gut-microbiota-brain axis in a number of neuropsychiatric disorders (Cryan and Dinan, 2012), these results have relevance for a novel mechanistic hypothesis by which n-3 PUFA exert their influence on brain function and behaviour. It is possible that the neuroprotective effects of omega-3 PUFA are not solely attributed to their direct incorporation into neural tissues but indirectly through their beneficial effects on gut microbiota composition and subsequent inflammatory status as described here and previously (Kaliannan et al., 2015).

In humans, major depressive disorder has been associated with altered inflammatory markers in blood in addition to elevated 16S rDNA in the bloodstream

(Kéri et al., 2014). This data demonstrates a link between gut microbiota and inflammatory-associated mental disorders. Gut permeability is downregulated by *bifidobacteria* (Cani et al., 2007) and conversely is upregulated by a higher firmicutes:bacteroidetes ratio (Lam et al., 2012). Hence, the translocation of bacteria and their associated endotoxins across the epithelial barrier, as regulated by microbiota composition, may contribute to immune-associated changes in depressive and other behaviours as reported here.

The data presented here provide novel evidence for the role of early-life omega-3 PUFA status on the progression of social, depressive and cognitive behavioural disorders. It has been shown previously that n-3 fatty acids can improve behavioural deficits in adults, however, less is known about prenatal and early postnatal n-3 PUFA status on the progression of certain mood, social and anxiety disorders. It has been speculated that the relative increase in the prevalence of certain neuropsychiatric disorders in the last 30 years is concurrent with drastically declining n-3 PUFA intake in place of n-6 PUFA. Fish consumption, a potent source of dietary long-chain n-3 PUFA, is class dependent in developed western countries. Lower socioeconomic classes consume less fish (Darmon and Drewnowski, 2008, Galobardes et al., 2001) and have greater mental illness prevalence particularly in adolescence (McLaughlin et al., 2012). Due to the essentiality of n-3 fatty acids in neuronal composition and function, especially in early development, it is likely, as shown here, that dietary deficiency in these fatty acids can substantially impair behavioural development which may be first recognised at adolescence. Sufficient intake of n-3 PUFA *in utero* and in early life, are essential for the adequate development of the complex gut-microbiota-brain axis, and therefore ensure the correct function of immunological, gastrointestinal (GI) and CNS systems. Moreover these interactions with GI pathways suggest a new target and mechanism by which n-

3 PUFA exert their beneficial effects on the brain. These data and others therefore warrant further research and public health advice on increasing maternal, perinatal and lifelong omega-3 PUFA intake for the prevention of neuropsychiatric illnesses during critical early-life developmental stages and in later life.

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Table 1. Fatty acid composition of mother's milk and selected brain regions

	Mother's Milk			Prefrontal Cortex			Amygdala			Hippocampus		
	Control	**O3+**	**O3-**	**Control**	**O3+**	**O3-**	**Control**	**O3+**	**O3-**	**Control**	**O3+**	**O3-**
SFA												
14:0	10.8 ± 1.2	6.8 ± 0.3	8.5 ± 1.5	0.1 ± 0	0.1 ± 0.1	0.1 ± 0	0.7 ± 0.1	1 ± 0.2	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0	0.9 ± 0
16:0	29 ± 0.4^{a}	33.6 ± 1.1^{b}	27.3 ± 0.7^{a}	39.3 ± 0.7^{a}	36.7 ± 1.2$^{a\ b}$	34.2 ± 0.6^{b}	36.8 ± 0.8^{a}	35.9 ± 0.5$^{a\ b}$	34.6 ± 0.6^{b}	31.8 ± 0.6	31.6 ± 0.7	31.3 ± 0.2
18:0	7.4 ± 0.5^{a}	13.3 ± 1.3^{b}	8.6 ± 0.6^{a}	30.9 ± 0.4^{a}	29.6 ± 0.7$^{a\ b}$	27.9 ± 0.3^{b}	29.8 ± 0.5^{a}	28.6 ± 0.3$^{a\ b}$	27.8 ± 0.3^{b}	26.4 ± 0.3	26 ± 0.4	25.6 ± 0.2
20:0	n.d.	n.d.	0.1 ± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.4 ± 0	0.3 ± 0	0.3 ± 0.1
Total SFA	47.3 ± 1.3$^{a\ b}$	53.8 ± 2.6^{a}	44.5 ± 2.7^{b}	70.2 ± 1.1^{a}	66.4 ± 1.9$^{a\ b}$	62.2 ± 0.9^{b}	67.3 ± 1.3^{a}	65.5 ± 0.6$^{a\ b}$	63.2 ± 0.8^{b}	59.4 ± 0.9	58.9 ± 1.1	58.1 ± 0.4
MUFA												
16:1	3.5 ± 0.1^{a}	2.4 ± 0.3^{b}	3.2 ± 0.3$^{a\ b}$	n.d.	n.d.	n.d.	0.4 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.8 ± 0.1	1 ± 0.2	0.8 ± 0.1
18:1t n-9	n.d.	n.d.	0.1 ± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
18:1c n-9	29.9 ± 1.6	26.6 ± 0.9	33.8 ± 3.4	11.1 ± 0.4	12.1 ± 0.5	11.7 ± 0.3	15.9 ± 0.2	15.6 ± 0.2	15.5 ± 0.1	16.3 ± 0.3^{a}	16.5 ± 0.3^{a}	15 ± 0.2^{b}
18:1 t n-7	2.6 ± 0.2^{a}	1 ± 0.1^{b}	3 ± 0.1^{a}	1.7 ± 0.1^{a}	1.7 ± 0.2^{a}	2.6 ± 0.1^{b}	1.6 ± 0.1^{a}	1.6 ± 0.1^{a}	2.1 ± 0.1^{b}	2.5 ± 0.1$^{a\ b}$	2.4 ± 0.1^{a}	2.7 ± 0.1^{b}
20:1	0.9 ± 0.1^{a}	0.1 ± 0.1^{b}	1.5 ± 0.2^{c}	n.d.^{a}	1.5 ± 0.6$^{a\ b}$	0.7 ± 0.3^{b}	n.d.	n.d.	n.d.	1.2 ± 0.2^{a}	3.1 ± 0.5^{b}	2.3 ± 0.4$^{a\ b}$
Total MUFA	36.9 ± 1.8$^{a\ b}$	30.1 ± 1.3^{a}	41.6 ± 3.7^{b}	12.8 ± 0.5^{a}	15.3 ± 0.5^{b}	15.1 ± 0.4^{b}	17.9 ± 0.3	17.6 ± 0.2	17.9 ± 0.2	21 ± 0.4^{a}	23.2 ± 0.4^{b}	21.1 ± 0.2^{a}
PUFA												
n-6 PUFA												
18:2 n-6	12.4 ± 0.7	12 ± 0.5	11.1 ± 0.8	n.d.	n.d.	n.d.	0.3 ± 0.2	0.3 ± 0.2	0.1 ± 0	1 ± 0.1	1.2 ± 0.2	0.9 ± 0.2
18:3 n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.2 ± 0	0.3 ± 0	0.3 ± 0
20:3 n-6	0.5 ± 0.1^{a}	0.1 ± 0^{b}	0.7 ± 0.1^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.3 ± 0.1$^{a\ b}$	0.4 ± 0^{a}	0.1 ± 0^{b}
20:4 n-6	1.1 ± 0.1^{a}	0.2 ± 0.1^{b}	1.2 ± 0.1^{a}	5.7 ± 0.3^{a}	4.9 ± 0.5^{a}	8.5 ± 0.1^{b}	5.6 ± 0.6^{a}	5.7 ± 0.3^{a}	8.2 ± 0.3^{b}	7.1 ± 0.2^{a}	5.3 ± 0.3^{b}	7.7 ± 0.2^{a}
22:4 n-6	0.2 ± 0^{a}	n.d.^{b}	0.4 ± 0^{c}	0.4 ± 0.1^{a}	0.2 ± 0.1^{a}	2 ± 0.1^{b}	0.8 ± 0.2^{a}	0.5 ± 0.1^{a}	1.9 ± 0.1^{b}	1.7 ± 0.1^{a}	0.8 ± 0.1^{b}	2.3 ± 0.1^{c}
22:5 n-6	n.d.	n.d.	0.1 ± 0	n.d.^{a}	n.d.^{a}	7.5 ± 0.2^{b}	n.d.^{a}	n.d.^{a}	5.3 ± 0.2^{b}	n.d.^{a}	n.d.^{a}	5 ± 0.2^{b}
Total n-6 PUFA	14.3 ± 0.8	12.3 ± 0.6	13.4 ± 0.9	6.1 ± 0.4^{a}	5.1 ± 0.6^{a}	18 ± 0.4^{b}	6.8 ± 0.8^{a}	6.4 ± 0.3^{a}	15.4 ± 0.5^{b}	10.2 ± 0.3^{a}	8 ± 0.3^{b}	16.4 ± 0.3^{a}
n-3 PUFA												
18:3 n-3	1 ± 0.1	0.5 ± 0.2	0.5 ± 0.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:5 n-3	0.1 ± 0	0.3 ± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:5 n-3	0.2 ± 0$^{a\ b}$	0.5 ± 0.3^{a}	n.d.^{b}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:6 n-3	0.3 ± 0^{a}	2.4 ± 0.5^{b}	n.d.^{a}	10.8 ± 0.5^{a}	13.3 ± 1.1^{a}	4.7 ± 0.2^{b}	8 ± 0.5^{a}	10.6 ± 0.4^{b}	3.5 ± 0.3^{c}	9.3 ± 0.4^{a}	9.9 ± 0.6^{a}	4.5 ± 0.2^{b}
Total n-3 PUFA	1.5 ± 0.2^{a}	3.8 ± 1.1^{b}	0.5 ± 0.3^{a}	10.8 ± 0.5^{a}	13.3 ± 1.1^{a}	4.7 ± 0.2^{b}	8 ± 0.5^{a}	10.6 ± 0.4^{b}	3.5 ± 0.3^{c}	9.3 ± 0.4^{a}	10 ± 0.6^{b}	4.5 ± 0.2^{c}
Total PUFA	15.8 ± 0.9	16.1 ± 1.6	13.9 ± 1	17 ± 0.8^{a}	18.3 ± 1.7^{a}	22.7 ± 0.4^{b}	14.8 ± 1.3^{a}	17 ± 0.7$^{a\ b}$	18.9 ± 0.7^{b}	19.6 ± 0.6$^{a\ b}$	18 ± 0.9^{a}	20.8 ± 0.4^{b}
n6:n3	9.6 ± 0.9	4.2 ± 1.1	424.7 ± 343.1	0.6 ± 0	0.4 ± 0	3.9 ± 0.2	0.8 ± 0.1	0.6 ± 0	4.6 ± 0.3	1.1 ± 0	0.8 ± 0	3.7 ± 0.2

SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; n.d., not detected Superscripts with different letters are significantly different between treatment group

FIGURES

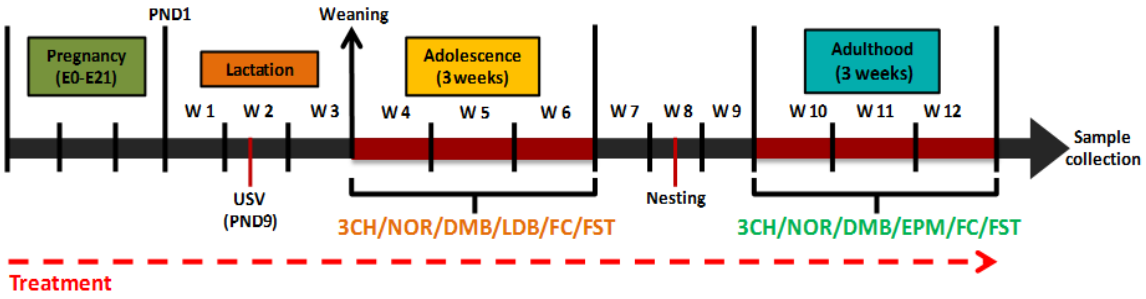


Figure 1. Experimental design. CON, O3+ or O3- diets were provided during the pre-natal and post-natal periods until adulthood. C57BL/6 (n=10 per group) male offspring underwent a battery of testing related to sociability, memory and learning, depression and anxiety. Isolation-induced ultrasonic vocalization (iUSV) was performed at PND9 and nesting behaviour was performed during week 8. At 13 weeks old, animals were sacrificed by cervical dislocation and samples collected.

Abbreviations: W, week; PND, Postnatal day; USV, Ultrasonic vocalization; N, 3CH, three chamber test; NOR, novel object recognition; DMB, defensive marble burying; LDB, light dark box; EPM, elevated plus maze; FC, fear conditioning; FST, forced swim test.

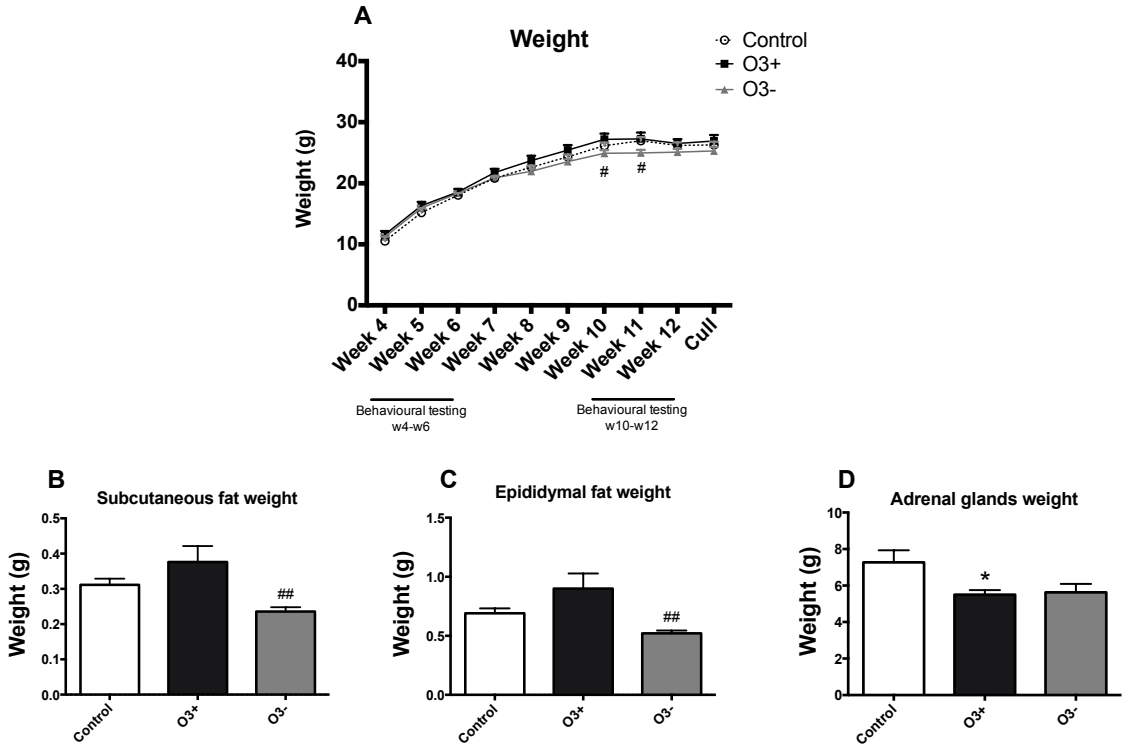


Figure 2. Effects of omega-3 status on body composition. **(A)** There were no significant differences between groups at sacrifice in body weight however O3- displayed reduced weight compared with O3+ during a battery of behavioural testing in adulthood (weeks 10-12)**.** O3- also resulted in significantly reduced subcutaneous **(B)** and epididymal **(C)** fat weight compared with O3+. **(D)** Adrenal gland weight was significantly reduced in O3+ compared with control. Data shown as mean ± SEM. n= 9-10 per group . *$p < 0.05$, CON vs. O3+ or O3- group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ O3+ vs. O3-.

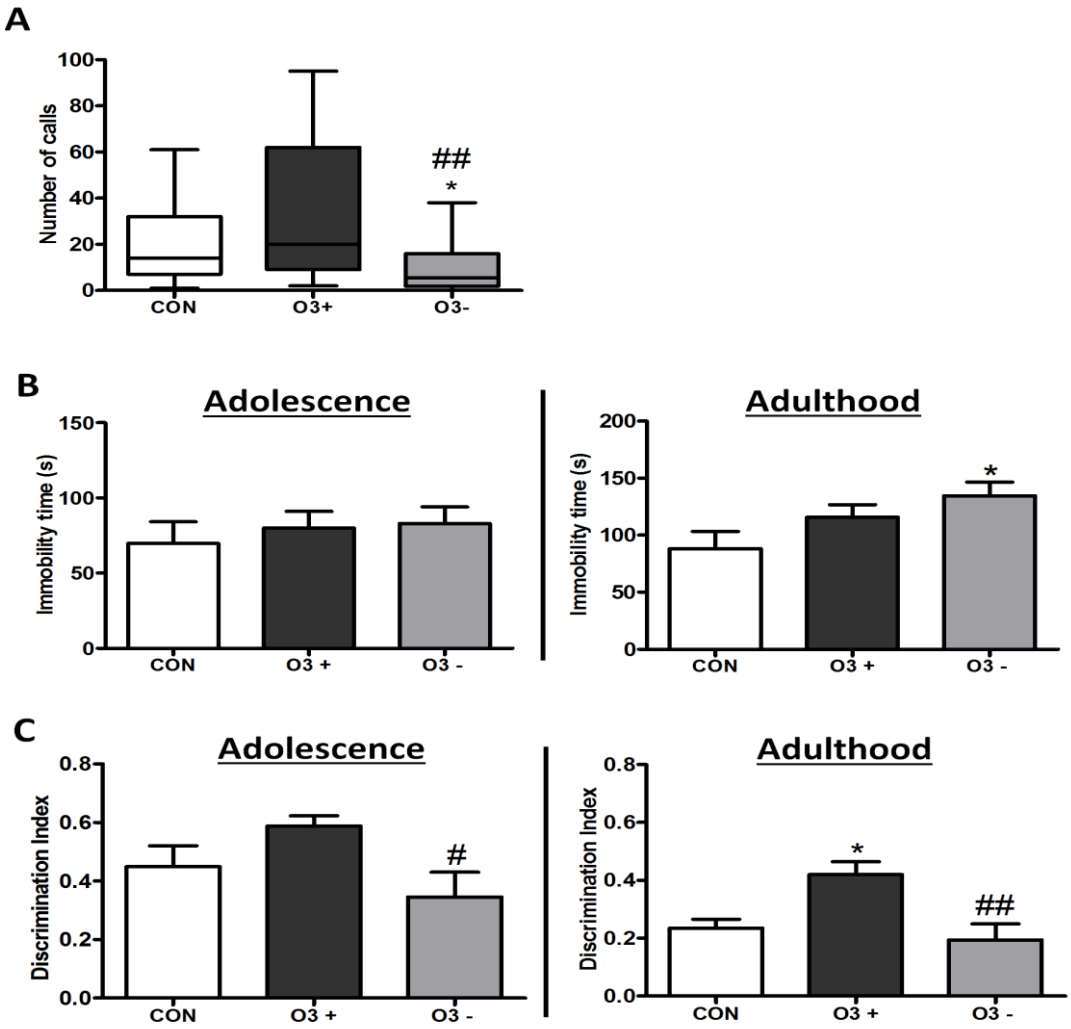


Figure 3. Effects of omega-3 dietary intervention on isolation-induced ultrasonic vocalizations, depression-like behaviour and cognition. **(A)** At PND9, O3- diet caused a significant reduction of number of calls in comparison to both the CON and the O3+ group (n= 9-10 per group). **(B)** Omega-3 dietary intervention caused no significant differences in immobility time during adolescence. However, dietary O3- caused a depression-like phenotype during the forced swim test compared with the CON group in adulthood (n=10 per group). **(C)** O3+ displayed enhanced memory compared with O3- in both adolescence and adulthood. Furthermore, O3+ enhanced memory compared with the CON in adulthood (n= 10 per group). Data shown as mean ± SEM. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ CON vs. O3- or O3+; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ O3- vs. O3+.

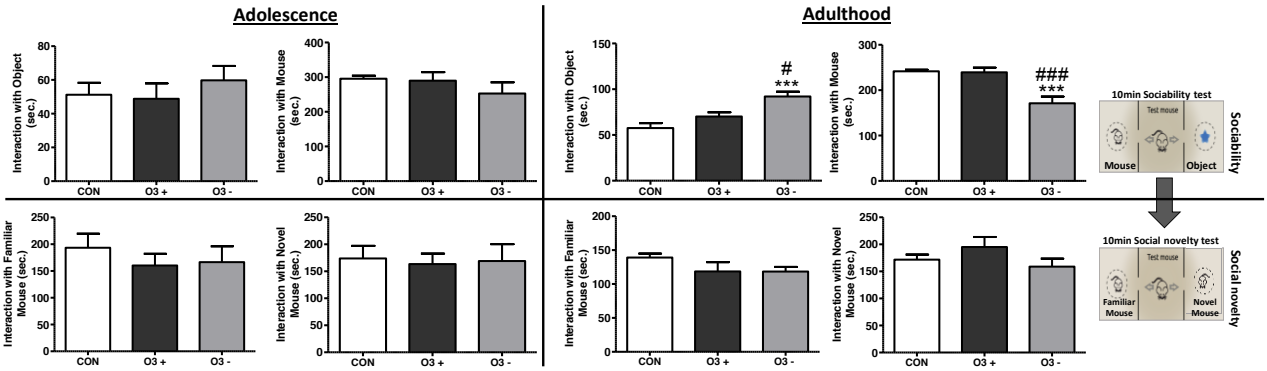


Figure 4. Effects of omega-3 dietary intervention on social behaviour. Omega-3 dietary intervention caused no significant differences on sociability and social novelty in adolescence. O3- caused sociability impairments compared with both the CON and O3+ groups in adulthood. However, there were no differences between different dietary interventions in social novelty during adulthood (n= 9-10 per group). Data shown as mean ± SEM. ***$p < 0.001$ CON vs. O3- or O3+; # $p < 0.05$, ### $p < 0.001$ O3- vs. O3+.

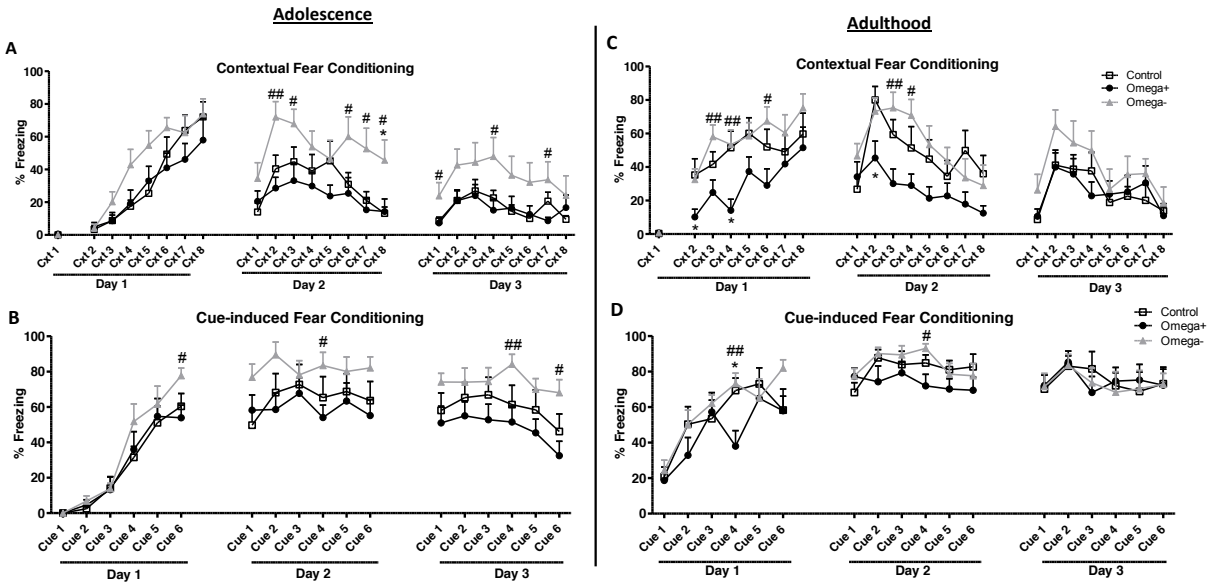


Figure 5. Fear Conditioning during Adolescence and Adulthood. During acquisition, day 1, in both context (A) and cue (B) components all the groups exhibited good learning at adolescence. There were no significant differences between groups in baseline freezing behaviour. During memory/extinction, days 2 and 3, O3- presented a significant higher % of freezing compared mainly to O3+ during both context (A) and cue (B) components. During acquisition, day 1 in adulthood, in context component (C), no differences were observed between groups in the baseline freezing behaviour. . Moreover, O3- froze significantly higher than O3+, but overall all groups presented good learning as shown in the cue component (D). During day 2 of memory/extinction, O3- group presented a significant higher % of freezing for few cues compared mainly to O3+ during both context (C) and cue (D) components. At day 3 of memory/extinction, all the groups presented the same % of freezing. Data shown as mean ± SEM. n= 9-10 per group. Two-way ANOVA repeated measures followed by Tukey HSD post-hoc test. *$p < 0.05$, CON vs. O3- or O3+ group; $^{\#}$ $p < 0.05$, O+ vs. O3-; $^{\#\#}$ $p < 0.01$ O3+ vs. O3-.

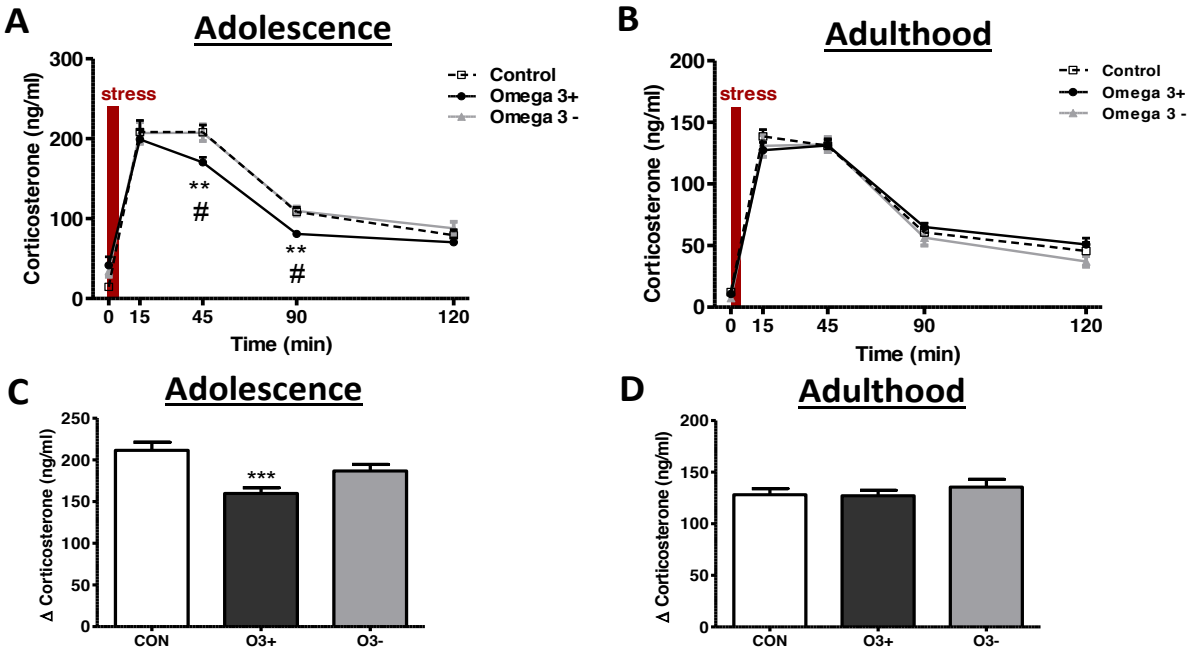


Figure 6. Effects of omega-3 dietary intervention on HPA-axis response to acute stress. (A) O3+ enhanced corticosterone recovery in response to acute stress compared to CON and O3- in adolescence. **(B)** However, there were no differences in corticosterone recovery between the different dietary interventions with respect to time during adulthood. **(C)** O3+ presented significantly decreased delta corticosterone in comparison with CON in adolescence. Nonetheless, dietary intervention showed no significant differences in delta corticosterone in adulthood **(D)**. Data shown as mean ± SEM. n=7-9 per group, ** $p < 0.01$, ***$p < 0.001$ CON vs. O3- or O3+; # $p < 0.05$ O3+ vs. O3-.

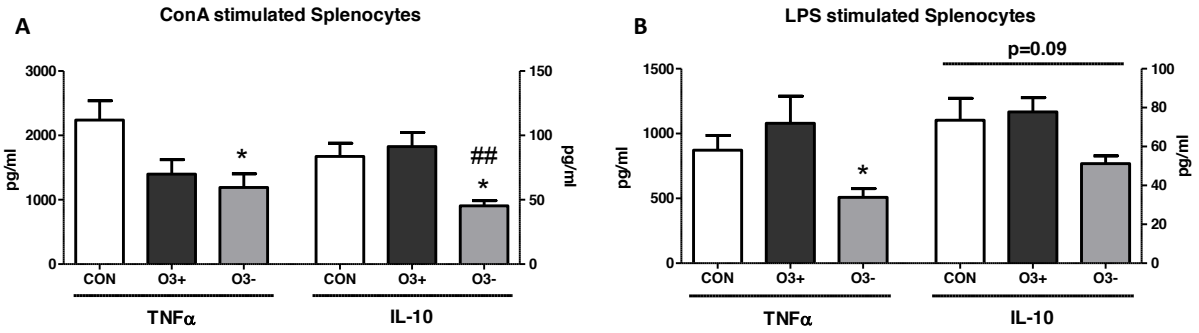


Figure 7. Effects of omega-3 dietary intervention on spleen cytokine production following stimulation with lipopolysaccharide (LPS) and concanavalin A (ConA). O3+ caused significant differences on cytokine release levels of tumor necrosis factor (TNFα) and Interleukin 10 (IL-10) after LPS and ConA stimulation. **(A)** O3+ caused no effects on the release of TNFα and IL-10 after ConA stimulation, while O3- significantly decreased spleen levels of TNFα and IL-10 after stimulation with ConA compared with the controls. Moreover, O3- presented a significant decrease of IL-10 after stimulation with ConA compared to the O3+ group. **(B)** O3- significantly decreased spleen levels of TNFα after stimulation with LPS in comparison to the CON group. There was a trend to decreased levels of IL-10 cytokine in the O3- group after stimulation with LPS (p=0.09). Data shown as mean ± SEM. n= 8-10 per group. *$p < 0.05$, CON vs. O3- or O3+ group; ## $p < 0.01$ O3+ vs. O3-.

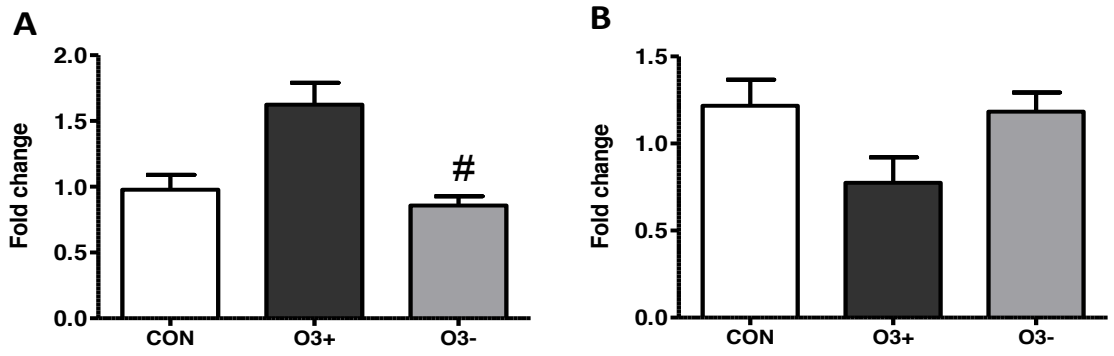


Figure 8. Effects of omega-3 dietary intervention on gene expression of *CD206* in Hippocampus and Amygdala. **(A)** O3- group had significant lower mRNA expression of *CD206*, a microglial anti-inflammatory marker, in the hippocampus compared to the O3+ group. **(B)** There was no effect of dietary intervention on the mRNA expression of *CD206* in Amygdala. Data shown as mean ± SEM. n= 7-8 per group. # $p < 0.05$ O3+ vs. O3-.

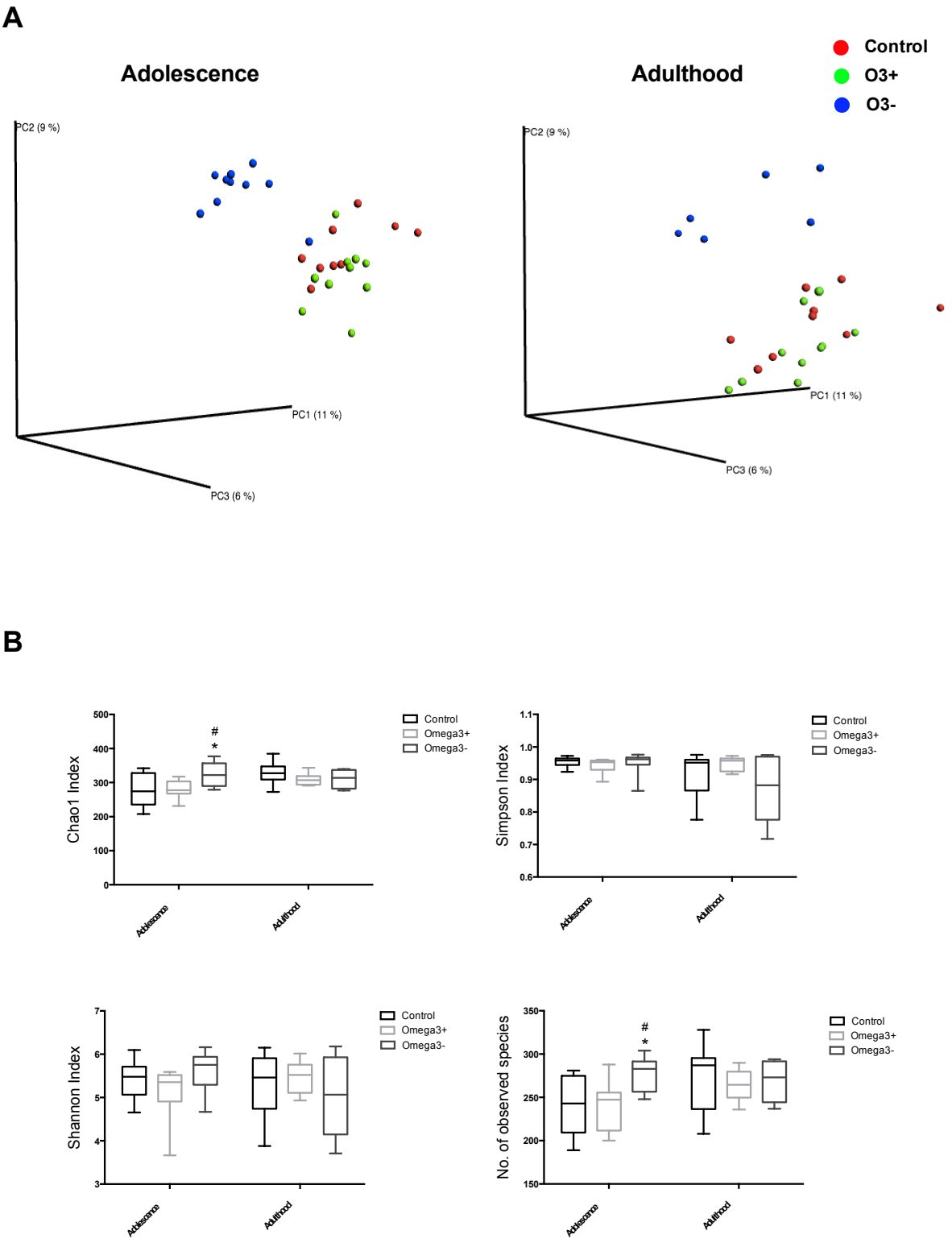


Figure 9. Effects of omega-3 status on fecal gut microbiota diversity. Principle coordinate analysis (PCoA) of gut microbiota composition based on unweighted UniFrac revealed distinct separation and clustering of the O3- group away from both CON and O3+ groups **(A)**. Analysis of adolescent fecal microbiota revealed significantly greater α diversity in the O3- groups compared with both CON and O3+ as measured by the chao1 and number of observed species indices ($p<0.05$) **(B)**. No significant differences in α diversity were observed in at adulthood. n=6-10 per group. Analysis by non parametric Kruskall Wallis test followed by multiple comparisons by Mann-Whitney. * significantly different to control. # significantly different to O3+.

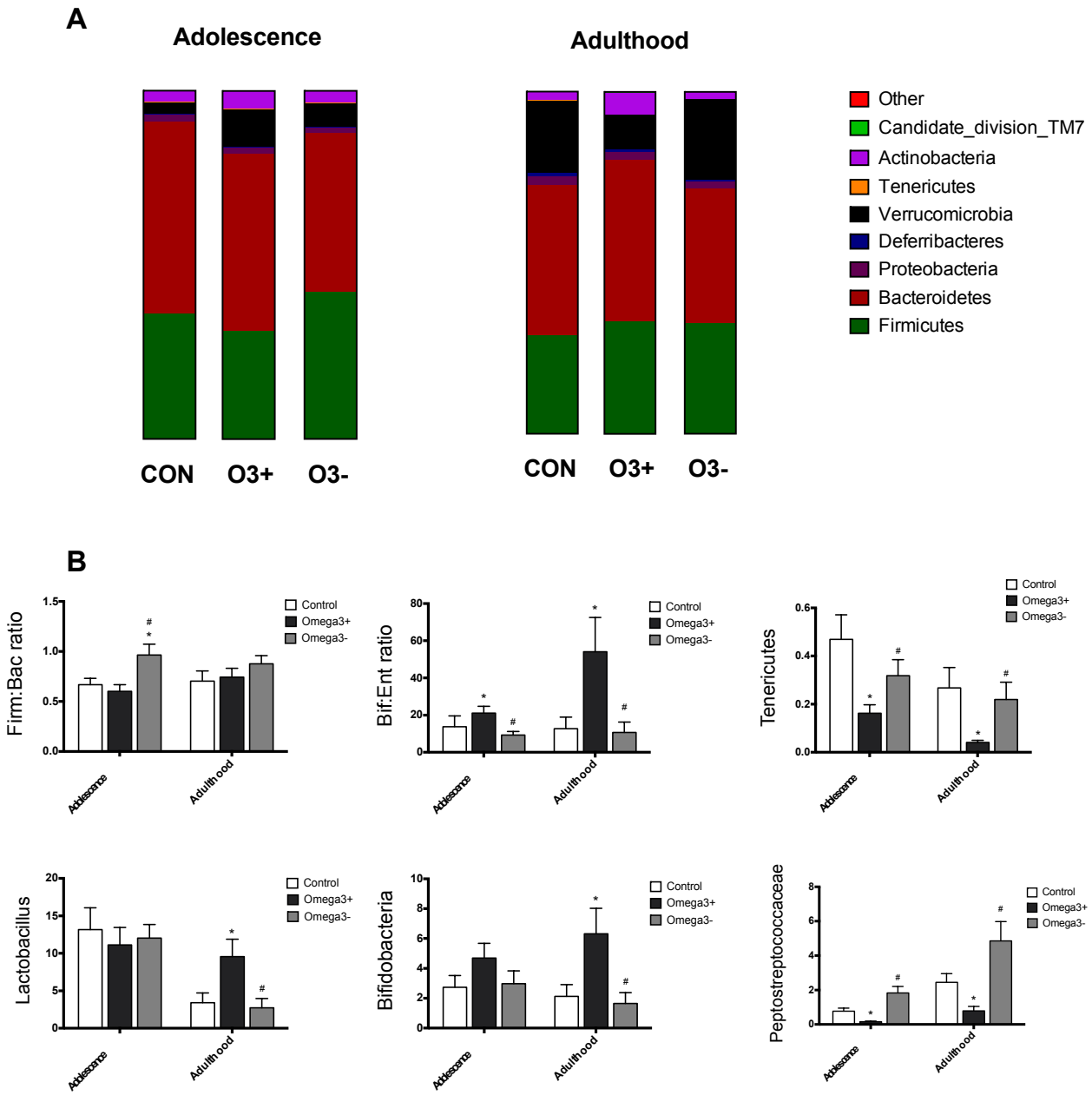


Figure 10. Effects of omega-3 status on fecal gut microbiota composition. Phylum level distributions were significantly altered by omega-3 status at both adolescence and adulthood **(A)**. Family and genus level distributions were also significantly altered at adolescence and these changes were further strengthened into adulthood **(B)**. Analysis by non parametric Kruskall Wallis test followed by multiple comparisons by Mann-Whitney. * significantly different to control. # significantly different to O3+.

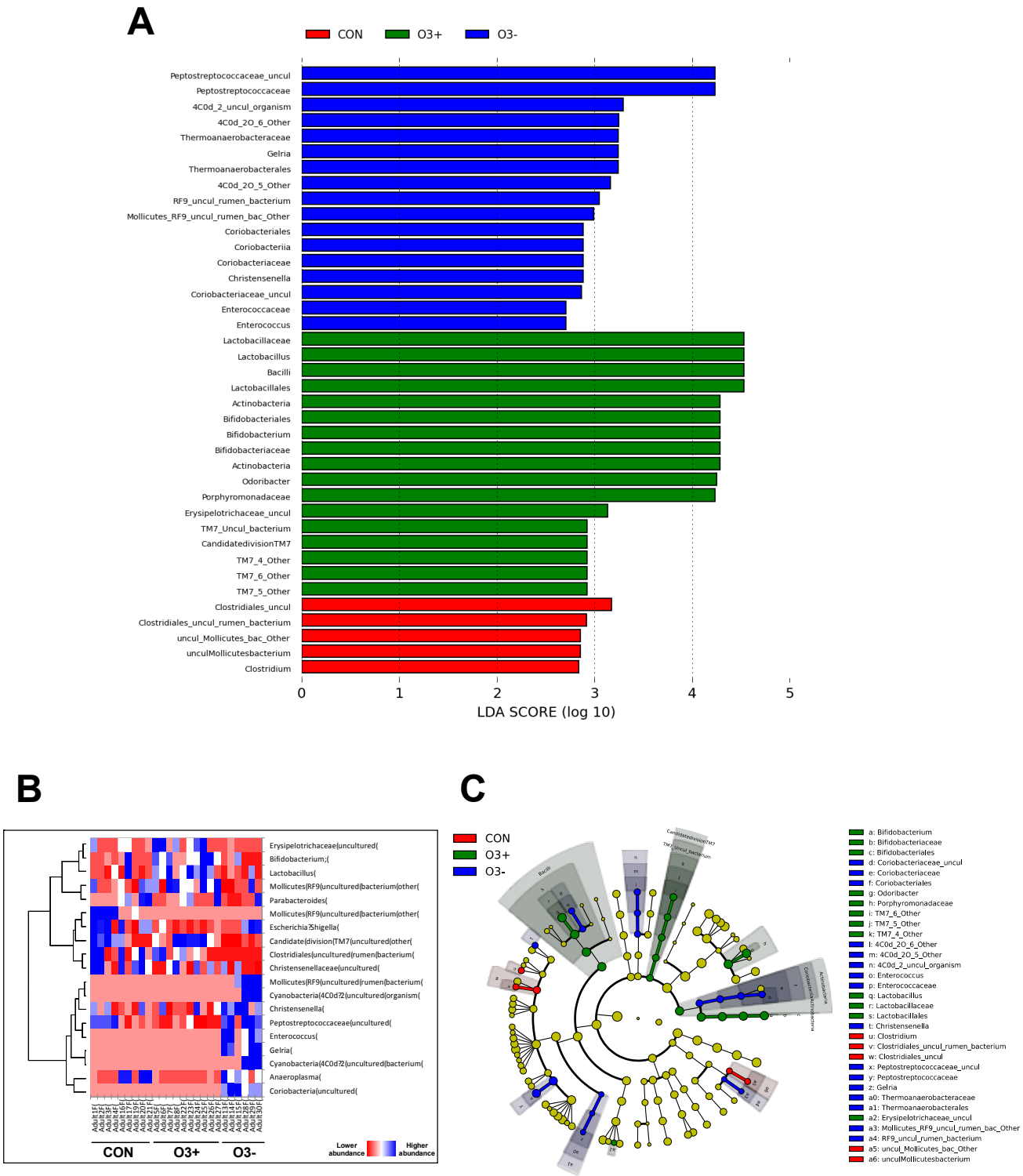


Figure 11. LDA scores of gut microbiota composition following omega-3 interventions. LDA scores of taxa significantly altered by omega-3 status in adulthood are shown in **(A)** (LDA scores >2 and significance of $p<0.05$ as determined by Wilcoxon signed-rank test). Heatmap distribution of significantly altered taxa in adulthood reveals distinct differences between treatment groups **(B)**. The most differentially abundant taxa in adulthood following dietary intervention are represented as a cladogram in **(C)** as generated from LEfSe data in **(A)**.

SUPPLEMENTARY

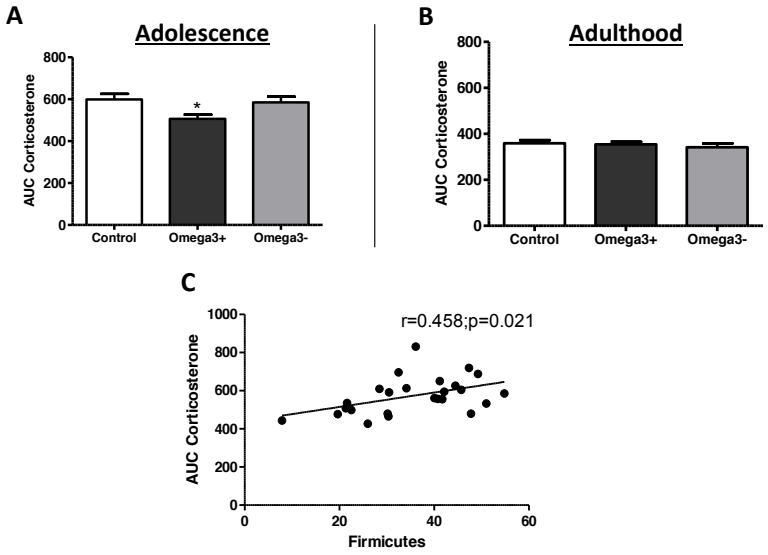


Figure S1. Correlations between AUC of plasma corticosterone levels after stress and fecal microbiota in adolescence. O3+ showed significantly decreased AUC in comparison to CON in adolescence **(A)**. However, dietary intervention showed no significant differences in AUC in adulthood **(B)**. Regression analysis revealed positive correlation between corticosterone AUC and Firmicutes **(C)**. One-way ANOVA followed by Tukey HSD post-hoc test.

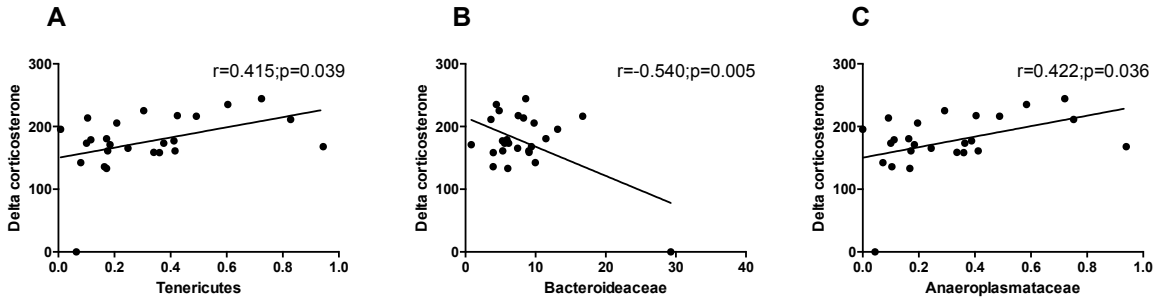


Figure S2. Correlations between delta corticosterone and fecal microbiota in adolescence. Regression analysis revealed positive correlation between delta corticosterone and Tenericutes **(A)** and Anaeroplasmataceae **(C)**. Furthermore, regression analysis revealed negative correlation between delta corticosterone and Bacteroideaceae **(B)**.

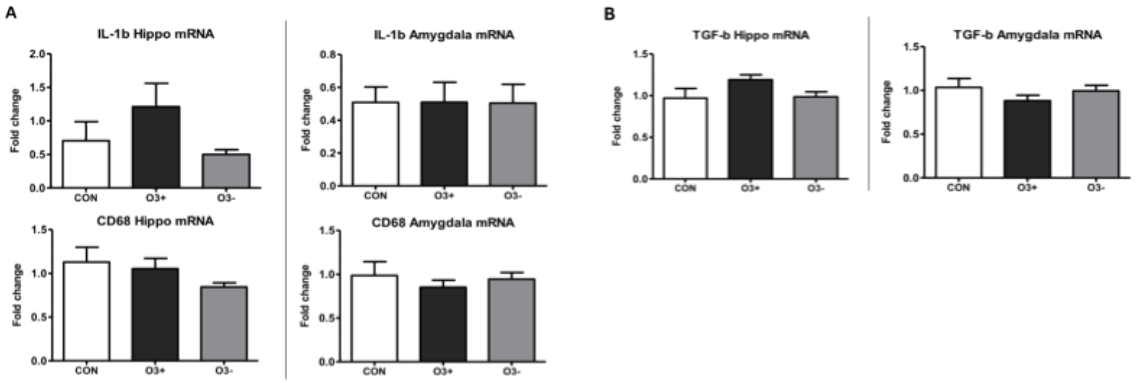


Figure S3. Effects of omega-3 dietary interventions on microglia gene expression in Hippocampus and Amygdala. **(A)** Omega-3 dietary intervention causes no significant differences on microglial pro-inflammatory markers mRNA expression and **(B)** no significant differences on the mRNA expression of TGF-b, a microglial anti-inflammatory marker, in both Hippocampus and Amygdala.

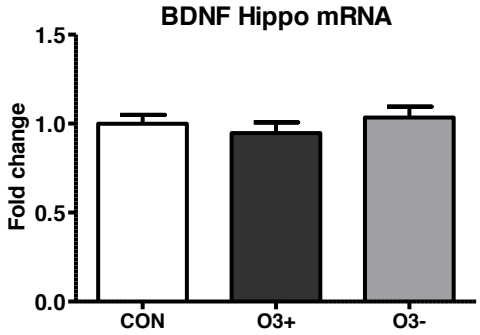


Figure S4. Effects of omega-3 dietary interventions on BDNF gene expression in the Hippocampus. No significant differences were observed for BDNF mRNA between groups.

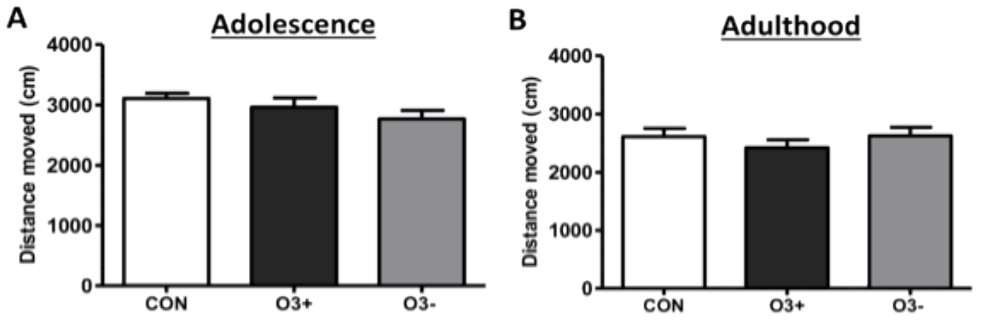


Figure S5. Effects of omega-3 dietary intervention on locomotor activity during adolescence and adulthood. There were no significant differences between groups in the total distance moved in the open field during both adolescence **(A)** and adulthood **(B).**

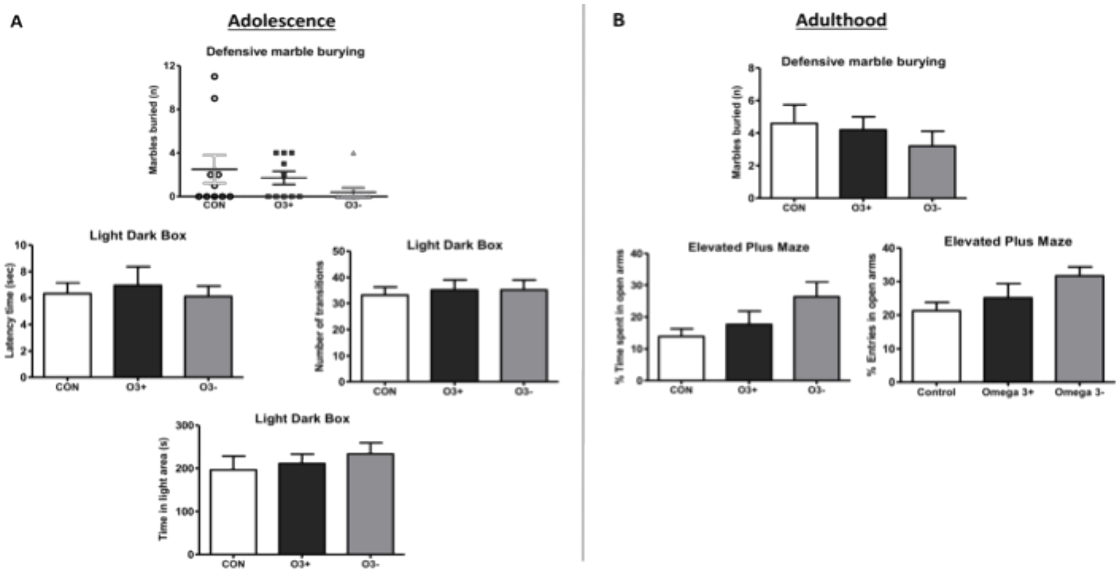


Figure S6. Effects of omega-3 dietary intervention on anxiety-like behaviours in both adolescence and adulthood. (A) In adolescence, omega-3 dietary intervention caused no significant differences on the number of marbles buried in the defensive marble burying (DMB) test. Moreover, no significant changes were seen in the latency time, number of transitions and time spent in light area in the light dark box (LDB). **(B)** In adulthood, no significant differences were found in DMB. Furthermore, there were no significant differences in the % of time spent and % of entries in open arms during EPM.

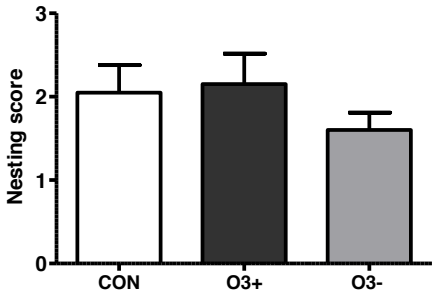


Figure S7. Effects of omega-3 dietary intervention on nesting. Omega-3 dietary intervention showed no effect on the nest quality at week 8.

Table S1. Diet macronutrient profiles

	Control	O3+	O3-
Protein (% by weight)	18.3	18.3	18.3
Carbohydrate (% by weight)	56.7	56.5	56.7
Fat (% by weight)	10	10	10
Protein (% of Kcal)	18.8	18.8	18.8
Carbohydrate (% of Kcal)	58.2	58	58.2
Fat (% of Kcal)	23.1	23.2	23.1
Kcal/g	3.9	3.9	3.9
LA (% by weight)	2.6	2.6	2.6
ALA (% by weight)	0.4	0.4	0
EPA (% by weight)	0	0.35	0
DHA (% by weight)	0	0.66	0
n-6 (% by weight)	2.6	2.6	2.6
n-3 (% by weight)	0.4	1.53	0

Abbreviations: O3+, Omega 3 polyunsaturated fatty acid supplemented diet; O3-, Omega-3 polyunsaturated fatty acid deficient diet; LA, Linoleic acid (C18:2 n-6) ; ALA, Alpha linolenic acid (C18:3 n-3); EPA, Eicosapentaenoic acid (C20:5 n-3); DHA, Docosahexaenoic acid (C22:6 n-3); n-6, n-6 polyunsaturated fatty acids; n-3, n-3 polyunsaturated fatty acids

Table S2. Fatty acid profile of diets

% Total fatty acids	Control	O3+	O3-
Saturated fatty acids (SFA)			
8:0	3.4	2.2	3.6
10:0	3.1	2	3.3
12:0	27	16.9	29
14:0	11.1	7.25	12.3
16:0	9	10.9	8.5
18:0	7.9	5.9	8.7
Total SFA	59.8	44	64.4
Monounsaturated fatty acids (MUFA)			
18:1	7.1	13.3	6.5
Total MUFA	7.3	13.3	6.7
Polyunsaturated fatty acids (PUFA)			
18:2 n-6	25.7	25.4	22.4
18:3 n-3	4.2	4.1	tr
20:4 n-6	0	0.26	0
20:5 n-3	0	3.2	0
22:5 n-3	0	1	0
22:6 n-3	0	5.7	0
n6	25.7	25.66	22.4
n3	4.2	14	0
Total PUFA	29.9	39.66	22.4

Table S3. Primer sequences and accession numbers.

	Accession number	Forward primer	Reverse primer
B-Actin	NM_007393.4	GTGCTTCTAGGCGGACTGTTAC	GCTCCAACCAACTGCTGTC
IL-1β	NM_008361.3	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
CD68	NM_001291058.1	GGGGCTCTTGGGAACTACAC	GTACCGTCACAACCTCCCTG
CD206	NM_008625.2	GTGGGGACCTGGCAAGTATC	CACTGGGGTTCCATCACTCC
TGF-β	NM_011577.1	CGTCAGACATTCGGGAAGCA	TGCCGTACAACTCCAGTGAC
CX3CR1	NM_009987.4	GGAAGCCCAAGAGCATCACT	GTTGTTCATGGAGTTGGCGG
CX3CL1	NM_009142.3	TCCCCAGAAACTGAGTGTGC	CTACCATTTCCCCCGCCATT

Table S4. Fatty acid composition of selected brain regions

	Frontal Cortex			Striatum			Hypothalamus			Cerebellum			Brain stem		
	Control	**O3+**	**O3-**	**Control**	**O3+**	**O3-**	**Control**	**O3+**	**O3-**	**Control**	**O3+**	**O3-**	**Control**	**O3+**	**O3-**
SFA															
14:0	$0.6 \pm 0^{a\ b}$	0.7 ± 0.1^{a}	0.6 ± 0^{b}	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0	1.1 ± 0	1.1 ± 0.2	0.8 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0
16:0	$28.2 \pm 0.1^{a\ b}$	29.1 ± 0.5^{a}	27.8 ± 0.3^{b}	34.5 ± 0.6	34.2 ± 0.6	33 ± 0.7	38.9 ± 0.3	38.8 ± 0.9	38.4 ± 0.8	28.7 ± 0.4	28.5 ± 0.1	27.9 ± 0.2	23.9 ± 0.4	24.6 ± 0.3	23.6 ± 0.3
18:0	$24.8 \pm 0.1^{a\ b}$	25.2 ± 0.3^{a}	24.2 ± 0.1^{b}	29.2 ± 0.3	28.7 ± 0.4	28.4 ± 0.3	31.1 ± 0.1	31.2 ± 0.4	30.8 ± 0.4	25.1 ± 0.2^{a}	24.5 ± 0.1^{b}	$24.6 \pm 0.1^{a\ b}$	23.7 ± 0.2	23.8 ± 0.2	23.8 ± 0.2
20:0	0.3 ± 0	0.3 ± 0	0.3 ± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1 ± 0	0.1 ± 0	0.2 ± 0	0.6 ± 0^{a}	0.6 ± 0^{a}	0.8 ± 0^{b}
Total SFA	$53.9 \pm 0.3^{a\ b}$	55.3 ± 0.8^{a}	52.8 ± 0.3^{b}	64.2 ± 0.9	63.3 ± 1	61.8 ± 1	71.2 ± 0.4	71.2 ± 1.3	70 ± 1.2	54.3 ± 0.6	53.4 ± 0.2	53 ± 0.3	48.8 ± 0.6	49.4 ± 0.4	48.5 ± 0.5
MUFA															
16:1	0.7 ± 0	0.8 ± 0	0.7 ± 0	n.d.	0.1 ± 0	0.1 ± 0	0.1 ± 0	n.d.	n.d.	0.1 ± 0	0.2 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0
18:1t n-9	$0.3 \pm 0^{a\ b}$	0.3 ± 0^{a}	0.2 ± 0^{b}	n.d.	n.d.	n.d.	0.1 ± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18:1^{c} n-9	15.5 ± 0.1^{a}	16 ± 0.2^{b}	14.9 ± 0.2^{c}	13.3 ± 0.3	14.3 ± 0.4	13.5 ± 0.3	15 ± 0.2	15.2 ± 0.6	15.6 ± 0.5	17.6 ± 0.2^{a}	19 ± 0.1^{b}	16.4 ± 0.1^{c}	24.6 ± 0.2^{a}	25.7 ± 0.1^{b}	23.3 ± 0.1^{c}
18:1 t n-7	3.4 ± 0^{a}	3 ± 0.1^{b}	3.6 ± 0^{c}	2.5 ± 0.1^{a}	2.4 ± 0.1^{a}	2.9 ± 0.1^{b}	2.1 ± 0	2.2 ± 0.1	2.4 ± 0.2	4.1 ± 0.1^{a}	3.7 ± 0^{b}	4.6 ± 0.1^{c}	4.7 ± 0.1^{a}	4.3 ± 0.1^{b}	4.9 ± 0.1^{c}
20:1	0.9 ± 0	0.9 ± 0.1	0.9 ± 0	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	n.d.	0.3 ± 0.1	0.2 ± 0.1	2.1 ± 0.1	2.3 ± 0.1	2.1 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	4.8 ± 0.1
Total MUFA	20.8 ± 0.1	21 ± 0.2	20.3 ± 0.2	16.4 ± 0.4	17.3 ± 0.6	17.2 ± 0.4	17.2 ± 0.3	17.7 ± 0.8	18.3 ± 0.6	24 ± 0.4^{a}	25.2 ± 0.2^{b}	23.3 ± 0.1^{c}	34 ± 0.3^{a}	35 ± 0.2^{b}	33.1 ± 0.3^{c}
PUFA															
n-6 PUFA															
18:2 n-6	0.9 ± 0.1^{a}	$0.8 \pm 0^{a\ b}$	0.7 ± 0^{b}	n.d.$^{a\ b}$	0.2 ± 0.1^{a}	n.d.^{b}	n.d.	n.d.	0.2 ± 0.1	0.4 ± 0^{a}	0.4 ± 0^{a}	0.3 ± 0^{b}	0.4 ± 0	0.4 ± 0	0.3 ± 0
18:3 n-6	0.1 ± 0	0.2 ± 0	0.1 ± 0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:3 n-6	0.4 ± 0^{a}	0.5 ± 0^{b}	0.2 ± 0^{c}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.1 ± 0^{a}	0.1 ± 0^{a}	n.d.^{b}	0.2 ± 0^{a}	0.3 ± 0^{a}	0.1 ± 0^{b}
20:4 n-6	7.9 ± 0.1^{a}	6.2 ± 0.1^{b}	9.1 ± 0.1^{c}	7.2 ± 0.2^{a}	5.9 ± 0.2^{b}	8.3 ± 0.2^{c}	$4.1 \pm 0.1^{a\ b}$	3.2 ± 0.2^{a}	4.8 ± 0.3^{b}	6.2 ± 0.1^{a}	4.2 ± 0.1^{b}	8.1 ± 0.1^{c}	5.3 ± 0.1^{a}	3.7 ± 0.1^{b}	6.2 ± 0.1^{c}
22:4 n-6	2 ± 0^{a}	1.2 ± 0^{b}	2.9 ± 0.1^{c}	1.4 ± 0.1^{a}	0.7 ± 0.1^{b}	2.3 ± 0.1^{c}	0.9 ± 0^{a}	0.4 ± 0.1^{b}	1.5 ± 0.1^{c}	1 ± 0.1^{a}	0.4 ± 0^{b}	2.3 ± 0^{c}	2.2 ± 0.1^{a}	1.1 ± 0^{b}	3.7 ± 0.1^{c}
22:5 n-6	n.d.^{a}	0.1 ± 0^{a}	8 ± 0.2^{b}	n.d.^{a}	n.d.^{a}	5.3 ± 0.2^{b}	n.d.^{a}	n.d.^{a}	2.1 ± 0.2^{b}	n.d.^{a}	n.d.^{a}	5.6 ± 0.2^{b}	n.d.^{a}	n.d.^{a}	3.9 ± 0.1^{b}
Total n-6 PUFA	11.4 ± 0.1^{a}	8.9 ± 0.2^{b}	21.1 ± 0.3^{c}	8.7 ± 0.3^{a}	6.8 ± 0.3^{b}	15.9 ± 0.5^{c}	5 ± 0.1^{a}	3.6 ± 0.3^{a}	8.5 ± 0.5^{b}	7.6 ± 0.2^{a}	5.2 ± 0.1^{b}	16.3 ± 0.2^{c}	8 ± 0.2^{a}	5.4 ± 0.2^{b}	14.1 ± 0.3^{c}
n-3 PUFA															
18:3 n-3	n.d.	n.d.	n.d.	n.d.	n.d.	0.3 ± 0.3	n.d.	n.d.	n.d.	n.d.	0.4 ± 0.2	0.5 ± 0.2	n.d.	n.d.	n.d.
20:5 n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:5 n-3	n.d.	0.1 ± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.2 ± 0	n.d.
22:6 n-3	14 ± 0.2^{a}	14.7 ± 0.5^{a}	5.8 ± 0.2^{b}	10.7 ± 0.3^{a}	12.6 ± 0.5^{b}	4.8 ± 0.2^{c}	6.5 ± 0.1^{a}	7.5 ± 0.4^{a}	3.2 ± 0.2^{b}	14.1 ± 0.1^{a}	15.8 ± 0.2^{b}	7 ± 0.2^{c}	9.2 ± 0.2^{a}	10.1 ± 0.2^{b}	4.3 ± 0.1^{c}
Total n-3 PUFA	14 ± 0.2^{a}	14.8 ± 0.6^{a}	5.8 ± 0.2^{b}	10.7 ± 0.3^{a}	12.6 ± 0.5^{b}	5 ± 0.3^{c}	6.5 ± 0.1^{a}	7.5 ± 0.4^{a}	3.2 ± 0.2^{b}	14.1 ± 0.1^{a}	16.3 ± 0.3^{b}	7.4 ± 0.3^{c}	9.2 ± 0.2^{a}	10.3 ± 0.2^{b}	4.3 ± 0.1^{c}
Total PUFA	25.4 ± 0.2^{a}	23.7 ± 0.7^{b}	26.8 ± 0.3^{a}	19.4 ± 0.5	19.4 ± 0.8	20.9 ± 0.6	11.6 ± 0.3	11.1 ± 0.7	11.7 ± 0.7	21.7 ± 0.3^{a}	21.4 ± 0.2^{b}	23.7 ± 0.3^{c}	17.2 ± 0.4^{a}	15.7 ± 0.3^{b}	18.4 ± 0.3^{c}
n6:n3	0.8 ± 0	0.6 ± 0	3.7 ± 0.2	0.8 ± 0	0.5 ± 0	3.2 ± 0.2	0.8 ± 0	0.5 ± 0	2.7 ± 0.2	0.5 ± 0	0.3 ± 0	2.2 ± 0.1	0.9 ± 0	0.5 ± 0	3.3 ± 0.1

Table S5. Relative abundance of genera from adolescent microbiota

	Adolescence				
Genus	**CON**	**O3+**	**O3-**	**p-value**	**q-value**
Alistipes	16.3	17.2	16.5	0.89	1
Bacteroidales S24-7 uncultured	15.8	16.2	10.7	**0.004**	**0.047**
Lactobacillus	13.2	11.1	12.0	0.772	1
Bacteroides	12.0	7.09	5.97	**0.034**	0.216
Lachnospiraceae uncultured	6.01	6.64	9.32	**0.046**	0.233
Odoribacter	5.91	4.80	5.9	0.287	0.574
Ruminococcaceae uncultured	4.76	4.03	6.03	0.242	0.529
Rikenellaceae RC9 gut group	3.36	3.56	3.97	0.827	1
Akkermansia	3.21	10.86	6.66	0.623	0.907
Allobaculum	3	3.51	4.78	0.776	1
Bifidobacterium;	2.74	4.69	2.98	0.13	0.406
Ruminococcaceae Incertae Sedis	1.8	1.46	2.52	0.177	0.477
Clostridium	1.43	0.2	1.07	**0.006**	0.06
Rikenella	1.21	1.53	1.93	0.205	0.526
Parasutterella	1.10	0.86	0.55	0.227	0.526
Turicibacter	0.790	0.307	0.722	0.321	0.607
Peptostreptococcaceae uncultured	0.763	0.156	1.828	**<0.001**	**0**
Lachnospiraceae Incertae Sedis	0.717	0.688	0.624	0.833	1
Oscillibacter	0.709	0.427	0.794	0.09	0.35
Parabacteroides	0.656	0.645	0.527	0.893	1
Clostridiales uncultured bacterium	0.643	0.782	0.528	0.253	0.537
Anaerotruncus	0.512	0.404	0.600	0.267	0.550
Anaeroplasma	0.457	0.153	0.292	**0.05**	0.233
Desulfovibrio	0.443	0.296	0.258	0.14	0.406
Family XIII Incertae Sedis	0.442	0.293	0.226	**0.022**	0.171
Coprococcus	0.352	0.203	0.362	**0.039**	0.228
Escherichia-Shigella	0.284	0.526	0.358	0.858	1
Mucispirillum uncultured	0.240	0.081	0.099	0.22	0.526
Roseburia	0.226	0.134	0.286	**0.034**	0.216
Peptococcaceae uncultured	0.139	0.103	0.133	0.454	0.722
Candidate division TM7 uncultured other	0.128	0.212	0.206	0.229	0.526
Ruminococcus	0.113	0.076	0.114	0.438	0.713
Enterorhabdus	0.110	0.129	0.148	0.674	0.944
Bilophila	0.101	0.130	0.365	**0.007**	0.061
Anaerovorax	0.0880	0.0708	0.122	0.086	0.350
Christensenellaceae uncultured	0.0627	0.0616	0.0372	0.233	0.526
Family XIII Incertae Sedis uncultured	0.0627	0.0984	0.0492	0.833	1
Thalassospira	0.0587	0.0676	0.0592	0.635	0.907
Marvinbryantia	0.0347	0.0012	0.002	0.145	0.406
Christensenella	0.0289	0.0388	0.0428	0.073	0.319
Peptococcus	0.0244	0.0196	0.0408	0.112	0.406
Unidentified	0.0187	0.0152	0.0232		
Mollicutes RF9 uncultured bacterium other	0.0124	0.0092	0.026	0.137	0.406
Erysipelotrichaceae uncultured	0.0116	0.0056	0.0048	0.499	0.759
Lachnospiraceae uncultured bacterium	0.0111	0.0116	0.018	0.705	0.968
Pseudobutyrivibrio	0.0076	0	0.1164	**<0.001**	**0**
Erysipelotrichaceae Incertae Sedis	0.0040	0.0164	0.0104	0.141	0.406
Ruminococcaceae uncultured bacterium	0.0027	0.0004	0.0032	**0.049**	0.233
Clostridiales uncultured rumen bacterium	0.0018	0.0016	0	0.307	0.597
Hydrogenoanaerobacterium	0.0009	0.0024	0.0016	0.495	0.759
Enterococcus	0.0004	0	0.0416	**0.003**	**0.042**

Streptococcus	0.0004	0.0004	0.0008	0.996	1
Clostridiaceae uncultured	0.0004	0.0016	0.0012	0.587	0.874
Mogibacterium	0.0004	0.0016	0.0016	0.389	0.648
Coriobacteria uncultured	0	0	0.0452	**<0.001**	**0**
Barnesiella	0	0.0008	0	0.139	0.406
Prevotella	0	0	0	1	1
Prevotellaceae uncultured	0	0	0	1	1
Flavobacterium	0	0	0	1	1
Cyanobacteria 4C0d-2 uncultured bacterium	0	0	0	1	1
Cyanobacteria 4C0d-2 uncultured organism other	0	0	0	1	1
Staphylococcus	0	0.0012	0.0008	0.387	0.648
Aerococcus	0	0	0	1	1
Lactococcus	0	0	0	1	1
Candidatus Arthromitus	0	0	0.0064	0.387	0.648
Anaerostipes	0	0	0	1	1
Blautia	0	0	0.0012	0.387	0.648
Gelria	0	0	0.0264	**<0.001**	**0**
alpha proteobacterium	0	0	0	1	1
Mollicutes RF9 uncultured bacterium other	0	0	0	1	1
Mollicutes RF9 uncultured rumen bacterium other	0	0	0.0004	0.387	0.648

Table S6. Relative abundance of genera from adulthood microbiota

	Adulthood				
Genus	**CON**	**O3+**	**O3-**	**p-value**	**q-value**
Akkermansia	21	10.2	23.4	0.309	0.585
Bacteroidales S24-7 uncultured	14.3	15.6	12.1	0.304	0.585
Alistipes	11.2	13.7	12.9	0.619	0.833
Odoribacter	8.55	8.61	5.95	0.096	0.292
Lachnospiraceae uncultured	7.43	7.25	7.66	0.816	0.952
Ruminococcaceae uncultured	4.93	5.14	6.27	0.651	0.853
Bacteroides	4.07	3.39	2.93	0.495	0.722
Lactobacillus	3.4	9.56	2.72	**0.015**	0.081
Ruminococcaceae Incertae Sedis	3.04	2.72	3.42	0.57	0.782
Allobaculum	2.62	4.33	2.75	0.178	0.429
Rikenellaceae RC9 gut group	2.60	2.53	3.03	0.883	0.966
Rikenella	2.53	2.47	2.01	0.67	0.853
Peptostreptococcaceae uncultured	2.44	0.78	4.85	**0.002**	**0.028**
Bifidobacterium	2.13	6.32	1.66	**0.004**	**0.047**
Oscillibacter	1.02	0.47	1.13	0.093	0.292
Mucispirillum uncultured	0.983	0.6	0.245	0.56	0.782
Parasutterella	0.868	1.208	1.12	0.385	0.654
Lachnospiraceae Incertae Sedis	0.790	0.471	0.624	0.196	0.429
Desulfovibrio	0.668	0.769	0.31	0.181	0.429
Anaerotruncus	0.652	0.56	0.653	0.666	0.853
Clostridiales uncultured bacterium	0.572	0.419	0.279	0.123	0.359
Parabacteroides	0.506	0.499	0.257	**0.028**	0.14
Bilophila	0.471	0.254	0.318	0.737	0.889
Family XIII Incertae Sedis	0.441	0.3	0.324	0.226	0.479
Escherichia-Shigella	0.438	0.143	0.323	0.08	0.292
Turicibacter	0.371	0.039	0.231	0.444	0.688

Ruminococcus	0.273	0.142	0.358	0.096	0.292
Coprococcus	0.245	0.169	0.273	0.268	0.548
Christensenellaceae uncultured	0.227	0.122	0.090	**0.041**	0.177
Anaeroplasma	0.226	0.022	0.205	**0.014**	0.081
Candidate division TM7 uncultured other	0.222	0.266	0.101	**0.007**	**0.049**
Anaerovorax	0.125	0.084	0.146	0.139	0.389
Peptococcaceae uncultured	0.104	0.114	0.137	0.392	0.654
Enterorhabdus	0.0951	0.1352	0.1447	0.174	0.429
Roseburia	0.0702	0.1160	0.1353	0.411	0.654
Family XIII Incertae Sedis uncultured	0.0684	0.2160	0.0353	0.168	0.429
Clostridium	0.0604	0.0164	0.0033	0.092	0.292
Clostridiales uncultured rumen bacterium	0.0551	0.0284	0	**0.011**	0.07
Peptococcus	0.0511	0.0556	0.0587	0.864	0.966
Thalassospira	0.0391	0.0472	0.0420	0.777	0.922
Mollicutes RF9 uncultured bacterium other	0.0316	0	0	0.06	0.233
Unidentified	0.0302	0.0164	0.0260		
Pseudobutyrivibrio	0.0284	0.0132	0.0300	0.274	0.548
Christensenella	0.0276	0.0264	0.0627	**0.041**	0.177
Prevotella	0.0116	0.0008	0	0.711	0.881
Mollicutes RF9 uncultured bacterium other	0.0098	0.0192	0.0060	**0.005**	**0.049**
Erysipelotrichaceae Incertae Sedis	0.0084	0.0136	0.0060	0.452	0.688
Lachnospiraceae uncultured bacterium	0.0076	0.0104	0.0053	0.88	0.966
Ruminococcaceae uncultured bacterium	0.0040	0.0024	0.0007	0.355	0.637
Erysipelotrichaceae uncultured	0.0036	0.0160	0.0007	**0.043**	0.177
Blautia	0.0031	0	0	0.411	0.654
Clostridiaceae uncultured	0.0027	0.0020	0.0020	0.927	0.998
Candidatus Arthromitus	0.0022	0.0064	0	0.196	0.429
Mogibacterium	0.0018	0.0016	0.0007	0.53	0.757
Hydrogenoanaerobacterium	0.0013	0.0012	0.0013	0.869	0.966
Cyanobacteria 4C0d-2 uncultured bacterium	0.0009	0	0.3367	**0.007**	**0.049**
Streptococcus	0.0009	0.0044	0.0027	0.325	0.599
Marvinbryantia	0.0009	0.0036	0.0020	0.188	0.429
Barnesiella	0.0004	0.0004	0	0.717	0.881
Prevotellaceae uncultured	0.0004	0	0	0.411	0.654
Coriobacteria uncultured	0	0	0.1360	**<0.001**	**0.000**
Flavobacterium	0	0	0	1.000	1
Cyanobacteria 4C0d-2 uncultured organism other	0	0	0.1000	**0.006**	**0.049**
Staphylococcus	0	0	0	1.000	1
Aerococcus	0	0	0	1.000	1
Enterococcus	0	0	0.0613	**<0.001**	**0**
Lactococcus	0	0	0	1.000	1
Anaerostipes	0	0.0176	0	0.472	0.703
Gelria	0	0	0.0133	**<0.001**	**0.000**
alpha proteobacterium	0	0	0	1.000	1
Mollicutes RF9 uncultured rumen bacterium other	0	0	0.0080	**0.001**	**0.018**

SUPPLEMENTARY MATERIALS AND METHODS

Nesting Behaviour

Nesting was carried out on PND 56 mice (week 8). Mice were single-housed over night into a clean cage and given a paper tissue as a nest-building material. In the morning after, nest quality was scored by a blinded researcher to the experimental conditions using a rating scale of 0-4 (0-paper tissue untouched; 4-perfect nest). After the nest scoring, mice were returned to their home cage.

Defensive marble burying

The defensive marble burying (DMB) test measures compulsive and anxious behaviour, indicating higher levels of anxiety at high number of marbles buried.

Mice were habituated to the room for 30min. prior to the test. After the habituation, mice were placed individually in small cages, in which 20 glass marbles were equally distributed on top of 4cm – deep bed of sawdust and a wire lid placed on top of the cage. Mice were left undisturbed for 30 min, after which the number of buried marble (more than 2/3 of their surface buried) was counted.

Light Dark Box

The light/dark box (LDB) test is a conflict-based anxiety test whereby the tendency to explore a novel environment contrasts against the natural aversion of mice to brightly lit spaces (Crawley 2000). The apparatus consisted of a Plexiglas enclosure divided into two compartments (one light and one dark) by a partition in which there was a small opening at the floor level. The light aversive compartment (two thirds) was open-roofed, with walls of transparent Plexiglas and was brightly illuminated by a 60-W desk lamp overhead (approximately 1,000 lx). The smaller, dark safe compartment

(one third) was closed-roofed and was constructed of black Plexiglas. Mice were habituated to the room for 30min. prior to the test. After the habituation, mice were individually placed in the center of the light compartment, facing away from the partition and allowed to explore the apparatus freely for 10 min. The apparatus was cleaned thoroughly between subjects. The number of light–dark transitions, time spent in the light compartment, and latency to enter the dark, were recorded by a blinded observer.

Elevated plus maze

The elevated plus maze (EPM) is used to assess anxiety-related behaviour and is based on the conflict mice experience between the attraction and the fear for a novel environment. The EPM was carried out as described previously (Cryan et al. 2003). The apparatus was made of an elevated cross plastic maze (1 m from the floor), comprising two open and two closed arms (50 cm × 5 cm × 15 cm walls), respectively constituting the fearful and safe areas. Mice were habituated to the room for at least 60 minutes prior to testing. Experiments occurred under red light (~9 lux.) used to illuminate the open arms. Mice were individually placed into the center of the maze facing an open arm to avoid direct entrance into a closed arm and leave the mice free choice for open or close arm and were allowed 6-min of free exploration. The apparatus was cleaned with 10% ethanol between subjects and mice were placed back into their home cage in the end of the trial. Experiments were videotaped and the time spent in each arm, and the number of entries, were scored (entrance in one arm being defined as all four paws inside the arm) by a blinded observer. Mice often exhibit increased defecation when placed in an anxiety-provoking environment (Barone, Barton et al. 2008). Therefore, faecal output was also measured in this test.

Open Field

The open field (OF) test is used to assess the response to a novel stressful environment and locomotor activity. In this study, we used the habituation phase of the NOR test as an open field, therefore the conditions (lux and dimensions) were not the appropriate ones to run an accurate OF. Mice were tracked and monitored for 10 minutes using Ethovision videotracking system (Noldus Information Technology). The distance moved, the velocity, time in the center zone and transitions between zones (center- outer) in the open field. Mice were placed back into the home cage after testing. The box is cleaned with 10% alcohol and allowed to dry prior to next test.

Chapter 3

Impact of dietary omega-3 polyunsaturated fatty acids on the cecal microbiome and metabolome

ABSTRACT

Background: Omega-3 polyunsaturated fatty acids (PUFA) are essential lipids that play crucial roles in immune-regulation, cardioprotection and neurodevelopment. Recent evidence suggests that the effects that different dietary fats have on such outcomes are mediated through changes in gut microbiota composition. However, little is known about how varying omega-3 status impacts gut microbiota between mother and offspring and the effect not only on microbiota composition by also on functionality.

Methods: Female C57BL/6 mice were assigned to one of three diets [control (CON), omega-3 supplemented (O3+) or omega-3 deficient (O3-)] during gestation and male offspring were continued on the same diet as previous maternal diet for 12 weeks. Following sacrifice, cecal content of mothers and offspring were collected for 16S sequencing and metabolomics analysis. Short chain fatty acid analysis was performed by gas-chromatography. Plasma and stimulated-spleen cytokine production were also assessed.

Results: O3- male displayed significantly less % fat mass and greater % lean mass than O3+ and CON. Omega-3 status induced subtle changes to gut microbiota composition such that O3+ mothers had significantly lower *Lachnospiraceae* and *Firmicutes:Bacteroidetes* ratio whereas O3- offspring had great abundance of *Tenericutes*, *Anaeroplasma* and *Coriobacteriaceae*. Metabolomics analysis revealed a significant increase in cecal metabolites involved in energy metabolism in O3+ including α-ketoglucaric acid, malic acid and fumaric acid. O3- animals displayed significantly reduced acetate, butyrate and cecal short chain fatty acid production. O3+ displayed a significantly higher IL-10/TNFα ratio in ConA-stimulated splenocytes.

Conclusions: Dietary omega-3 PUFA subtly regulate gut microbiota homeostasis such that omega-3 deficiency induces a state of compositional and functional disturbance. These gut microbiota alterations induced by omega-3 PUFA may mediate changes in immune status, adiposity and other markers of chronic disease.

1. INTRODUCTION

The gut microbiota is a virtual 'organ' referring collectively to all of the microorganisms residing in the gastrointestinal tract (GIT). In recent times, much research has indicated the essentiality of this microbial biomass for human health from birth to old age due to the role it plays in host immunity and metabolic health (Claesson et al., 2012, O'Toole, 2012). Furthermore, disruption to the composition and function of the gut microbiota has been associated with a wide range of physiological and even mental health disorders (Seira Oriach et al., 2016, Cryan and Dinan, 2012). Due to the 'flexible' nature of the gut microbiota, it is highly susceptible to change by a number of environmental stressors to the host including infection, medication use, stress, birth mode and diet (Bäckhed et al., 2015). Alongside increasing incidence of c-section births, antibiotic use and stress, the western lifestyle is uniquely characterised by a diet rich in fat and refined sugar and low in fibre. The metabolic outcomes of this modernised diet have been well characterised, however the rates of obesity, diabetes and chronic metabolic disease have continued to increase (Cordain et al., 2005). Expanding knowledge of the role of the gut microbiota in such disorders indicates the potential for this microbial 'organ' as a new mechanism and target by which nutritional interventions may prevent onset of diet-related metabolic disorders. Therefore it is essential to understand how nutritional deficiencies impact on gut microbiota composition and conversely how nutritional interventions can restore normal composition and function.

Despite much evidence pointing to the role of excess sugar and saturated fatty acid (SFA) intake in the epidemic of diet-induced metabolic disease, one of the most drastic changes in the western diet over the last five decades has been the intake of omega-3 polyunsaturated fatty acids (n-3 PUFA) relative to omega-6 polyunsaturated fatty acids (n-6 PUFA) (Simopoulos, 2002). It is widely acknowledged that humans

evolved on an n-6/n-3 PUFA ratio of around 1-2:1, however the modern diet, which is high in n-6 PUFA-rich vegetable oils, has contributed to a modern n-6/n-3 PUFA ratio of 20-30:1 (Simopoulos, 2002). This drastic increase in n-6 PUFA combined with declining n-3 PUFA intake, which are found in oily fish, nuts and seeds, has contributed to relative n-3 deficiency which has likely spanned 2-3 generations since the 1970s (Simopoulos, 1999). n-3 deficiency has hence been associated with progression of a number of chronic metabolic disorders. Maternal nutritional deficiencies have also been proven to increase offspring disease risk in later life, as shown by the Barker Hypothesis (Barker et al., 1993). Hence, it is possible that trans-generational nutritional inadequacies, such as n-3 PUFA deficiency, across 2-3 generations may further compound the effects on later life disease risk.

Both n-6 and n-3 PUFA are integral components of cell membranes and their metabolism is dependent upon competition within the same enzymatic pathway. Hence, increased availability of n-6 PUFA leads to increased n-6 metabolism and incorporation into cell membranes in place of n-3. n-3 and n-6 PUFA however, have differential bioactivities with respect to inflammation (Calder, 2006), cardioprotection (Simopoulos, 2008) and adiposity (Simopoulos, 2016). It has previously been shown that rats fed a high dietary ratio of n-3/n-6 PUFA gain less weight and are protected against obesity-linked inflammation through TLR-4-related mechanisms than those fed a diet high in SFA or with low n-3/n-6 ratio (Liu et al., 2013). Similarly, maternal diet with elevated n-3/n-6 ratio in mice is associated with protection against adipose inflammation, liver triglyceride deposition and offspring obesity compared to a low dietary n-3/n-6 ratio (Heerwagen et al., 2013).

It has been observed that the gut microbiota plays a critical role in metabolic health and immune regulation (Cani et al., 2009). For example, chronic low grade inflammation, a hallmark of chronic metabolic disease, is characterised by elevated

inflammatory cytokine production and increased circulation of the bacteria-derived endotoxin lipopolysaccharide (LPS), which is a TLR-4 agonist, resulting in downstream immune activation through NF-kB (Cani et al., 2008). Concentrations of LPS in circulation are dependent upon the integrity of tight junctions at the intestinal epithelial barrier, which is critically regulated by interaction between endothelial cells and the gut microbiota (Cani et al., 2007). It has also been observed that a high n-6 dietary intake induces gut microbiota dysbiosis resulting in increased intestinal permeability, metabolic dysfunction and adipose tissue inflammation, which is reversed by n-3 feeding (Lam et al., 2012). Similarly, Caesar at al. have shown that a diet containing fish oil, rich in long-chain n-3 PUFA, reverses adipose tissue inflammation through TLR signalling which is mediated through comprehensive changes in gut microbiota composition (Caesar et al., 2015). Similarly, it has been suggested that the beneficial effects of long-chain n-3 fatty acids on metabolic endotoxemia are mediated through increased production of the LPS-scavenging antimicrobial peptide, intestinal alkaline phosphatase (IAP) (Kaliannan et al., 2015).

The effects of n-3 and n-6 PUFA on mature microbiota composition have recently been investigated (Patterson et al., 2014), however less data are available on the role of maternal and early-life n-3 PUFA intake on the development of microbiota from birth. Gibson et al suggested that maternal fish oil supplementation in pharmacological excess (18 % energy) led to the overgrowth of opportunistic pathogens such as *Bilophila wadsworthia* and *Enterococcus faecium* in offspring microbiota and altering of macrophage homeostasis (Gibson et al., 2015). Similarly, it has been shown that parental long-chain n-3 PUFA intake at high levels altered offspring gut microbiota composition and dampened the immune response in mice offspring (Myles et al., 2013). These studies however, provided n-3 PUFA in pharmacologically excessive doses, which does not reflect normal dietary intake, and in

addition did not examine the progression of microbiota development in the offspring from birth to adulthood.

Despite a number of reports describing the role of dietary fat on host microbiota composition, there is a lack of data on the effect on cecal metabolite production. Recently Daniel et al provided a comprehensive report of the cecal metabolomic alterations induced by a high fat diet in mice, which provided novel insights into the functional metabolic changes induced by a high-fat diet induced microbiota (Daniel et al., 2014). The effect of n-3 PUFA on liver metabolite production has previously been outlined (Depner et al., 2013) however there is a lack of data of their effect on cecal metabolomics and the functional effects this may have on host physiology.

We previously reported results from this study in which varying n-3 PUFA availability altered microbiota composition (Robertson et al., 2016). Here, additional data from the same study design, we aimed to investigate the impact of omega-3 deficiency and omega-3 supplementation on the microbiota composition of mothers and their male offspring in mice. In addition, we sought to examine the impact on microbiota functionality by examining cecal metabolite production and associations with inflammatory status.

2. MATERIALS AND METHODS

2.1. Animals

Eight-week old male and female C57BL/6J mice were purchased from Harlan Laboratories, UK. Animals were kept in the University College Cork animal facility on a 12:12 h light-dark cycle at 20 ± 1°C. After acclimatisation, female and males breeding pairs were combined and assigned to one of three diets; control (CON),

omega-3 supplemented (O3+), omega-3 deficient (O3-) diet from gestational day 0. Male offspring (n=10 per group) were randomised within treatment groups, group-housed (3-4 per cage) and weaned on to the same mothers corresponding diets.

Food intake and body weight were recorded weekly during 13 weeks of dietary treatment. Fat and lean body mass were measured using a Minispec mq bench-top NMR spectrometer (Bruker Instruments) before sacrifice. All experiments were conducted in accordance with the Directive 2010/63/EU and were approved by the Animal Experimentation Ethics Committee of University College Cork #2012/036.

2.2. Diets

Dietary intervention was provided during pre-natal (pregnancy and lactation) and through post-natal period until adulthood. The three dietary treatments were matched for macronutrient content and differed only in fatty acid profile, as follows: 1) control standard chow (CON); 2) Omega 3 polyunsaturated fatty acid supplemented diet (O3+) - 1g Eicosapentaenoic acid + Docosahexaenoic acid /100g diet; 3) Omega 3 polyunsaturated fatty acid deficient diet (O3-). The O3+ diet was prepared with microalgal oil rich in EPA/DHA (*life'sOmega^{TM}45* – DSM, Netherlands). Diets were formulated and supplied by Harlan Laboratories, Inc. (Madison, WI, USA), which were matched for macronutrient content and differed only in fatty acid profile. The detailed compositions and fatty acid profiles of the three diets are shown in Table S1 and S2.

2.3. Experimental design

Fig. S1 illustrates the experimental design. Briefly, C57BL/6J females were assigned one of the three diets at gestational day 0. Upon confirmation of pregnancy, males were removed and the pregnant dams were continued on the dietary treatment for 6 weeks,

during pregnancy and lactation. Male offspring were subsequently weaned onto the same diets as their mothers until adulthood (12 weeks). Fecal samples were collected from mothers during lactation. Fecal samples were collected from offspring at both adolescence (4 weeks old) and adulthood (12 weeks). Mother's were sacrificed at weaning. At 13 weeks old, offspring were sacrificed by decapitation and samples collected.

2.4. Tissue Harvesting

Mice were sacrificed by decapitation and trunk blood was collected in potassium EDTA tubes. Plasma was prepared by collecting supernatant following centrifugation of blood samples for 15 min at 8000 g. The red blood cell pellet was washed 3 times with a five-fold volume of PBS and centrifugued at 1000 g for 5 min prior to fatty acid analysis. Dissected tissues were weighed, snap frozen in liquid nitrogen and stored at -80ºC until further analysis.

2.5. Tissue fatty acid analysis

Fatty acid analysis was performed on mothers milk, epididymal fat, red blood cell and liver tissues. Briefly, lipid extraction was performed using chloroform:methanol (2:1 v/v) as previously described (Folch et al., 1957). Methylation of lipid extracts was performed using 10 ml 0.5 N NaOH in methanol and heating at 90 °C for 10 min, followed by the addition of 10 ml 14% BF3 in methanol for 10 min at 90 °C (Park and Goins, 1994). Fatty acid methyl esters (FAME) were resuspended in hexane and analysed by gas chromatography (GC).

Separation of FAME was performed via GC (Agilent 7890B, Agilent Technologies, Santa Clara, CA, USA) with flame ionisation detection (Chrompack CP Sil 88 column, Chrompack, JVA Analytical, 100 m × 0.25 mm internal diameter, 0.25

μm film thickness) and helium as carrier gas. The GC column oven cycle was programmed as follows: 80 °C for 8 min followed by an increase by 8.5 °C/min to a final column temperature of 200 °C. Sample injection (0.5 μl) was automated (CTC Autosampler, Model 80) on an Aglient Multimode Inlet on-column temperature programmable injector. Peak integration was performed using the Agilent OpenLAB Chemstation software (Version A.01.06.111). Identification of individual FAME was done by comparison of retention times with pure FAME standards (Nu-Chek Prep, Elysian, MN, USA). Results are expressed as g/100 g total FAME.

2.6. Plasma and spleen cytokine analysis

For cytokine analysis, plasma was prepared from whole blood and stored at -80 °C prior to analysis. Spleens were collected immediately following sacrifice and stored in RPMI cell culture medium prior to splenocyte culturing. For splenocytoe culturing, the spleen of each animal were homogenised in media [RPMI (with L-glutamine and sodium bicarbonate, R8758 Sigma) + FBS (F7524, Sigma) + Penicillin/Streptomycin] and the homogenate was filtered over a 70um strainer, centrifuged at 200 g for 5 min and resuspended in media. Cells were counted and cultured in 6-well plates (4,000,000 cells/mL media). After 2.5h of incubation at 37 °C, cells were stimulated with lipopolysaccharide (LPS-2 μg/ml) or concanavalin A (ConA-2.5 μg/ml) for 24h and incubated at 37 °C. Following stimulation, the supernatants were collected and stored at -80 °C until analysis. Cytokine production in the supernatants was assessed using a chemoilluminescence V-PLEX Kit (Proinflammatory Panel 1 - mouse) (Meso Scale Discovery, Maryland, USA) for TNFα, IL-10, IL-1β and IL-6 according to manufacturers instructions. The plate was read using a MESO QuickPlex SQ 120, SECTOR Imager 2400, SECTOR Imager 6000, SECTOR S 600.

2.7. DNA extraction and 16S sequencing

Total bacterial metagenomic DNA was extracted from the cecal content of mothers following sacrifice after 6 weeks dietary treatment. DNA from the cecal content of offspring was also extracted after sacrifice after 12 weeks DNA was extracted using a commercial DNA extraction kit (QIAmp DNA Stool Mini Kit, Qiagen, UK). An initial repeated bead-beating step was performed prior to DNA extraction in order to increase final DNA yield. DNA quantification was performed using a Nanodrop 1000 (Thermo Scientific, Ireland) and stored at -20°C until analysis.

16S MiSeq sequencing was performed using the 16S metagenomic sequencing library protocol (Illumina) as previously described (Fouhy et al., 2015). The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA using two PCR reactions and clean-up steps. The initial PCR reaction utilised primers specific for amplification of the V3-V4 region of the 16S rRNA gene (Forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). Confirmation of PCR product size was performed by visualisation on an agarose gel via gel electrophoresis. PCR product clean-up and purification was achieved using AMPure XP magnetic beads (Labplan, Dublin, Ireland). The second PCR reaction was subsequently done to index and barcode individual samples in order for samples to be pooled and sequenced together in one flow cell. Samples were demultiplexed for analysis after sequencing. A unique indexing primer pair was used for each sample (Illumina Nextera XT indexing primers, Illumina, Sweden). PCR product size was again confirmed via gel electrophoresis and samples were cleaned and purified a second time using AMPure. Samples were quantified using the Qubit broad range DNA quantification assay kit (Bio-Sciences, Dublin, Ireland). Following

quantification, samples were normalized to an equal concentration (4nM) and pooled together for analysis. The sample pool was denatured and diluted to 4pM and MiSeq sequencing was performed according to Illumina guidelines by the Eurofins sequencing facility (Eurofins, Germany) using a 2 x 300 cycle V3 kit.

2.8. Bioinformatics

300 bp paired-end reads were assembled using FLASH with minimuma and maximum overlap parameters of 20 bp and 120 bp respectively (Magoč and Salzberg, 2011). Further processing of paired-end reads, including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was performed using the QIIME suite of tools, v1.8.0 (Caporaso et al., 2010). Denoising, chimera detection and operational taxonomic unit (OTU) grouping were performed in QIIME using USEARCH v7 (Edgar, 2010). Taxonomic ranks were assigned by alignment of OTUs using PyNAST to the SILVA SSURef database release 111 (Quast et al., 2013). Alpha and beta diversities were generated in QIIME and calculated based on weighted and unweighted Unifrac distance matrices (Lozupone et al., 2011). Principal coordinate analysis (PCoA) plots were visualised using EMPeror v0.9.3-dev (Vázquez-Baeza et al., 2013).

2.9. Metabolomics

Metabolomics analysis of cecal water was performed as follows. Cecal water was prepared by the addition of sterile water to cecal content (~ 100mg) followed by vortexing. Bacteria and other solids were pelleted by centrifugation at 16,000 g for 30 mins. The supernatant was removed, placed in a clean tube and centrifuged again at 16,000 g for 30 min. The supernatant was centrifuged once more before being filtered

through a VectaSpin microcentrifuge 0.2µm filter (Sigma Aldrich) at 10,000 g. The resulting filtrate was stored at –20°C until analysis.

Derivatisation of cecal water samples for gas chromatography mass spectrometry (GC-MS) was carried out using methylchloroformate (MCF) as previously described (Smart et al., 2010) with slight modifications. Analysis was carried out using GC (Agilent 6890N) coupled with a quadropole detector (Agilent 5975B). The system was controlled by ChemStation (Agilent technologies). Data were converted to netCDF format using Chemstation (Agilent), before being imported into Matlab R2014b (Mathworks Inc, Natick, MA, United States) and processed using PARAFAC2 to obtain relative concentrations for each peak. PARAFAC2 was applied using the PARADISe software. Samples were analysed in randomised order.

2.10. Short chain fatty acid analysis

Short chain fatty acids (SCFA) were analysed by gas chromatography (GC). 1ml of sterile Milli-Q water was added to a known amount of mice cecal content (approx. 100mg). The sample was vortexed thoroughly and allowed to stand at room temperature for 10 min. Bacteria and other solids were pelleted by centrifugation at 10,000 g for 5 min and the supernatant was filtered into clean tubes. An internal standard (10mM 2-ethylbutyric acid (Sigma)) was subsequently added to each sample in duplicate. Samples were vortex mixed and centrifuged again at 10,000 g for 3 min then transferred to clean vials. SCFA were analysed by GC (Varian 3800 flame ionization) fitted with a ZB-FFAP column (30m x 0.32mm x 0.25, Phenomenex, Torrance, CA, USA). Helium was used as a carrier gas at a flow rate of 1.3ml/min. The GC oven cycle was programmed at 100 °C for 0.5min which increased by 8 °C/min to 180 °C, held for 1 min, then increased by 20 °C/min to a final temperature of 200 °C, which was held for 5 min. The detector and injector were programmed at 250 °C and

240 °C, respectively. Peak integration was performed using the Varian Star Chromatography Workstation Software version 6.0. A standard curve of SCFA standard solutions was used for comparison of retention times and for identification and quantification of individual sample peaks.

2.11. Statistical Analysis

Parametric statistical analyses were performed using GraphPad Prism version 6 (La Jolla, CA, USA). Non-parametric analyses of microbiota and metabolomic data were performed usign SPSS version 20 (Armonk, NY, USA). Results are presented as mean ± standard error of the mean (SEM). Significant differences between treatment groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison *post-hoc* test. Significant differences between non-parametric data were assessed by Kruskal-Wallis tests followed by Mann-Whitney to determine differences between individual groups. False Discovery Rate analysis was calculate at $p<0.05$. Significance was accepted at $p<0.05$.

3. RESULTS

3.1.Body composition and dietary intake

Offspring weights (males and females) were significantly lower (p=0.031) in O3- animals compared with O3+ animals at weaning (week 4) (Fig. 1A), however there were no significant differences in final body weights or dietary intakes between groups (Fig. 1 B and C).

At sacrifice, % total fat mass was significantly lower (P=0.0004) and % lean body mass significantly higher (p=0001) in the O3- group compared with both other groups (Fig. 1 D and E).

3.2.Fatty acid analysis

Fatty acid analysis revealed distinct differences in tissue fatty acid profiles between groups (Table 1). In epididymal fat, liver and red blood cells, n-6 ARA content was significantly higher in O3- animals and significantly lower in O3+ animals compared to CON. n-3 PUFA were not detected in the epididymal fat or red blood cells of O3- offspring and contributed a minor proportion of fatty acids in the liver (0.3%). Contrastingly, in epididymal fat, liver and red blood cells, DHA accounted for 2.2%, 17.3% and 6.5% of fatty acids in O3+ mice respectively, whereas in CON mice DHA accounted for 0.1%, 8% and 3.8% of total fatty acids in these tissues. Linoleic acid (LA) was significantly lower in O3- epididymal tissue compared to both other groups.

3.3.Plasma and stimulated-spleen cytokine production

There were no significant differences between treatment groups in circulating plasma TNF-α, IL-1β, IL-10 and IL-6 levels (Fig. S2). One-way ANOVA revealed a

significant effect of diet on the IL-10/TNF-α ratio in ConA-stimulated splenocytes [$F(2,26)=4.614$, $p=0.019$] (Fig. 2). Tukey HSD post-hoc testing showed that O3+ group displayed a significantly increased IL-10/TNF-α ratio compared with CON ($p<0.05$) and a non-significant increase compared with O3- group ($p=0.082$).

3.4.Composition of cecal microbiota

16S sequencing of the cecal microbiota resulted in a total of 3.1 million sequenced reads, corresponding to an average of 68,534 reads per sample. To remove any bias from variation in sample read number, samples were rarefied to 25,000 reads.

Phylogenetic alpha diversities revealed some difference between treatment groups (Fig. 3). Cecal microbiota diversity was significantly lower in both O3+ and O3- mothers compared with CON as assessed by the Simpson index. Similarly, alpha diversity was significantly lower in both O3+ and O3- mothers compared with CON group as assessed by the Shannon index, however O3- group also displayed significantly lower diversity compared with O3+ group. No significant differences in alpha diversity were observed in any of the offspring groups.

Principle coordinate analysis (PCoA) revealed no distinct clustering of treatment groups in mothers however in offspring, there was clear clustering and separation of the O3- group away from both other groups (Fig. 3).

The overall phylum level distribution was similar between mothers and their offspring (Fig. 4 and Fig. 5), however relative abundance analysis of different taxa at phylum level revealed some differences between treatment groups. *Firmicutes* and *Bacteroidetes* were the most abundant phyla in each group in both mothers and offspring. O3+ mothers displayed greater proportional abundance of *Bacteroidetes* and a lower *Firmicutes:Bacteroidetes* ratio compared with CON. These differences were not observed in offspring, however O3- offspring displayed significantly greater

proportional abundance of *Tenericutes* and *Cyanobacteria* compared with both other groups. *Deferribacteres* was significantly greater in both O3+ and O3- offspring compared with CON group.

A small number of significant differences were also observed at family and genus level distributions, many of which showed similar trends between mothers and offspring. There was a trend towards greater *Coriobacteriaceae* and *coriobacteria* abundance in O3- mothers which became significant in O3- offspring. Similarly, O3- mothers displayed non-significant increases in both *Gelria* and *Anaeroplasma,* which were significantly different in their offspring compared with both other groups. *Lachnospriaceae* and *coprococcus* were present in significantly lower abundance in O3+ mothers, compared with both other groups. In addition, *Enterobacteriaceae* were significantly less abundant in both O3+ and O3- offspring compared with CON.

3.5.Composition of cecal metabolome

Supervised modeling using principle least squares discriminant analysis (PLS-DA) of the cecal metabolome revealed separation of the O3+ group from both O3- and CON (Fig. 6). These observed differences in the O3+ animals were driven by significantly greater abundance of metabolites involved in energy metabolism. Notably α-ketoglucaric acid was significantly increased in O3+ compared with CON groups. Fumaric acid and malic acid were significantly increased in O3+ compared with both CON and O3-. Contrastingly, O3+ animals revealed significantly lower abundance of a number of other metabolites including valine containing compound peptide and glycine containing compound peptide. O3- group revealed significantly lower abundance of benzeneacetic acid compared with CON and significantly increased oxyoxathiolanylpentanoic acid compared with O3+group.

3.6.Cecal short chain fatty acid production

Cecal short chain fatty acid (SCFA) production was significantly altered by diet as displayed in Fig 7. Overall, the O3- group displayed significantly reduced production of total SCFA compared with CON ($p<0.05$) (Fig. 7), which was driven by significant reductions in acetate ($p<0.05$) and butyrate ($p<0.05$) production. However, O3+ group also demonstrated a significant reduction in butyrate production compared with CON ($p<0.05$). There were no significant differences observed between groups for content of propionate or isobutyrate.

DISCUSSION

The gut microbiota is a complex ecosystem that has a significant impact on metabolic health through interactions with the diet. Here, we report the impact of varying dietary omega-3 on cecal microbiota composition and metabolite production, which may provide novel information for future microbiota-targeted nutritional therapies. Briefly, we have shown that maternal omega-3 supplementation produces offspring of greater weight than omega-3 deficiency. This is further evident in the offspring that continued on the same dietary regime through to adulthood, such that dietary omega-3 deficiency resulted in reduced % fat mass and greater % lean mass in adolescence and adulthood, compared with omega-3 supplementation or CON diet. Omega-3 supplementation or deficiency also induced subtle changes to the cecal microbiota composition in both mothers and their offspring. This change in gut microbiota composition is also reflected in metabolite production such that omega-3 supplementation results in greater abundance of energy metabolites in cecal water. Conversely, cecal short chain fatty acid production appeared to be reduced by omega-3 deficiency. Finally these changes induced to the cecal microbiome and metabolome appear to be associated with minor

changes in inflammatory status such that dietary omega-3 increases the IL-10/TNFα ratio in splenocytes in response to ConA stimulation.

A number of intervention and large observational studies have shown that both greater n-3 PUFA intake and fish intake, especially fatty fish, significantly reduce incidence of preterm birth and increase infant birth weight, (Carlson et al., 2013, Leventakou et al., 2014, Salvig and Lamont, 2011). Here we have shown that, at weaning, offspring of O3+ mothers had significantly greater weight than O3-, although this was not significantly different to CON. Infant weight has important implications for infant development and has been associated with later life disease risk (Huxley et al., 2007). Low birth weight, for example, is associated with poor long term-health outcomes (Frankel et al., 1996). Lower umbilical cord DHA is associated with lower birth weight and vice versa (Elias and Innis, 2001), therefore omega-3 deficiency may impair various aspects of development including neurodevelopment (Coti Bertrand et al., 2006). The reason for omega-3 induced increases in infant weight are unclear but it has been suggested that n-3 PUFA may prolong gestation by interfering with the production of certain prostaglandins, notably PGF2a and PGE2, which play a role in uterine contractions (Olsen et al., 1986). However, in this study, birth weight solely differed between the treatment groups, which were not significantly different to the control.

Interestingly, at sacrifice, O3- mice displayed significantly reduced % fat mass and increased % lean mass compared with both other groups. n-3 PUFA have consistently reported anti-obesigenic properties through white adipose tissue thermogenesis and other mechanisms (Kim et al., 2015, Simopoulos, 2016). The animals in this study, however, were not obese and hence the observed loss in % fat mass in the O3- mice compared with CON is of note. Tissue linoleic acid (LA) is positively associated with weight gain and fat mass in both humans and animals (Wang

et al., 2016, Naughton et al., 2016, Simopoulos, 2016). In this study, however, LA and other n-6 fatty acids were not increased in the O3- diet in place of n-3 PUFA. Moreover, O3- LA was in fact not different to CON in the liver, tended to be lower than CON and O3+ in red blood cells ($p=0.07$) and was significantly lower than CON in epididymal adipose tissue. Total n-6 PUFA, however, were not different between groups in epididymal tissue and all other n-6 fatty acids were present in concentrations of <0.5%. The unexpected reduction of LA in O3- epididymal tissue is likely due to it's rapid conversion into AA and other longer chain n-6 PUFA due to n-3 deficiency and hence a lack of competition for desaturase and elongase enzymes in the n-3 pathway. Therefore, interestingly, these results suggest that LA plays a more significant role in the regulation of fat mass than other n-6 PUFA, and in cases of n-3 deficiency, where dietary n-6 PUFA are not increased and tissue LA is converted to its longer n-6 substrates, reduced tissue LA may lead to loss of fat mass. This may explain the observed difference in body composition and is supported by previous work showing that LA has a positive correlation with weight and fat mass (Wang et al., 2016). In the O3+ animals, as expected, peripheral tissues all displayed elevated levels of omega-3 fatty acids, which were absent or present in minor proportions in O3- animals.

n-3 PUFA have potent anti-inflammatory activities through the production of eicosanoids and resolvins (Calder, 2006). By interfering with signalling pathways such as NF-kB, n-3 PUFA and their products of metabolism dampen the production of pro-inflammatory cytokines and inflammatory gene expression. The results presented here suggest that dietary O3 increased the ratio of IL-10/TNFα production in ConA-stimulated splenocytes. IL-10 is an anti-inflammatory cytokine produced by T regulatory cells that is involved in the resolution of inflammation and hence inflammatory homeostasis, especially in the gut. Contrastingly, TNFα is a key promoter of inflammation and is elevated in a number of inflammatory disease states.

Using the same experimental design, it has previously been shown that absolute production of IL-10 and TNFα is dampened in O3- animals suggesting muted systemic responsiveness to LPS (Robertson et al., 2016). In the present study however, the ratio of these two opposing cytokines is significantly lower in O3- following ConA stimulation and trends towards reduction in unstimulated cells (p=0.097). The IL-10/TNFα ratio is an established marker of inflammatory balance and has been associated with cardiovascular disease and diabetes outcomes (Hashem et al., 2008, Moreli et al., 2015). In this study, the elevated n-6/n-3 ratio of the O3- animals may have stimulated a relative increase in the production of proinflammatory eicosanoids, which are produced by AA, which was significantly increased in peripheral tissues. This is a probable cause of the pro-inflammatory profile observed in splenocytes in this study.

The mechanisms by which n-3 PUFA interact with the gut microbiota are slowly being resolved (Kaliannan et al., 2016). It has recently been suggested that n-6 PUFA stimulate a pro-inflammatory intestinal environment characterized by increased gram-negative bacteria such as *Enterobacteriaceae*, increased gut permeability and subsequent translocation of LPS across the epithelial barrier. It has been suggested that n-3 PUFA resolve this cascade of events through the stimulation of *Bifidobacterium* growth and intestinal alkaline phosphatase (IAP) production, an antimicrobial peptide that scavenges LPS (Kaliannan et al., 2015). Interestingly, this was examined in a transgenic model of endogenous n-3 production suggestion that n-3 PUFA may have an indirect as well as direct interaction with the commensal microbiota. However, no differences were observed in jejenum IAP in the present study (Fig. S2). n-3 PUFA also modulate gut microbiota composition in infants. Previously it has been shown that certain species within the *Bacteroidetes* phyla are elevated following fish oil supplementation compared with sunflower oil in infants (Andersen et al., 2011). This

was partially reflected in our data in which maternal *Bacteroidetes* were significantly increased in O3+ and the Firmicutes:Bacteroidetes ratio significantly reduced. This may have relevance for later life health consequences due to the observation of an elevated Firmicutes:Bacteroidetes ratio in obesity (Murphy et al., 2010) and its association with chronic inflammation which was also reflected here in stimulated splenocytes. PCoA and LeFSe analysis revealed that differential abundance of a number of species within the O3- offspring microbiota contributed to the separation from O3- and CON. These included a significant increase in taxa within the *Tenericutes* phylum as well as elevated *coriobacteriaceae* and *anaeroplasma*. *Tenericutes* have previously been shown to be elevated in high fat diet fed mice and are correlated with obesity-associated metabolic parameters (Lecomte et al., 2015). Similarly, *coriobacteria* were elevated in obese subjects, an effect that was normalized by gastric bypass surgery suggesting that this species is present as an effect or may even contribute to metabolic deficiencies (Zhang et al., 2009). Furthermore, *Anaeroplasma*, a species within the *Tenericutes* phylum, was significantly increased in mice following l-carnitine feeding. This was associated with elevated trimethylamine-N-oxide (TMAO), a key metabolite involved in cardiovascular disease progression (Koeth et al., 2013). Interestingly, this suggests that the role of n-3 PUFA status in cardiovascular disease may be somewhat mediated through changes in the gut microbiota that stimulate pro-atherogenic metabolite production. Interestingly, *Lachnospiraceae* was significantly reduced in O3+ mothers. It has been shown that a mixture of fish oil and flaxseed oil feeding significantly reduced *Lachnospiraceae* abundance in mice compared with a high saturated fat diet (Patterson et al., 2014).

The overall microbiota composition was very similar between mothers and offspring, with the most abundant taxa being *Lachnospiraceae uncultured, Alistipes, Rumunococcaeae incertae sedis* and *uncultured, Odoribacter* and *Bacteroidales S24-7*

at both time points. However through PCoA analysis, it appeared that compositional differences between groups were more evident in offspring, which may be due to longer dietary exposure. Interestingly, these cecal compositional data appeared to differ from previously published fecal data from the same treatment groups (Robertson et al., 2016). It was previously shown that the fecal microbiota from these treatment groups appeared to be more enriched in *Actinobacteria, Peptostreptococcaceae* and *Lactobacillus*. In the previous study, fecal *Bifidobacterium*, a member of the phylum *Actinobacteria,* and *Lactobacillus* were significantly higher in O3+. Overall, the composition of all other major taxa were similar, however more differences were evident between treatment groups in the fecal compositional analysis. This comparison of data suggests that the cecal microbiota may differ significantly from the fecal microbiota due to altering composition within different regions of the intestinal tract. Segmental differences have been previously observed in the gut microbiota (Marteau et al., 2001). This is of particular note in this study as a majority of PUFA are absorbed in the upper intestinal tract (Niot et al., 2009). Therefore, these data suggests that a certain proportion of dietary PUFA reach the caecum and interact directly with the cecal microbiota differently to the colonic microbiota or alternatively exert differing indirect effects on the intestinal environments of these two regions, which modifies microbial composition.

We further analysed the composition of the cecal metabolome to assess whether the observed differences in microbiota composition induced by varying n-3 PUFA status resulted in modifications to the metabolic activity of the cecal microbiota. This is the first study to examine the effect of n-3 PUFA supplementation or deficiency on the cecal metabolome. However, previously, the liver metabolome was assessed in the context of n-3 PUFA (Depner et al., 2013). Indeed, the cecal metabolome has been examined in the context of high fat diet feeding in which there were distinct effects on

markers of fat and carbohydrate metabolism. The interaction between the metabolic activity of the microbiota and tissue fatty acid composition has also previously been examined (Wall et al., 2012). It has been shown that modulation of the microbiota through feeding of a *Bifidobacterium* probiotic can significantly increase both adipose tissue and brain n-3 PUFA composition in mice and pigs (Wall et al., 2009;). These results suggest that there is a close relationship between dietary fat intake and the metabolic activity of the gut microbiota. In this study, PCoA loading plots in the 16S sequencing data revealed distinct clustering of the O3- group away from O3+ and CON. Interestingly, in the PLS-DA analysis of the metabolomics data, it appeared that differences between groups were driven by alterations to the O3+ group. Further analysis of this data revealed that the metabolites strongly contributing to this separation were those involved in energy metabolism. Fumaric acid and malic acid were significantly elevated in O3+ compared with both other groups. α-ketoglucaric acid was also increased in O3+ as well as O3- compared with CON group. The microbiota has previously proven essential for energy metabolism. Malic acid, fumaric acid and other energy metabolites involved in the tricarboxylic acid (TCA) cycle were previously shown to be significantly increased in the serum of conventionally raised animals compared with germ free animals (Velagapudi et al., 2010). Furthermore, using predicted functional metagenomics, it has been shown that the TCA cycle is downregulated on a high fat diet, an effect which is reversed with high intensity exercise (Denou et al., 2016). Hence these results may suggest that O3+ has a beneficial effect on energy metabolism through compositional changes in the gut microbiota and its subsequent metabolic activity particularly regarding the TCA cycle. These results, however, should be interpreted with caution due to the relative flux of the TCA cycle in different disease states and as significant differences exist between the cecal, serum and urinary metabolomes. For example, TCA metabolites, such as

those observed here, are decreased in the urinary metabolome of the obese Zucker rat but increased in *ob*/*ob* mice (Williams et al., 2005, Zhao et al., 2010, Salek et al., 2007). Therefore no strict conclusions can be made about this effect of O3+ on the cecal metabolome but these novel data may provide useful information for future studies about the role of n-3 PUFA in the metabolic activity of the commensal microbiota.

A more targeted analysis of cecal short chain fatty acid (SCFA) production revealed that O3- significantly reduced the production of acetate, butyrate and total SCFAs. O3+ also significantly reduced butyrate production. SCFAs are typically the fermentation products of indigestible dietary fibres and function as the key energy source for colonocytes (Byrne et al., 2015). A number of studies have shown their importance in the regulation of cholesterol, glucose and lipid metabolism, therefore, impaired SCFA production has been associated with metabolic disruption and chronic disease progression (den Besten et al., 2013). Interestingly, the combined effect of DHA and butyrate has previously been shown to induce Ca^{2+}-dependent apoptosis in coloncytes, a process which may reduce colon cancer progression (Kolar et al., 2007). Therefore the observed impairment of SCFA production in O3- suggests that such a deficiency in n-3 PUFA disrupts the homeostatic intestinal environment, which may have implications for metabolic disease. Indeed, certain taxa involved in SCFA production, namely members of the *Clostridiaceae* family, were not detected in O3- microbiota suggesting that n-3 PUFA deficiency in the diet resulted in absence of SCFA-producing bacteria among the gut microbiota. Interestingly however, the observed disruptions to SCFA production in O3- were not due to differences in abundance of other well-known butyrate producers such as *Roseburia* and *Pseudobutyvibrio*.

Long-chain n-3 PUFA have consistently reported beneficial effects for cardiovascular, immune and metabolic health, however the role they play in modulating the gut microbiota composition has remained unclear and their role on the metabolic activity of the microbiota has not been examined previously. These results suggest that dietary n-3 PUFA play a crucial role in modulating host body composition and inflammatory status. Furthermore, these physiological outcomes may be partially induced by significant alterations to gut microbiota composition and metabolic activity. Dietary omega-3 deficiency appears to induce gut microbial imbalance, which may contribute to impaired SCFA production. Conversely, greater omega-3 intake induces a more anti-inflammatory profile and enhances cecal production of energy metabolites possibly through modulation of gut microbiota composition. Further research is required to elucidate the long-term impact of such altered microbiota and metabolomic profiles on chronic disease progression. Due to the global trends of increasing omega-6 intake in place of omega-3, these results warrant the recommendation for greater omega-3 PUFA intake for gut health.

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FIGURES

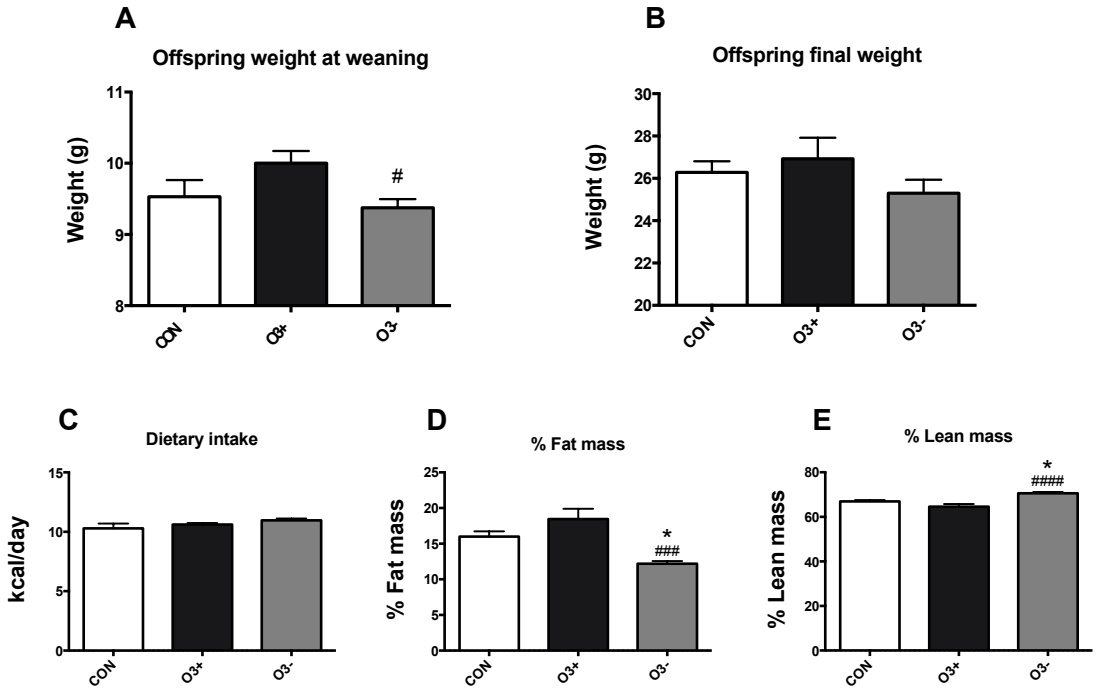


Figure 1. Effects of omega-3 status on body composition. (**A**) Male and female offspring of O3- mothers weighed significantly less than O3+ offspring at weaning (week 4). There were no significant differences between groups in final body weight **(B)** or dietary intake **(C)**. O3- caused significantly decreased fat mass **(D)** and increased lean mass **(D)** compared with CON and O3+. Data shown as mean ± SEM. n= 9-10 per group **(A, C-H)**. n= 3 cages per group for dietary intake **(B)**. *$p < 0.05$, CON vs. O3+ or O3- group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ O3+ vs. O3-.

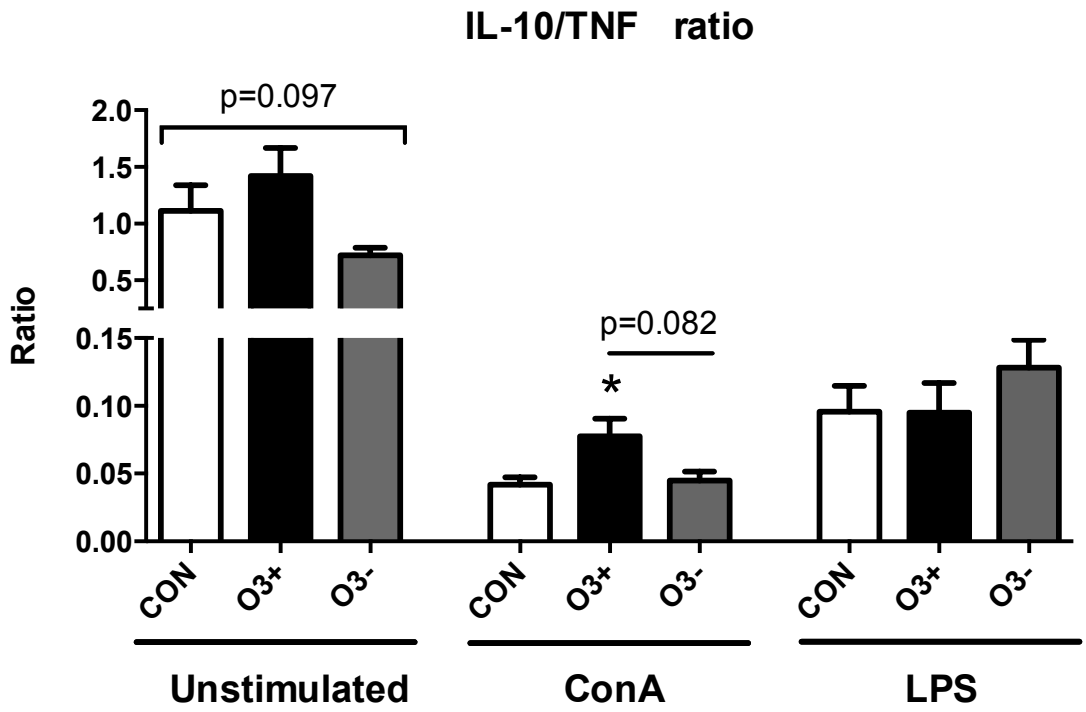


Figure 2. Effects of omega-3 dietary intervention on cytokine levels after lipopolysaccharide (LPS) and concanavalin A (ConA) spleen stimulation. Omega-3 dietary intervention causes significant differences on cytokine release levels of tumor necrosis factor (TNFα) and Interleukin 10 (IL-10) after LPS and ConA stimulation. **(A)** O3+ caused no effects on the release of TNFα and IL-10 after ConA stimulation, while O3- significantly decreased spleen levels of TNFα and IL-10 after stimulation with ConA compared to the CON. Moreover, **(A)** the deficient group presented a significant decrease of IL-10 after stimulation with ConA compared to the O3+ group. Thus, **(B)** the O3- significantly decreased spleen levels of TNFα after stimulation with LPS in comparison to the CON group. **(B)** There was a trend to decreased levels of IL-10 cytokine in the O3- group after stimulation with LPS. O3+ induced a significantly greater IL-10/TNFα ratio following ConA stimulation **(C)**. Data shown as mean ± SEM. n= 8-10 per group. *p < 0.05, CON vs. O3- or O3+ group; ## p < 0.01 O3+ vs. O3-.

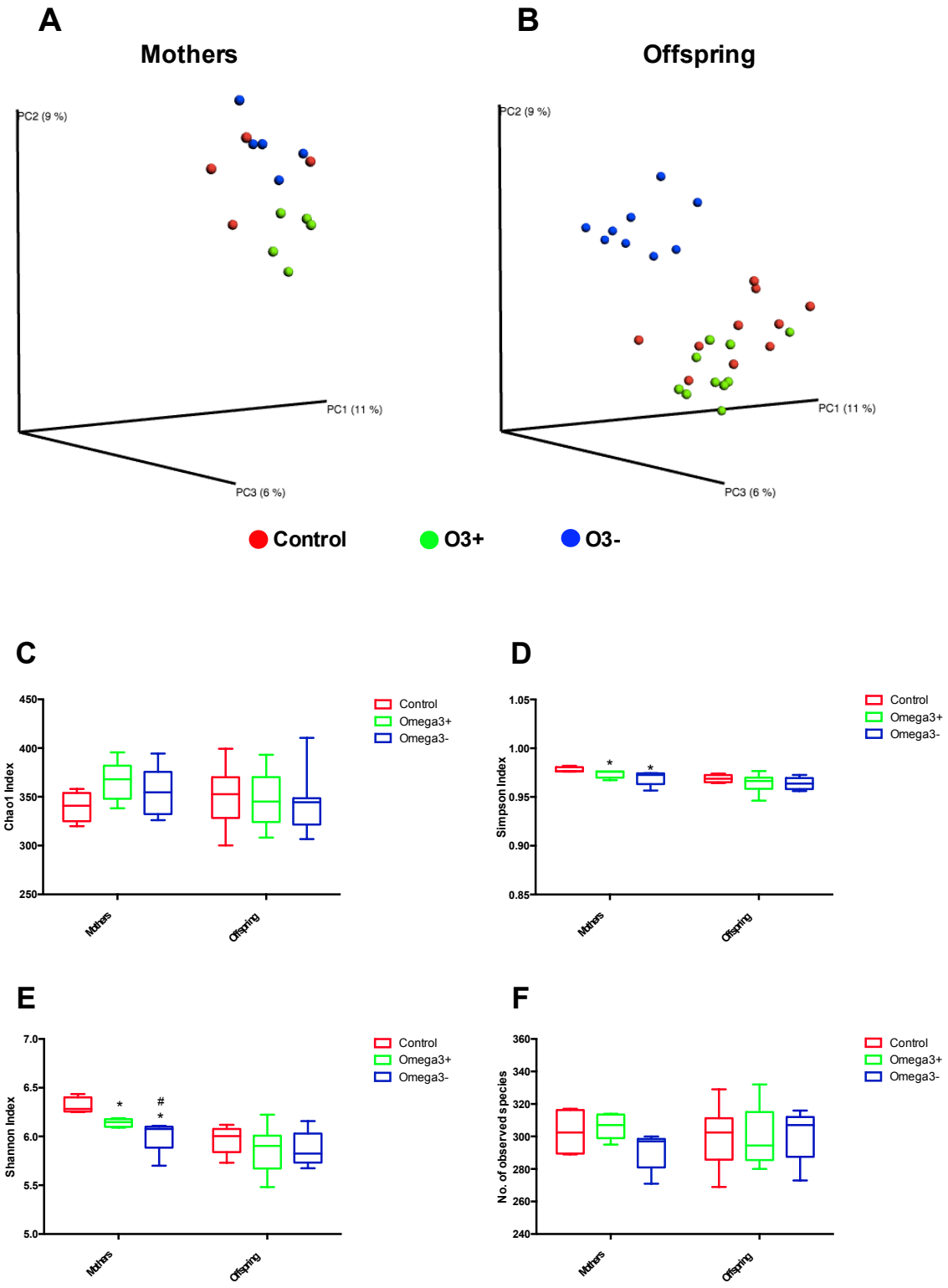


Figure 3. Effects of omega-3 PUFA on cecal gut microbiota diversity. Principle coordinate analysis (PCoA) revealed no distinct clustering of treatment groups in mothers **(A)**. In offspring, there was distinct clustering of the O3- group away from both O3+ and CON **(B)**. Alpha diversity of cecal microbiota was significantly reduced in both O3+ and O3- mothers as measured by the Simpson **(D)** and Shannon **(E)** indices. O3- alpha diversity was significantly lower than O3+ as measured by the Shannon index **(E)**. There were no significant differences in alpha diversity between offspring treatment groups. In addition no differnces were observed in mothers or offspring using the Chao1 index **(C)** or number of observed species **(F)**. Data shown as mean ± SEM. n= 4-5 per group for mothers and 9-10 per group for offspring. *$p < 0.05$, CON vs. O3- or O3+ group; # $p < 0.05$ O3+ vs. O3-.

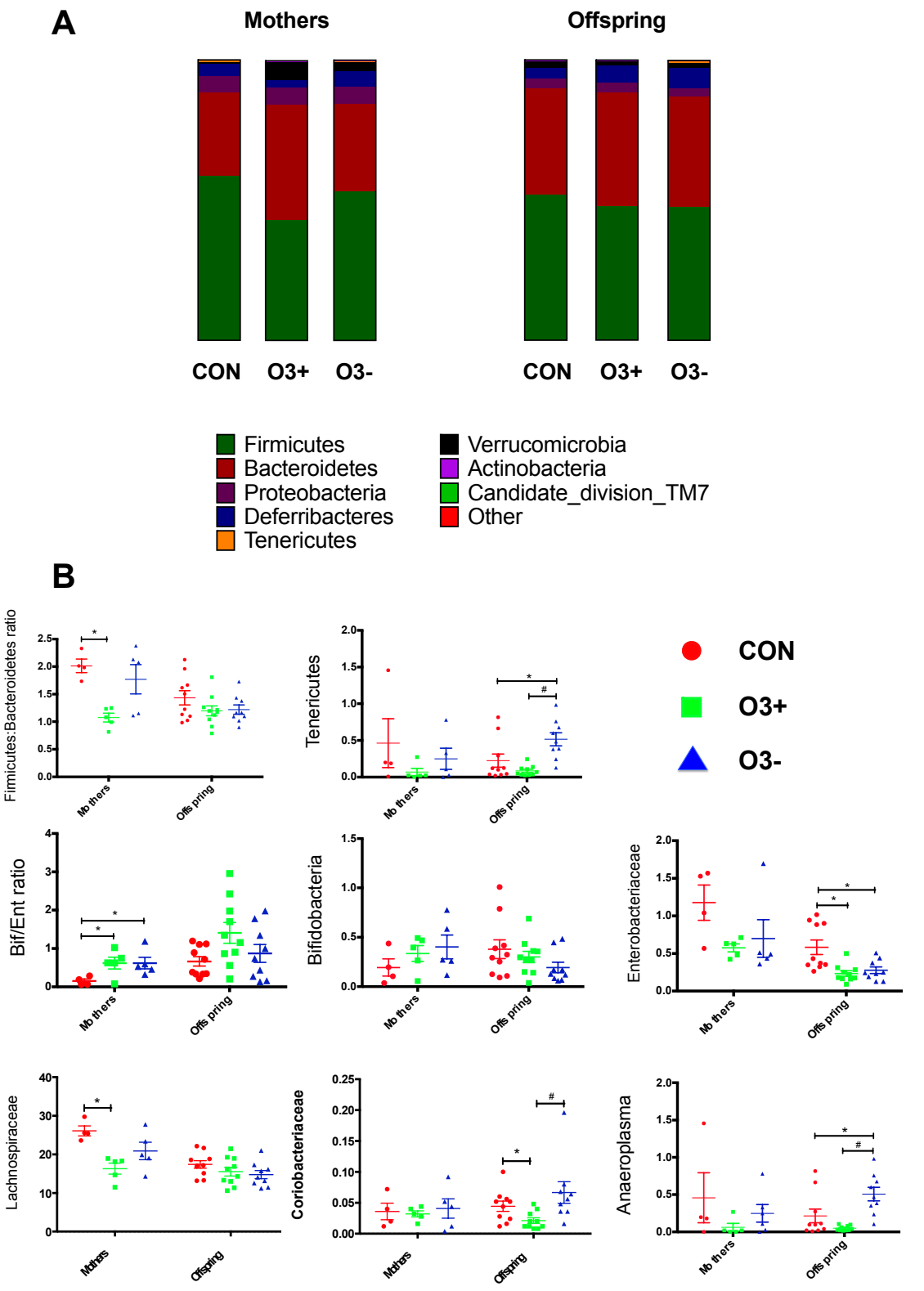


Figure 4. Effects of omega-3 PUFA on taxonomic composition of the cecal microbiota. At phylum level O3+ mothers displayed significantly greater *Bacteroidetes* and a significantly lower *Firmicutes:Bacteroidetes* ratio, where as *Tenericutes* were significantly higher in O3- offpspring **(A)**. At family and genus level, *lachnospiraceae* was significantly lower in O3+ mothers while *coriobacteriaceae* and *anaeroplasma* were significantly greater in O3- offspring **(B)**. Data shown as mean ± SEM. n= 4-5 per group for mothers and 9-10 per group for offspring. *p < 0.05, CON vs. O3- or O3+ group; # p < 0.05 O3+ vs. O3-.

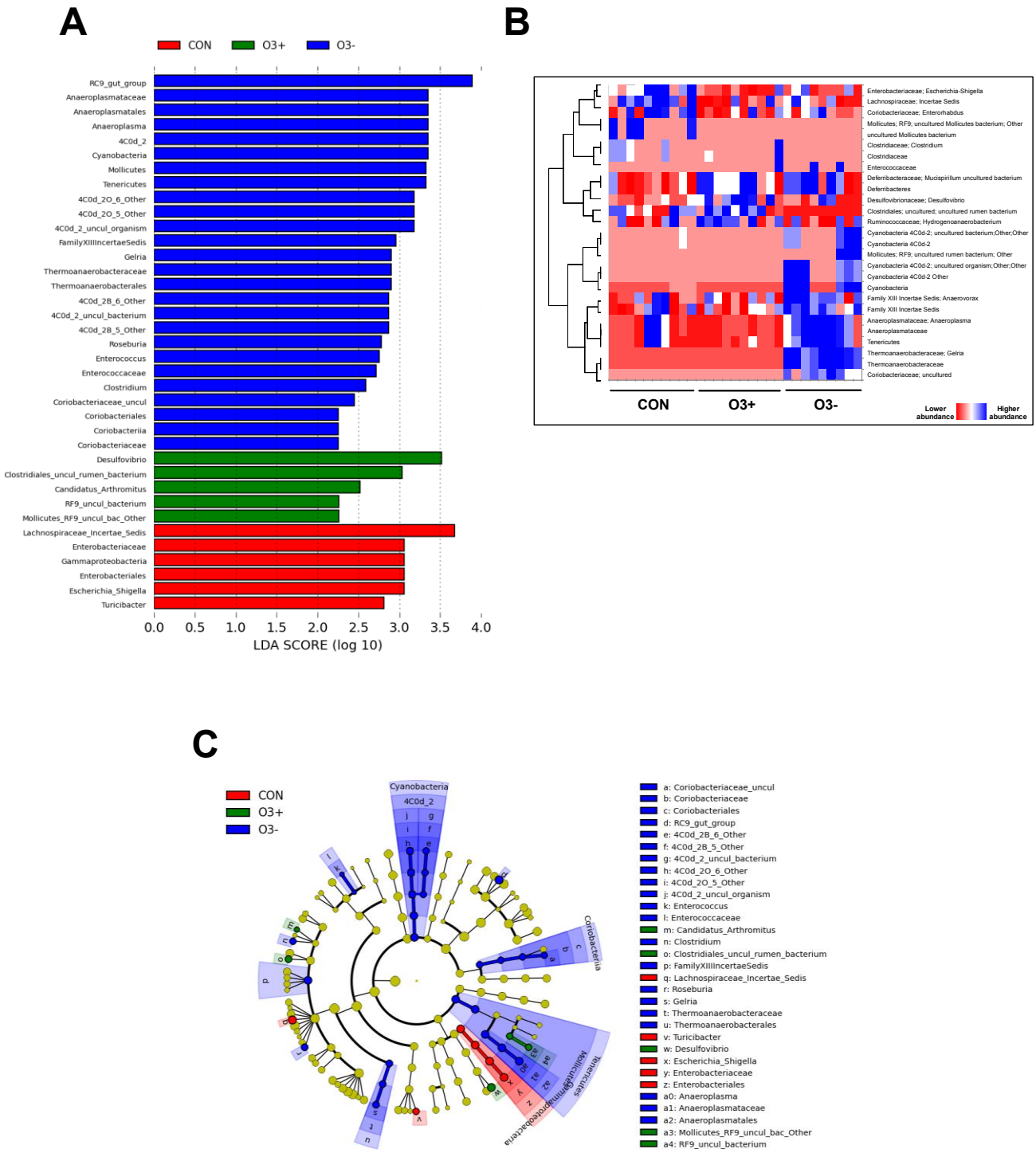


Figure 5. Differential abundance of gut microbiota taxa following omega-3 intervention. LDA scores of taxa significantly altered by omega-3 status in offspring are shown in **(A)** (LDA scores >2 and significance of $p<0.05$ as determined by Wilcoxon signed-rank test). The most differentially abundant taxa in adulthood following dietary intervention are represented as a cladogram in **(C)** as generated from LEfSe data in **(A)**. Heatmap distribution of significantly altered taxa in adulthood reveals distinct differences between treatment groups **(B)**. n=9-10 per group. Analysis by non parametric Kruskall Wallis test followed by multiple comparisons by Mann-Whitney. * significantly different to CON. # significantly different to O3+.

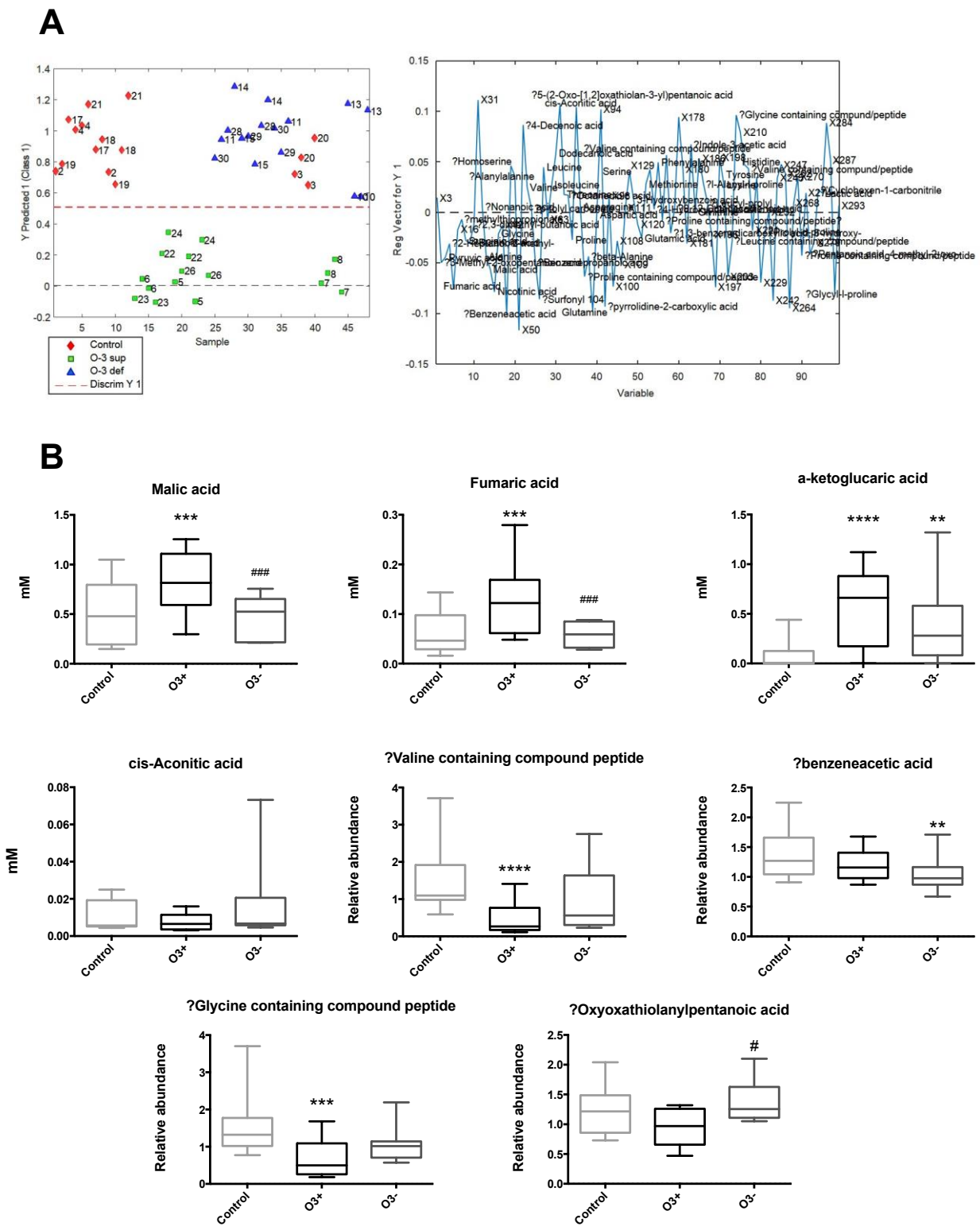


Figure 6. Omega-3 PUFA-induced changes to the cecal metabolome. Partial least squares discrimination analysis (PLS-DA) revealed differences in the cecal metabolomic profile between groups that were driven by changes in O3+ **(A)**. The metabolites contributing most strongly to the separation were those involved in energy metabolism and others **(B)**. "?" denotes compounds that were identified based on a library database without a standard.

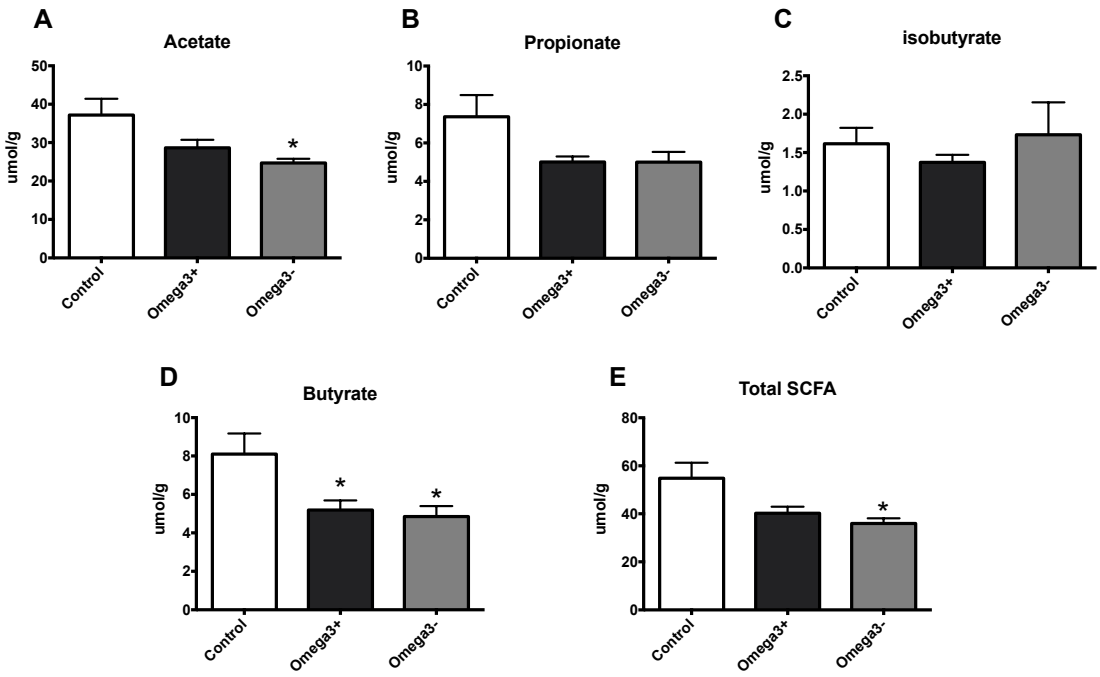


Figure 7. Cecal short chain fatty acid production. O3- displayed significantly reduced cecal acetate **(A)**, butyrate **(D)** and total short chain fatty acid (SCFA) **(E)** production. O3+ also displayed significantly reduced butyrate production **(D)**. There were no significant differences in propionate **(B)** or isobutyrate **(C)** production. Data shown as mean ± SEM. n= 9-10 per group. *$p < 0.05$ vs. CON.

Table 1. Tissue fatty acid profile

	Epididymal fat			Liver			Red blood cells		
	CON	**O3+**	**O3-**	**CON**	**O3+**	**O3-**	**CON**	**O3+**	**O3-**
SFA									
14:0	6.3 ± 0.1[a]	5.1 ± 0.1[b]	6.5 ± 0.1[a]	1.2 ± 0.2[a]	0.8 ± 0.1[b]	1.3 ± 0[a]	2 ± 0.2	2 ± 0.2	1.7 ± 0.1
16:0	20.7 ± 0.3[a]	24.8 ± 0.2[b]	21 ± 0.4[a]	24.6 ± 0.5[a]	25.2 ± 0.4[a]	22.4 ± 0.2[b]	35.3 ± 1	36.3 ± 1	35.1 ± 0.6
18:0	2.7 ± 0.1	2.7 ± 0.1	2.8 ± 0.1	11.9 ± 0.4	12.7 ± 0.5	11.3 ± 0.2	19.1 ± 1.1	17.5 ± 1.2	20 ± 0.9
20:0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.4 ± 0	0.3 ± 0.1	0.2 ± 0	0.3 ± 0.1	0.3 ± 0	0.3 ± 0
Total SFA	29.8 ± 0.4[a]	32.7 ± 0.3[b]	30.4 ± 0.6[a]	38.1 ± 0.7[a]	39 ± 0.8[a]	35.2 ± 0.2[b]	56.6 ± 1.9	56.1 ± 2.1	57.1 ± 1.4
MUFA									
16:1	8.8 ± 0.3	8 ± 0.3	8.3 ± 0.3	3.5 ± 0.2[a]	2.1 ± 0.2[b]	3.7 ± 0.1[a]	2.2 ± 0.3	2.3 ± 0.4	1.5 ± 0.2
18:1t n-9	0.1 ± 0[a]	0.1 ± 0[b]	0.1 ± 0[a]	n.d.	n.d.	n.d.	0.1 ± 0	n.d.	n.d.
18:1c n-9	2.2 ± 0.1	4.1 ± 2.7	3.2 ± 0.1	16.1 ± 0.5[a]	12.4 ± 0.9[b]	18.7 ± 0.5[a]	13.6 ± 1.2	15.2 ± 1.5	11.5 ± 0.7
18:1 t n-7	31.4 ± 0.3[a b]	25.2 ± 2.9[a]	33.6 ± 0.4[b]	2.7 ± 0.1[a]	1 ± 0.1[b]	4.3 ± 0.3[c]	1.7 ± 0.1[a]	1.2 ± 0[b]	1.8 ± 0.1[a]
20:1	0.6 ± 0[a]	0.4 ± 0[b]	0.7 ± 0[c]	0.5 ± 0.1[a]	0.3 ± 0[a]	0.7 ± 0[b]	0.6 ± 0.2	2.1 ± 1	3.1 ± 1.3
Total MUFA	43.1 ± 0.6[a]	37.4 ± 0.6[b]	46 ± 0.8[c]	22.8 ± 0.7[a]	15.8 ± 1.1[b]	27.4 ± 0.7[c]	18.1 ± 1.6	20.8 ± 1.8	17.9 ± 1.3
PUFA									
n-6 PUFA									
18:2 n-6	24.7 ± 0.3[a]	24 ± 0.4[a b]	23 ± 0.4[b]	18 ± 0.3[a]	16.2 ± 0.3[b]	17.6 ± 0.3[a]	10 ± 0.7	9.7 ± 0.9	7.7 ± 0.5
18:3 n-6	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0[a b]	n.d.[a]	0.2 ± 0.1[b]	0.1 ± 0	n.d.	n.d.
20:3 n-6	0.1 ± 0[a]	0 ± 0[b]	0.2 ± 0[a]	1.2 ± 0.2[a]	0.6 ± 0.1[b]	1.3 ± 0[a]	0.8 ± 0.1[a]	0.3 ± 0[b]	0.8 ± 0.1[a]
20:4 n-6	0.2 ± 0[a]	0.1 ± 0[b]	0.4 ± 0[c]	10.1 ± 0.2[a]	4.7 ± 0.2[b]	14.1 ± 0.2[c]	9.4 ± 0.6[a]	3.6 ± 0.3[b]	12 ± 0.5[c]
22:4 n-6	0 ± 0[a]	0 ± 0[a]	0 ± 0[b]	0.1 ± 0.1[a b]	n.d.[a]	0.3 ± 0.1[b]	0.8 ± 0.1[a]	n.d.[b]	1.7 ± 0.2[c]
22:5 n-6	0 ± 0[a]	0.1 ± 0[b]	0.1 ± 0[c]	0.1 ± 0.1[a]	n.d.[a]	3.6 ± 0.1[b]	n.d.[a]	n.d.[a]	2.8 ± 0.3[b]
Total n-6 PUFA	25.1 ± 0.2	24.4 ± 0.4	23.6 ± 0.5	29.6 ± 0.4[a]	21.5 ± 0.3[b]	37.1 ± 0.5[c]	21 ± 0.6[a]	13.6 ± 0.7[b]	25 ± 1.3[c]
n-3 PUFA									
18:3 n-3	1.9 ± 0[a]	2.3 ± 0.1[b]	0 ± 0[c]	0.8 ± 0.1[a]	1 ± 0[a]	n.d.[b]	0.3 ± 0.1[a b]	0.3 ± 0.1[a]	n.d.[b]
20:5 n-3	0 ± 0[a]	0.4 ± 0[b]	0 ± 0[a]	0.5 ± 0.1[a]	4.1 ± 0.2[b]	n.d.[a]	0.1 ± 0[a]	2.1 ± 0.2[b]	n.d.[a]
22:5 n-3	0 ± 0[a]	0.4 ± 0[b]	0 ± 0[a]	0.2 ± 0.1[a]	1.4 ± 0.2[b]	n.d.[a]	0.2 ± 0.1[a b]	0.6 ± 0.2[a]	n.d.[b]
22:6 n-3	0.1 ± 0[a]	2.2 ± 0.1[b]	0 ± 0[a]	8 ± 0.3[a]	17.3 ± 0.4[b]	0.3 ± 0.2[c]	3.8 ± 0.3[a]	6.5 ± 0.4[b]	n.d.[c]
Total n-3 PUFA	2.1 ± 0[a]	5.4 ± 0.2[b]	0 ± 0[c]	9.5 ± 0.2[a]	23.7 ± 0.6[b]	0.3 ± 0.2[c]	4.3 ± 0.3[a]	9.5 ± 0.6[b]	n.d.[c]
Total PUFA	27.2 ± 0.2[a]	29.9 ± 0.5[b]	23.6 ± 0.5[c]	39.1 ± 0.6[a]	45.2 ± 0.6[b]	37.4 ± 0.7[a]	25.3 ± 0.9	23.1 ± 1	25 ± 1.3
n6:n3	12.1 ± 0.3	4.5 ± 0.1	n/a	3.1 ± 0.1	0.9 ± 0	n/a	5 ± 0.3	1.5 ± 0.1	n/a

SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids, PUFA, Polyunsaturated fatty acids

SUPPLEMENTARY

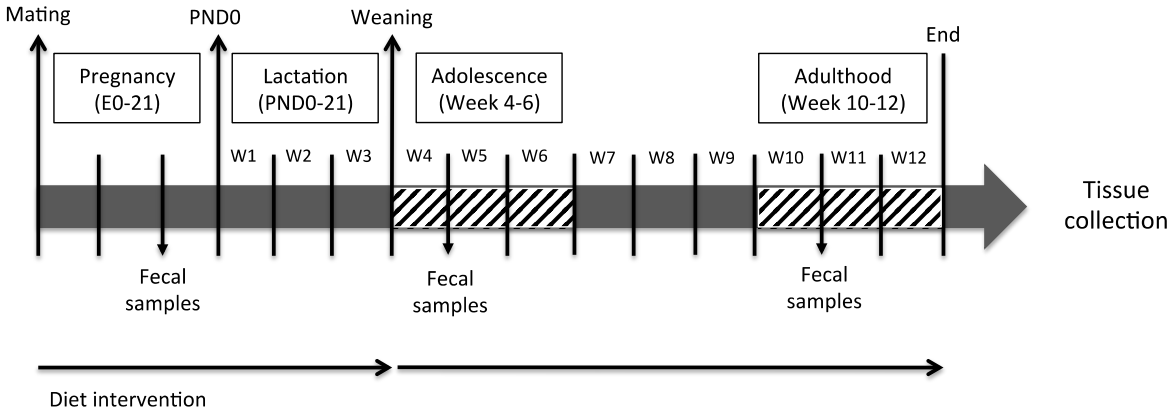


Figure S1. Experimental timeline. Following confirmation of mating, pregnant C57BL/6 dams were assigned to one of three diets; CON, O3+ or O3- for the duration of gestation (19-21 days) and lactation (21 days). Male offspring (n=10 per group) were weaned onto the same diet as their mothers at PND21 until 12 weeks. Fecal samples were collected from mothers during gestation and from offspring at both adolescence (week 4) and adulthood (week 10). At 13 weeks old, animals were sacrificed by cervical dislocation and samples collected.

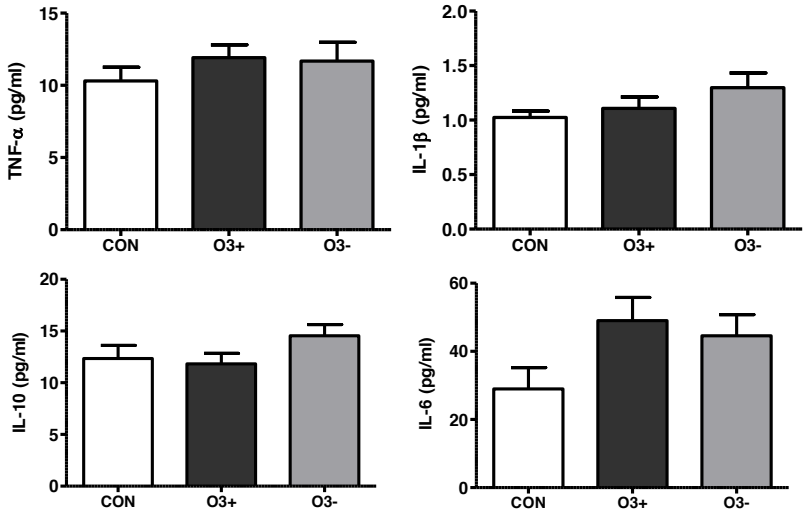


Figure S2. Plasma cytokine levels during adulthood. Dietary interventions revealed no significant differences on the levels of TNF-α, IL-1β, IL-10 and IL-6 in plasma.

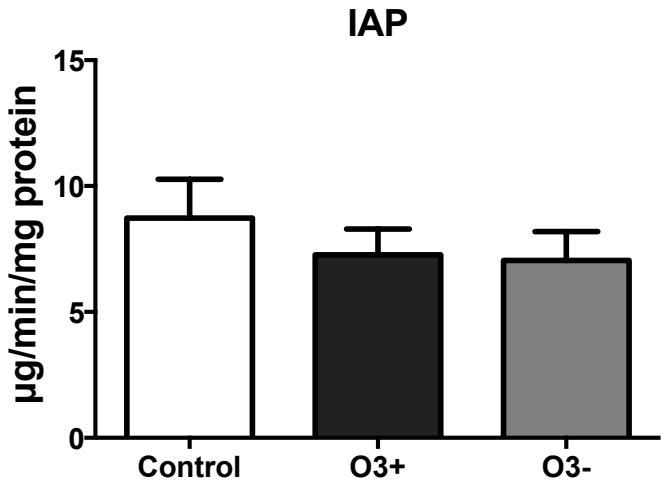


Fig S3. Jejenum intestinal alkaline phosphatase production. There were no significant differences in jejenum IAP production between groups

Table S1. Diet macronutrient profiles

	CON	O3+	O3-
Protein (% by weight)	18.3	18.3	18.3
Carbohydrate (% by weight)	56.7	56.5	56.7
Fat (% by weight)	10	10	10
Protein (% of Kcal)	18.8	18.8	18.8
Carbohydrate (% of Kcal)	58.2	58	58.2
Fat (% of Kcal)	23.1	23.2	23.1
Kcal/g	3.9	3.9	3.9
LA (% by weight)	2.6	2.6	2.6
ALA (% by weight)	0.4	0.4	0
EPA (% by weight)	0	0.35	0
DHA (% by weight)	0	0.66	0
n-6 (% by weight)	2.6	2.6	2.6
n-3 (% by weight)	0.4	1.53	0

Abbreviations: O3+, Omega 3 polyunsaturated fatty acid supplemented diet; O3-, Omega-3 polyunsaturated fatty acid deficient diet; LA, Linoleic acid (C18:2 n-6) ; ALA, Alpha linolenic acid (C18:3 n-3); EPA, Eicosapentaenoic acid (C20:5 n-3); DHA, Docosahexaenoic acid (C22:6 n-3); n-6, n-6 polyunsaturated fatty acids; n-3, n-3 polyunsaturated fatty acids

Table S2. Fatty acid profile of diets

% Total fatty acids	CON	O3+	O3-
Saturated fatty acids (SFA)			
8:0	3.4	2.2	3.6
10:0	3.1	2	3.3
12:0	27	16.9	29
14:0	11.1	7.25	12.3
16:0	9	10.9	8.5
18:0	7.9	5.9	8.7
Total SFA	59.8	44	64.4
Monounsaturated fatty acids (MUFA)			
18:1	7.1	13.3	6.5
Total MUFA	7.3	13.3	6.7
Polyunsaturated fatty acids (PUFA)			
18:2 n-6	25.7	25.4	22.4
18:3 n-3	4.2	4.1	tr
20:4 n-6	0	0.26	0
20:5 n-3	0	3.2	0
22:5 n-3	0	1	0
22:6 n-3	0	5.7	0
n6	25.7	25.66	22.4
n3	4.2	14	0
Total PUFA	29.9	39.66	22.4
Saturated fatty acids (SFA)			
8:0	3.4	2.2	3.6
10:0	3.1	2	3.3
12:0	27	16.9	29
14:0	11.1	7.25	12.3
16:0	9	10.9	8.5
18:0	7.9	5.9	8.7
Total SFA	59.8	44	64.4
Monounsaturated fatty acids (MUFA)			
18:1	7.1	13.3	6.5
Total MUFA	7.3	13.3	6.7
Polyunsaturated fatty acids (PUFA)			
18:2 n-6	25.7	25.4	22.4
18:3 n-3	4.2	4.1	tr
20:4 n-6	0	0.26	0
20:5 n-3	0	3.2	0
22:5 n-3	0	1	0
22:6 n-3	0	5.7	0
n6	25.7	25.66	22.4
n3	4.2	14	0
Total PUFA	29.9	39.66	22.4

Table S3. Relative abundance of genera from mothers microbiota

	Mothers				
Genus	**CON**	**O3+**	**O3-**	**P-value**	**q-value**
Lachnospiraceae uncultured	22	14	17	**0.024**	0.399
Ruminococcaceae uncultured	13	11	10	0.104	0.568
Bacteroidales S24-7 uncultured	9.7	16.3	8.7	0.083	0.568
Alistipes	9.6	11.1	10.6	0.607	0.917
Ruminococcaceae Incertae Sedis	7.8	6.7	8.8	0.188	0.577
Odoribacter	4.6	5.4	4.6	0.997	1
Mucispirillum uncultured	4.4	2.9	5.5	0.566	0.893
Anaerotruncus	3.1	2.6	2.6	0.26	0.577
Bilophila	2.8	2.9	4	0.248	0.577
Lactobacillus	2.7	1	3.8	0.15	0.577
Oscillibacter	2.5	1.5	2.7	0.089	0.568
Lachnospiraceae Incertae Sedis	2.3	1.4	2.2	0.116	0.577
Bacteroides	2.1	3.6	4.4	0.58	0.895
Rikenellaceae RC9 gut group	2	2.9	2.2	0.516	0.833
Rikenella	1.3	1.5	0.9	0.3	0.609
Desulfovibrio	1.3	0.6	0.5	0.206	0.577
Escherichia-Shigella	1.2	0.6	0.7	0.176	0.577
Coprococcus	1.1	0.7	1.5	**0.006**	0.399
Clostridiales uncultured bacterium	1	2.9	2	**0.037**	0.399
Allobaculum	0.73	0.42	0.64	0.401	0.722
Akkermansia	0.66	6.17	3.18	0.249	0.577
Anaeroplasma	0.46	0.06	0.25	0.363	0.716
Peptococcaceae uncultured	0.45	0.41	0.38	0.713	0.993
Parasutterella	0.35	1.71	1.04	0.224	0.577
Turicibacter	0.33	0.06	0.11	0.233	0.577
Roseburia	0.24	0.16	0.21	0.514	0.833
Bifidobacterium;	0.19	0.34	0.4	0.285	0.595
Parabacteroides	0.19	0.23	0.19	0.904	1
Anaerovorax	0.17	0.07	0.12	0.248	0.577
Ruminococcus	0.16	0.09	0.02	**0.045**	0.399
Peptococcus	0.14	0.11	0.08	0.174	0.577
Peptostreptococcaceae uncultured	0.14	0.33	0.13	0.867	1
Candidate division TM7 uncultured other	0.12	0.11	0.06	0.165	0.577
Clostridium	0.1	0.01	0	0.424	0.734
Christensenellaceae uncultured	0.1	0.15	0.09	0.373	0.716
Family XIII Incertae Sedis	0.079	0.093	0.054	0.269	0.579
Enterorhabdus	0.036	0.032	0.024	0.677	0.961
Christensenella	0.031	0.027	0.041	0.482	0.815
Lachnospiraceae uncultured bacterium	0.027	0.029	0.068	0.846	1.000
Thalassospira	0.026	0.183	0.047	0.096	0.568
Other	0.026	0.038	0.057	**0.03**	0.399
Family XIII incertae sedis uncultured	0.022	0.018	0.011	0.212	0.577
Aerococcus	0.011	0.005	0.01	0.824	1
Erysipelotrichaceae Incertae Sedis	0.009	0.004	0.009	0.256	0.577
Hydrogenoanaerobacterium	0.008	0.006	0.006	0.87	1
Mogibacterium	0.006	0.007	0.002	0.191	0.577
Ruminococcaceae uncultured bacterium	0.006	0.007	0.006	0.673	0.961
Mollicutes RF9 uncultured bacterium other	0.005	0.007	0	**0.017**	0.399
Erysipelotrichaceae uncultured	0.003	0.003	0	0.144	0.577
Gelria	0.002	0	0.013	0.122	0.577
Staphylococcus	0.001	0.002	0.017	0.839	1
Enterococcus	0.001	0.002	0.008	0.231	0.577

Pseudobutyrivibrio	0.001	0.002	0.006	0.089	0.568
Coriobacteria uncultured	0	0	0.017	**0.043**	0.399
Barnesiella	0	0.001	0.002	0.645	0.954
Prevotella	0	0	0	1	1
Prevotellaceae uncultured	0	0	0	1	1
Flavobacterium	0	0	0	1	1
Cyanobacteria 4C0d-2 uncultured bacterium	0	0	0	1	1
Cyanobacteria 4C0d-2 uncultured organism other	0	0	0.009	**0.043**	0.399
Lactococcus	0	0	0	1	1
Streptococcus	0	0	0	1	1
Candidatus Arthromitus	0	0	0	1	1
clostridiaeceae uncultured	0	0	0	1	1
Anaerostipes	0	0	0	1	1
Blautia	0	0	0	1	1
Marvinbryantia	0	0	0.001	0.407	0.722
Clostridiales uncultured rumen bacterium	0	0.001	0	0.407	0.722
alpha proteobacterium	0	0	0	1	1
Mollicutes RF9 uncultured bacterium other	0	0	0	1	1
Mollicutes RF9 uncultured rumen bacterium other	0	0	0	1	1

Table S4. Relative abundance of genera from offspring microbiota

	Offspring				
Genus	**CON**	**O3+**	**O3-**	**P-value**	**q-value**
Lachnospiraceae uncultured	17	16	15	0.195	0.433
Alistipes	12	11	12	0.653	0.861
Ruminococcaceae Incertae Sedis	9.7	8.6	9.6	0.545	0.774
Ruminococcaceae uncultured	9.7	9.2	8.5	0.514	0.745
Odoribacter	9.5	13.1	12.2	0.211	0.454
Bacteroidales S247 uncultured bacterium	6.9	6.2	5.2	0.184	0.433
Mucispirillum uncultured bacterium	3.6	6.2	6.9	**0.032**	0.162
Bacteroides	3.4	3.7	3.7	0.964	1
Rikenella	3	2.8	3	0.88	1
Oscillibacter	3	2	3	0.077	0.288
Rikenellaceae RC9 gut group	2.6	2.7	3.2	0.174	0.426
Lachnospiraceae Incertae Sedis	2.6	1.4	1.6	**0.008**	0.063
Clostridiales uncultured uncultured bacterium	2.6	3	3.1	0.772	0.962
Anaerotruncus	2.4	2.9	2.5	0.135	0.398
Akkermansia	2.1	2.5	1.9	0.449	0.664
Lactobacillus	1.2	0.5	0.8	0.973	1
Bilophila	1.1	1.1	1.2	0.99	1
Parasutterella	0.94	0.91	0.77	0.928	1
Allobaculum	0.74	0.79	0.4	0.246	0.499
Coprococcus	0.66	0.44	0.58	0.303	0.552
Parabacteroides	0.6	0.97	0.58	0.723	0.917
EscherichiaShigella	0.58	0.23	0.28	**0.004**	**0.036**
Desulfovibrio	0.51	1.12	0.57	**0.023**	0.148
Christensenellaceae uncultured	0.43	0.41	0.41	0.564	0.785
Bifidobacterium	0.38	0.3	0.19	0.271	0.534
Anaerovorax	0.3	0.26	0.41	**0.031**	0.162
Ruminococcus	0.26	0.47	0.48	0.655	0.861
Peptococcaceae uncultured	0.23	0.24	0.25	0.918	1
Anaeroplasma	0.21	0.05	0.51	**0.001**	**0.014**
Peptostreptococcaceae uncultured	0.2	0.1	0.19	0.169	0.426
Clostridiales uncultured rumen bacterium	0.16	0.2	0	**<0.0001**	**0.002**

TM7 uncultured bacterium Other	0.15	0.19	0.1	0.105	0.339
Family_XIII_Incertae_Sedis__Incertae_Sedis	0.13	0.11	0.15	0.287	0.551
Roseburia	0.13	0.17	0.17	0.072	0.284
Pseudobutyrivibrio	0.11	0.07	0.08	0.828	0.985
Turicibacter	0.11	0.01	0.11	0.092	0.325
Peptococcus	0.1	0.12	0.11	0.894	1
Thalassospira	0.1	0.19	0.12	0.157	0.413
Enterorhabdus	0.04	0.02	0.03	**0.038**	0.180
Other	0.04	0.1	0.05	0.246	0.499
Erysipelotrichaceae Incertae Sedis	0.03	0.05	0.04	0.832	0.985
Lachnospiraceae uncultured bacterium	0.03	0.02	0.02	0.195	0.433
Clostridium	0.03	0.08	0	**0.003**	**0.030**
Family XIII Incertae Sedis uncultured	0.02	0.03	0.02	0.947	1
Christensenella	0.02	0.02	0.03	0.331	0.585
A 4C0d2 uncultured bacterium Other Other	0.008	0	0.208	**0.003**	**0.030**
Hydrogenoanaerobacterium	0.008	0.016	0.008	**0.045**	0.200
RF9 uncultured bacterium Other	0.007	0.036	0.007	**0.072**	0.284
A 4C0d2 uncultured organism Other Other	0.006	0	0.275	**0.001**	**0.014**
Mogibacterium	0.005	0.005	0.004	0.976	1.000
Ruminococcaceae uncultured bacterium	0.004	0.005	0.005	0.704	0.909
uncultured_Mollicutes_bacteriumOther	0.004	0	0	**0.015**	0.107
Prevotellaceae uncultured	0.003	0	0	0.14	0.398
Erysipelotrichaceae uncultured	0.003	0.001	0	0.136	0.398
Blautia	0.002	0	0	0.387	0.585
Prevotella	0.001	0	0	0.387	0.585
Coriobacteriaceae uncultured	0	0	0.038	**<0.0001**	**0.002**
Barnesiella	0	0.002	0.004	**0.096**	0.325
Flavobacterium	0	0	0	0.387	0.585
Staphylococcus	0	0.001	0	0.387	0.585
Aerococcus	0	0	0	1	1
Enterococcus	0	0	0.001	0.296	0.552
Lactococcus	0	0.006	0	0.387	0.585
Streptococcus	0	0	0	0.996	1
Clostridiaceae Candidatus Arthromitus	0	0.006	0	0.146	0.399
Clostridiaceae uncultured	0	0.001	0	0.786	0.962
Anaerostipes	0	0.158	0	0.341	0.585
Marvinbryantia	0	0	0	0.627	0.856
Gelria	0	0	0.177	**<0.0001**	**0.002**
wr0007 uncultured alpha proteobacterium	0	0	0	0.387	0.585
RF9 uncultured rumen bacteriumOther	0	0	0.003	**0.028**	0.162

SUPPLEMENTARY METHODS

IAP enzyme activity

Jejenum intestinal alkaline phosphatase (IAP) enzyme activity was carried out using a commercial alkaline phosphatase assay kit (Abcam, Cambridge, UK) as per manufacturers instructions and results expressed as ug IAP/minute reaction time/mg protein. Protein concentration of jejenum tissue was assessed using Bradford reagent as per manufacturers instructions (Sigma Aldrich, Wicklow, Ireland).

Chapter 4

Maternal omega-3 and omega-6 fatty acids differentially regulate offspring susceptibility to obesity through modulation of gut microbiota

ABSTRACT

Background: Dietary omega-3 polyunsaturated fatty acids (n-3 PUFA) have anti-obesigenic properties compared with n-6 PUFA. The pathogenesis of obesity however has its origins in the fetal and neonatal periods, therefore maternal PUFA status may influence offspring susceptibility to obesity. Furthermore, little is known about the role of the gut microbiota in the interaction between maternal n-3 PUFA status and offspring weight gain.

Methods: Wild type (WT) offspring of WT and transgenic *fat-1* mice, that endogenously produce n-3 PUFA, were placed on a high fat diet (HFD) to assess the role of maternal n-3 PUFA status on obesity susceptibility. Offspring were also fostered to mothers of the opposite genotype at birth to compare the role of prenatal and postnatal n-3 PUFA status. Weight gain, body composition, gut permeability, inflammatory markers and gut microbiota composition were all assessed following 3 months HFD feeding.

Results: Offspring born to a *fat-1* mother or fostered to *a fat*-1 mother during lactation gained significantly less weight than those of WT mothers (189-196% vs. 232%) suggesting that maternal n-3 PUFA *in utero* or during lactation reduce HFD-induced weight gain in offspring. Offspring n-3 PUFA dietary exposure during lactation, via milk, resulted in greater n-3 tissue accumulation than *in utero* n-3 exposure ($p<0.05$). Similarly, the effects on the gut microbiota were primarily driven by the mother's phenotype during lactation such that *fat-1* mothers reduced gut permeability ($p<0.05$), *Firmicutes*, *Clostridia*, *Lachnospiraceae* and increased *Akkermansia* and *Bacteroidetes* in offspring. The changes in microbiota composition induced by maternal n-3 PUFA status persisted throughout life after HFD-feeding and after differences in tissue PUFA status disappeared.

Conclusions: Elevated maternal n-3 PUFA status during pregnancy and/or lactation led to significantly reduced weight gain in offspring fed HFD following weaning. This is associated with comprehensive alterations to the offspring microbiota, which persist throughout life. Maternal n-3 PUFA supplementation poses potential as a therapeutic intervention to prevent obesity, gut microbiota dysbiosis and associated chronic disease.

1. INTRODUCTION

Obesity is one of the biggest global public health issues, which negatively impacts the long-term health of millions of people around the world. Obesity comorbidities include insulin resistance, hypertension and dyslipidemia, which contribute to increased risk of chronic diseases such as diabetes, cardiovascular disease and dementia (Ogden et al., 2007). In recent times, the age of obesity onset has progressively declined such that childhood obesity has now become a major public health concern (de Onis et al., 2010). Childhood obesity is expected to affect 9.1% of pre-school children globally by the year 2020 which will significantly increase long-term chronic metabolic disease risk. As a result, much research has begun to investigate how maternal and early-life nutrition impacts offspring health outcomes in later life. Evidence from the Barker Hypothesis and the Dutch Hunger Winter suggests that maternal nutritional inadequacies adversely affect fetal metabolic programming in the offspring leading to negative health consequences in later life such as greater cardiovascular disease risk (Barker et al., 1993). Indeed, the adoption of the modern Western Diet has revealed that maternal obesity and over-nutrition also contribute to elevated long-term metabolic disease risk for infants (Boney et al., 2005, Laitinen et al., 2001, Gaillard, 2015). A number of mechanisms have been proposed to contribute to this phenomenon such as maternal or placental inflammation (Heerwagen et al., 2013, Catalano and deMouzon, 2015) and epigenetics (Heerwagen et al., 2010) however, a clear understanding is lacking. Consequently, there is limited evidence about which maternal dietary interventions may prevent such metabolic disruptions to the fetus or neonate and reduce later-life disease risk.

In recent times, obesity and a number of associated chronic diseases have been characterized by alterations to the gut microbiota (Patterson et al., 2016). Gut ‘dysbiosis’ or imbalance is often characterized by elevated abundance of pathogenic bacteria that impair gut homeostasis. For example *Enterobacteriaceae* produce

lipopolysaccharide (LPS), an endotoxin that stimulates TLR-4-induced inflammation. Low-grade chronic inflammation is the hallmark of metabolic endotoxemia, an inflammatory condition that may play a causal role in obesity pathogenesis (Cani et al., 2008). By stimulating the growth of pathogenic microorganisms, hypercaloric and pro-obesigenic diets impair the integrity of the gut epithelial barrier leading to subsequent translocation of bacterial-derived LPS across the intestinal wall and into circulation. As an agonist of TLR-4, LPS initiates an inflammatory cascade that involves the upregulation of transcription factors such as NF-kB, inflammatory gene expression and inflammatory cytokine production such as IL-6, TNFa and IL-1 β. This cascade of events occurs not only in circulation but also in adipocytes promoting adipogenesis, adipose tissue 'whitening' and subsequently, obesity (McArdle et al., 2013). However, selective targeting of the microbiota by prebiotics to restore microbial balance can prevent/correct this cascade of events, which dampens the inflammatory response, restores metabolism and subsequently reduces obesity (Cani et al., 2007, Cani et al., 2009, Everard et al., 2014).

Disruption of normal commensal gut microbiota composition can occur during early-life which may have implications for future metabolic health. The composition of the infant gut microbiota is mediated by maternal health status, antibiotics, birth delivery mode and early-life feeding practices (Bäckhed et al., 2015). Hence, disruptions to early-life microbiota development can induce a state of dysbiosis that disrupts metabolic, immunological and even central nervous system functioning, which elevates chronic disease risk in later life. Indeed, a parental western diet is associated with elevated plasma LPS and heightened colonic immune responses in standard chow-fed mice offspring (Myles et al., 2013). These immune modulating responses were dependent upon the comprehensive changes induced to the offspring microbiota, as a result of the parental diet. Consequently, as the maternal microbiota correlates with that of the infant (Bäckhed et al., 2015), optimization of maternal diet and microbiota

composition may therefore enhance infant microbiota development. Recently, it was shown that selective modulation of the obese murine maternal microbiota, through prebiotic feeding, significantly reduced offspring adiposity, which was associated with beneficial changes to the offspring microbiota such as enhanced *Bifidobacteria* abundance (Paul et al., 2016).

Although it has been shown that high fat diets contribute to perturbation in gut microbial balance and inflammation-induced obesity, recent evidence suggests that saturated and polyunsaturated fats (PUFA) differ in their interaction with the gut microbiota and subsequent metabolic outcomes (Patterson et al., 2014, Caesar et al., 2015). Omega-3 (n-3) and omega-6 (n-6) play opposing roles in the inflammatory response (Calder, 2006). n-6 PUFA typically up-regulate inflammation by acting as precursors to pro-inflammatory eicosanoids. Alternatively, n-3 PUFA resolve inflammation by competing within the same enzymatic pathway, thereby reducing n-6 metabolism and the subsequent inflammatory cascade. The western diet however is characterized by high n-6/n-3 PUFA ratio, which many have suggested has contributed to the epidemic of chronic inflammatory diseases such as obesity (Simopoulos, 2002). The evolutionary ratio of n-6/n-3 PUFA has been estimated at 1:1, however this has increased to 10-50:1 in the modern western diet (Simopoulos, 2002). The importance of this ratio in metabolic disease has been widely studied in human and animals, and there is convincing evidence that lowering the n-6/n-3 ratio can restore disrupted metabolism in the context of chronic disease (Simopoulos, 2008, Simopoulos, 2016). From an early-life perspective, lowering the n-6/n-3 ratio in obese mothers can reduce offspring weight gain and associated inflammatory outcomes in mice (Heerwagen et al., 2013). Similar results have been reported for improving insulin sensitivity (Hussain et al., 2013). Furthermore, observational and intervention studies in humans have found negative correlations for n-3 PUFA status and positive correlations for n-6 status and offspring adiposity (Donahue et al., 2011, Moon et al., 2013).

Indeed, n-6 and n-3 PUFA appear to have opposing effects on gut homeostasis. Relative to dietary n-6 PUFA, n-3 PUFA reduce *Enterobacteriaceae* abundance, elevate *Bifidobacterium* abundance and dampen intestinal inflammation partially through the up-regulation of certain anti-microbial peptides (Kaliannan et al., 2015). Similarly, unlike n-6 PUFA, n-3 PUFA prevent weight gain and metabolic disruptions induced by early-life antibiotic exposure (Kaliannan et al., 2016). The result of early-life n-3 PUFA status on the developing microbiota has also been explored (Robertson et al., 2016). We have recently reported that early-life n-3 PUFA deficiency induces a state of gut dysbiosis and behavioural impairments even before adolescence, suggesting a role for n-3 PUFA within the developing gut-brain axis (Robertson et al., 2016).

Data on the role of maternal n-3 PUFA on offspring microbiota are limited. Recent evidence suggests that high doses of maternal fish oil supplementation impair the immune response in offspring and lead to the overgrowth of pathogenic bacteria (Gibson et al., 2015). However, these studies utilized crude fish oil in pharmacologicaly excessive doses (18% energy) and hence further studies are warranted to examine the relationship between nutritionally relevant maternal n-3 PUFA intake and offspring microbiota. In addition, the role of maternal n-3 PUFA intake in the context of offspring obesity and its association with gut microbiota has not been previously examined. Furthermore, there is a lack of evidence as to the differences between pre and post-natal n-3 PUFA status for offspring metabolic health.

Based on previous evidence reporting the critical opposing roles that n-3 and n-6 PUFA play in microbiota-associated metabolic endotoxemia (Kaliannan et al., 2015), we aim to investigate this in the context of maternal n-6/n-3 PUFA status and subsequent effects on obese offspring. We hypothesize that, in comparison to a high maternal n-6/n-3 ratio, a balanced maternal n-6/n-3 ratio can reduce weight gain and inflammatory outcomes in obese offspring through modulation of the gut microbiota. To examine this, we utilize the *fat-1* transgenic mouse model, which is capable of

endogenous conversion of n-6 PUFA to n-3 PUFA (Kang et al., 2004). Therefore, using a single diet for both treatment groups, this model allows the generation of two phenotypes: (a) *fat-1* mice with a balanced tissue n-6/n-3 ratio (~1:1) and (b) wild-type (WT) mice with a high n-6/n-3 ratio similar to the Western diet (>10:1). Our results show that a lower maternal n-6/n-3 ratio during gestation and/or lactation significantly reduces weight gain in offspring fed a HFD, which appears to be mediated through alterations in gut microbiota.

2. MATERIALS AND METHODS

2.1. Animals and diets

Generation of transgenic *fat-1* mice was performed as previously described (Kang et al., 2004) followed by backcrossing onto a C57BL/6 background. *Fat-1* phenotype was confirmed by gas-chromatography flame ionization detection (GC-FID) following identification of increased tissue n-3 PUFA compared with WT. *Fat-1* genotype was confirmed by RT-PCR. Mice were housed in the Massachusetts General Hospital (MGH) animal facility in a biosafety room (Level 2) in hard top cages with filtered air. Mice were maintained in a temperature-controlled room (22– 24°C) with a 12h light/dark diurnal cycle. Food and water were provided *ad libitum*. A subset of 3-month old female C57BL/6 WT mice was purchased from Charles River Laboratories and allowed to acclimatize to the facility conditions for 1 week prior to mating. *Fat-1* and WT mating pairs were fed a diet high in n-6 PUFA (AIN-76A with 10% corn oil) from LabDiet in order to maintain *fat-1* and WT phenotypes. At postnatal day (PND) 28, male and female offspring were weaned onto a high fat diet (HFD) with 60% kcal from fat (D12492, Research Diets Inc). Detailed fatty acid profiles of both diets are outlined in Table S1. Animals were sacrificed using CO_2. Dissected tissues were flash frozen in

liquid nitrogen. All animal procedures in this study were performed in accordance with the guidelines approved by the MGH Subcommittee on Research Animal Care.

2.2. Breeding and Cross fostering

Three month-old female *fat-1* and WT mice were mated with age-matched WT males. Mating pairs were housed in individual cages and males were separated from the females following confirmation of pregnancy. Within 48-hours of parturition, newborn litters were fostered to new mothers until weaning, at PND 28. Briefly, the newborn litter was removed from the biological mother's cage then mixed with the bedding of the foster mother in the hand of the investigator. The litter was then placed in the empty nest of the new foster mother. The foster mother was held above the new litter until she urinated on the litter in order to disguise their scent. Foster mother pairs were chosen such that offspring were fostered to mothers whom had given birth within 48 hours to a litter of similar size. The cross-fostering procedure was carried out in order to generate offspring of four distinct experimental groups as follows: *fat-1*/WT *group* – *fat-1* biological mother, cross-fostered to WT mother; WT/*fat-1* group – WT biological mother, cross-fostered to *fat-1* mother; *fat-1*/*fat-1 group* – *fat-1* biological mother, cross-fostered to new *fat-1* mother; WT/WT *group* – WT biological mother, cross-fostered to new WT mother. Cross-fostering was carried out in the *fat-1*/*fat-1* and WT/WT groups as a control to the cross-fostering procedure in the other two groups. The study design is outlined in Fig. 1.

At PND 10, the tails of the offspring were clipped with a scissors and genotyping was performed on the tail tissue by RT-PCR. Following confirmation of genotype, *fat-1* mice were removed from the litter such that only WT offspring remained. At PND28, WT offspring were separated from their mothers, grouped in separate cages and weaned onto the HFD.

2.3. Body weight, body composition and food intake

Body weight and food intake were measured weekly using an electrical balance. Body composition (fat mass, lean mass, water mass) was assessed on the day of sacrifice using a Minispec mq bench-top NMR spectrometer (Bruker Instruments).

2.4. Fatty acid analysis

Fatty acid analysis of tail and liver tissues was performed as previously described (Kang and Wang, 2005). Briefly, frozen tissue samples were ground to a powder under liquid nitrogen using a mortar and pestle. Lipid extraction and fatty acid methylation was performed by the addition of 14% (w/v) boron trifluoride (BF3)-methanol reagent (Sigma-Aldrich) followed by heading at 100°C for 1h. Fatty acid methyl esters (FAME) were analyzed using a fully automated HP5890 gas chromatography system equipped with a flame-ionization detector (Agilent Technologies, Palo Alto, CA). The fatty acid peaks were identified by comparing their relative retention times with the commercial mixed standards (NuChek Prep, Elysian, MN), and area percentage for all resolved peaks was analyzed by using a PerkinElmer M1 integrator.

2.5. Intestinal permeability

Intestinal permeability was performed as described previously (Kaliannan et al., 2015). Briefly, mice were fasted for 6 h and then FITC-dextran (70kDA, Sigma-Aldrich, in PBS solution) was administered to mice by oral gavage at a dose of 600 mg/kg body weight. Following gavage, blood samples were collected from the facial vein after 90 min. Serum was diluted with an equal volume of PBS, and fluorescence intensity was measured using a fluorospectrophotometer (Perkin-Elmer) with an excitation wavelength of 480nm and an emission wavelength of 520nm. Serum FITC-dextran concentration was calculated from a standard curve generated by serial dilution of FITC-dextran in PBS.

2.6. **Serum LPS**

Concentration of circulating serum LPS was measured using a ToxinSensor Chromogenic Limulus Amebocyte Lysate (LAL) Endotoxin Assay Kit (GenScript) according to manufacturers instructions. Prior to the assay, samples were diluted 50-fold with endotoxin-free water. Kit reagents were added to the serum samples followed by incubation at 37°C according to the assay protocol. Absorbance was read at 545nm and results generated from a standard curve.

2.7. qPCR

RNA extraction from tissue samples was carried out using TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY), following the manufacturer's instructions. RNA concentration (ng/ul) and purity (A260/280) of each sample was assessed using a plate reader. cDNA synthesis of RNA was performed using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturers instructions. qPCR was performed using SYBR Green reagent in a PRISM 9000 Light Cycler (Applied Biosystems). Primer sequences are detailed in Table S7. qPCR reactions were performed in triplicated and normalized to a house-keeping gene (β-actin). Gene expression was calculated using the ΔΔCt method.

2.8. Stool DNA extraction and 16s sequencing

Bacterial genomic DNA was extracted from mice fecal pellets using the QIAmp DNA Stool Mini Kit (Qiagen, UK) according to manufacturers instructions. DNA was quantified and purification was subsequently assessed by measuring absorbance and determining the A260/A280 ratio. DNA was stored at -20°C until analysis.

16s sequencing library preparation was performed on DNA samples according to the Illumina 16S metagenomic sequencing library protocol in order to generate V3-V4 amplicons. DNA samples were subjected to an initial PCR reaction utilising primers

specific for amplification of the V3-V4 region of the 16S rRNA gene (Forward primer 5’ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5’ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTA ATCC). Clean-up and purification of the PCR product was performed using the Agencourt AMPure XP system (Labplan, Dublin, Ireland). Following clean-up and purification, a second PCR reaction was performed in order to incorporate a unique indexing primer pair to each sample (Illumina Nextera XT indexing primers, Illumina, Sweden). The PCR products were purified a second time using the Agencourt AMPure XP system. Quantification of samples was performed using the Qubit broad range DNA quantification assay kit (Bio-Sciences, Dublin, Ireland). Following quantification, samples were pooled in equimolar amounts (4nM) and sequenced at Clinical Microbiomics (Copenhagen, Denmark) using Illumina MiSeq 2x300 bp paired end sequencing.

2.9. Bioinformatics

The 64-bit version of USEARCH (Edgar, 2013) and mothur (Schloss et al., 2009) is used in combination with several in-house programs for bioinformatic analysis of the sequence data.

Following tag identification and trimming, all sequences from all samples are pooled. Paired end reads are merged, truncating reads at a quality score of 4, requiring at least 100 bp overlap and a merged read length between 300 and 600 bp in length. Sequences with ambiguous bases, without perfect match to the primers, or homopolymer length greater than 8 are discarded and primer sequences trimmed. Reads are quality filtered, discarding reads with more than 5 expected errors and sequences are strictly dereplicated, discarding clusters smaller than 5.

Sequences are clustered at 97 % sequence similarity, using the most abundant strictly dereplicated reads as centroids and discarding suspected chimeras based on internal comparison. Additional suspected chimeric OTUs are discarded based on comparison with the Ribosomal Database Project classifier training set v9 (Cole et al., 2014) using UCHIME (Edgar et al., 2011). Taxonomic assignment of OTUs is done using the method by Wang et al. (Wang et al., 2007) with mothur's PDS version of the RDP training database v14.

2.10. Statistics

Statistical analysis was performed using SPSS (v19, NY, USA) and Graphpad Prism (v6, CA, USA). One-way analysis of variance (ANOVA) was performed to assess differences between groups followed by Tukey's or LSD post-hoc test. For the 16S sequencing data, non-parametric analysis was performed using the Kruskall-Wallis test followed by Mann-Whitney test. False Discovery Rate (FDR) analysis was calculated at $p<0.05$. Results are presented as mean ± standard error (SEM). Significance was accepted at $p<0.05$.

3. RESULTS

3.1. Weights and Dietary Intake

There were no significant differences in offspring weights at week 4 prior to introduction of the high fat diet (HFD) (Fig 2). In males, there were no significant differences in body weight gain between groups for the first five weeks on a HFD, however at week 6, WT/WT had gained significantly more weight than WT/*fat-1* ($p<0.05$). This effect was strengthened with continuation of the HFD and WT/WT continued to gain significantly more weight than all other groups throughout the experimental period. At sacrifice, following 3 months of HFD, WT/WT mice had

gained 232% body weight, which was significantly greater (p=0.012) than all other groups (*fat-1*/WT – 189%; WT/*fat-1* – 195%; *fat-1*/*fat-1* – 197%). There was no significant difference in male food intake.

Interestingly, there were no differences in female body weight gain between groups following HFD feeding (Fig. 2), suggesting a gender difference of maternal n-3 PUFA status on offspring weight gain. There was no significant difference in female food intake.

3.2. Body composition

Analysis of male body composition revealed that WT/WT displayed strong trends towards increased fat mass (p=0.08), reduced lean mass and increased subcutaneous fat weight (p=0.09) (Fig 3). There were no significant differences between female groups in body composition (Fig. S3).

3.3. Fatty acid analysis

The tail fatty acid profiles of mothers, offspring pre-HFD and offspring post-HFD are presented in table S3 and Fig 4. As expected, the n-6/n-3 PUFA ratio in tail tissue was significantly greater in WT mothers than *fat-1* mothers (p<0.0001). Following four weeks of lactation, WT/WT male offspring displayed the highest tail n-6/n-3 ratio, which was significantly greater than all other groups (p<0.0001). *Fat-1*/*fat-1* offspring displayed the lowest tail n-6/n-3 ratio. Interestingly, in the crossover groups, WT/*fat-1* offspring had a significantly lower n-6/n-3 ratio than *fat-1*/WT offspring.

Differences in adolescent tail n-6/n-3 ratio as a result of maternal genotype were eliminated following 3 months of HFD feeding. There were however, a small number of differences in individual tail fatty acids such that arachidonic acid (AA) was significantly greater in WT/WT offspring compared with all other groups (p=0.0004), which contributed to an increasing trend in total n-6 PUFA (p=0.07). There were no

differences in total n-6 PUFA, n-3 PUFA or the n-6/n-3 PUFA ratio in offspring liver (Table S4). Tail n-6/n-3 PUFA ratio pre-HFD positively correlated with total weight gain (R=0.24; p=0.006). Tail DHA pre-HFD negatively correlated with total weight gain (R=0.23; p=0.007).

3.4. Microbiota

16S Sequencing of the mothers, pre-HFD and post-HFD fecal microbiota generated a total of 23 million sequenced reads. Assessment of phylogenetic diversity using the Shannon Index revealed no significant differences between WT and *fat-1* mothers or between post-HFD groups (Fig. 5A). However, microbial diversity as measured by the Shannon Index was significantly reduced in the WT/WT group compared with WT/*fat-1* prior to HFD feeding. Number of OTUs, Chao1 and Simpson indices were also assessed as measures of phylogenetic diversity (Fig. S9). Principle coordinate analysis (PCoA) revealed distinct clustering of WT and *fat-1* mothers based on microbiota composition (Fig. 5B). Whole microbiome significance testing using PERMANOVA with Bray-Curtis similarity index showed significant differences between *fat-1* and WT mothers (p=0.0024). In the offspring, clustering was evident among treatment groups, as assessed using PERMANOVA with Bray-Curtis similarity index both pre HFD (p=0.0004) and post-HFD (p=0.0001). Both prior to and after HFD feeding, offspring clustered according to the lactating/foster mother's genotype such that the *fat-1*/WT and WT/WT groups clustered together, and the *fat-1*/*fat-1* and WT/*fat-1* groups clustered together, according to Bray-Curtis similarity index.

Relative abundances of phylum level taxa revealed distinct differences between groups (Fig. 6). *Firmicutes* and *Bacteroidetes*, the two most abundant phyla, differed significantly in mothers and offspring groups. In mothers, *Firmicutes* and *Defferibacteres* were significantly higher in WT mice compared with *fat-1*, whereas *Proteobacteria* were significantly lower. As seen in PCoA, phylum, family and genus

level taxa distribution appeared to be most significantly influenced by lactating/foster mother's genotype as opposed to biological mother's genotype. Hence, *Firmicutes* and the *Firmicutes:Bacteroidetes ratio* were also significantly greater in *fat-1*/WT offspring both pre-HFD and post-HFD. *Firmicutes* were also significantly greater in WT/WT offspring post-HFD. Consistent with the foster mother's microbiota, *fat-1*/WT and WT/WT displayed significantly reduced *Proteobacteria* at both time points.

A number of significant differences were observed at family and genus levels also (Fig. 7 and 8). PCA analysis of family taxa revealed that *Verrucomicrobiaceae* contributed most strongly to the separation seen between *fat-1* and WT mothers (Fig. S11). Due to the significant PCoA clustering between groups based on foster mother's genotype, offspring were grouped according to the foster mother's genotype and linear discriminant analysis effect size (LEfSe) was performed on these two groups (1. WT/*fat-1* + *fat*-1/*fat*-1; 2. *fat*-1/WT + *fat*-1/*fat*-1) (Fig. 9 and 10). LEfSe analysis elucidated the observed phylum level differences such that offspring whom had a WT foster mother were more abundant in species within the *Firmicutes* phylum and offspring whom had a *fat-1* foster mother were more abundant in species of the *Bacteroidetes* and *Proteobaceria* phyla. The elevated *Proteobacteria* were driven primarily by differences in *Deltaproteobacteria*, whereas G*ammaproteobacteria* appeared to be reduced (Fig. S10). *Lachnospiraceae*, a member of the class *Clostridia*, which is a member of the Firmicutes phyla, was significantly greater in WT mothers and in *fat-1*/WT and WT/WT offspring post-HFD. A similar trend was observed for *Clostridia*. Genera within the *Lachnospiraceae* family were also significantly elevated in *fat-1*/WT and WT/WT offspring, including *Johnsonella*, *Coprococcus* and *Marvinbryantia*. Members of the *Proteobacteria* phyla including *Bilophila* and *Helicobacter* were elevated in WT/*fat-1* and *fat-1*/*fat-1* groups. *Verrucomicrobia* appeared to be significantly elevated in *fat-1*/*fat-1* offspring post-HFD, an effect which appeared to be driven by a significant increase in *Akkermansia*.

A number of the differences in post-HFD microbiota correlated with the previously observed phenotypes (Fig. 11). *Firmicutes* and *Clostridia* abundance displayed significantly positive correlations with offspring weight. *Firmicutes* and *Lachnospiraceae* abundance also positively correlated with the tail n-6/n-3 ratio prior to HFD feeding. Furthermore, *Lachnospiraceae* abundance positively correlated with intestinal permeability.

3.5. Intestinal permeability

The *fat-1*/*fat-1* group exhibited the lowest intestinal permeability ($p=0.05$). *Fat*-1/*fat-1 and* WT/*fat-1* had significantly lower intestinal permeability compared with *fat-1*/WT (Fig. 11A)

3.6. LPS

Serum LPS was significantly lower in *fat-1*/WT compared with both *fat-1*/*fat-1* and WT/WT (Fig. 11A).

3.7. Adipose tissue inflammation

There were no differences in mRNA expression of *TNFa, F4*/*80* or *CCL2* in subcutaneous adipose tissue however *TLR4* expression appeared to be lower ($p<0.05$) in *fat-1*/WT compared with both *fat-1*/*fat-1* and WT/WT (Fig S5).

3.8. Cytokines

There were no significant differences between groups in circulating serum IL-10, IL-1β, IL-6, MCP-1 or TNFα levels. (Fig S6).

4. DISCUSSION

Much evidence exists indicating that obesity and its associated disorders have their origins in the fetal and neonatal periods (Oken and Gillman, 2003). As the gut microbiota plays a critical role in the pathogenesis of these disorders and the chronic low grade-inflammation that defines them, nutrition research must now focus on maternal and early-life interventions that target the gut microbiota. The excessive global consumption of n-6 PUFA in place of n-3 PUFA has contributed to a trans-generational epidemic of microbiota-associated chronic disease. Hence, increased n-3 PUFA consumption provides huge potential as an effective therapeutic measure to stem the rise in the modern chronic disease epidemic. Here, we have shown that the maternal n-6/n-3 ratio significantly regulates offspring weight gain during high fat feeding. We have shown for the first time, using a transgenic model, that maternal n-3 PUFA significantly reduce weight gain in high fat fed offspring in contrast to n-6 PUFA. We have also uniquely demonstrated that the differences in weight gain are associated with intestinal permeability and a modified gut microbiota in both mothers and offspring. Specifically, postnatal maternal n-3 PUFA appear to reduce the abundance of *Firmicutes*, *Lachnospiraceae* and *Clostridia* in offspring, which correlates with body weight, an effect that persists into adulthood. These results suggest that maternal tissue fatty acid content during gestation and milk fatty acid composition during lactation can significantly affect offspring weight, independent of offspring diet after lactation. Consequently, maternal n-3 PUFA intake may act as a suitable intervention to prevent excessive weight gain in offspring and the associated gut microbiota modifications that may have a causal effect.

Maternal n-3 PUFA status has previously been associated with offspring weight and adiposity in humans (Donahue et al., 2011). Maternal n-6 PUFA are positively associated and n-3 PUFA negatively associated with weight gain and fat mass accumulation (Muhlhausler et al., 2011, Moon et al., 2013). A number of mechanisms

may contribute to this. Firstly, maternal n-3 PUFA correlate with umbilical cord and infant n-3 PUFA (Donahue et al., 2009), therefore the beneficial effects on weight may be attributed to direct nutrient transport from mother to infant. This was demonstrated in the current study, in which *fat-1*/*fat-1* mice had the lowest tail n-6/n-3 ratio at weaning demonstrating that the n-3 PUFA produced by the *fat-1* mothers were vertically transmitted to the offspring both *in utero* and through the milk. It has previously been shown that the n-6/n-3 ratio is significantly lower in *fat-1* milk than WT (Kang et al., 2004). This reduced n-6/n-3 ratio in the offspring may therefore have had a direct impact on reduced weight gain through mechanisms involving reduced adipogenesis as has been described previously (Kim et al., 2015). The *fat-1*/WT group had a higher n-6/n-3 ratio than the WT/*fat-1* group. Interestingly, this suggests that the offspring n-3 status is more influenced by the dietary n 3 PUFA during lactation, via milk, rather than maternal n 3 PUFA status during gestation. The lactation period in the current study was however slightly longer (4 weeks) than gestation (3 weeks), which may have contributed to the differences observed in fatty acid status. However, as discussed below, the obese phenotypes and microbiota of the offspring appeared to be much more heavily influenced by the foster mothers fatty acid status rather than the biological mothers fatty acid status suggesting that maternal n-3 PUFA status is more influential during the lactation period rather than gestation.

Interestingly, there appeared to be a strong gender difference between male and female offspring regarding weight gain as a result of maternal n-3 status. WT/WT males gained significantly more weight on a HFD than all other groups suggesting that maternal n-3 PUFA either *in utero* or during lactation could reduce weight gain in obese offspring. However, no significant differences were observed between groups in female offspring. Gender-dependent differences have been reported before regarding n-3 status and weight (Howe et al., 2014, Thorsdottir et al., 2007). It has been shown that fish oil supplementation or fatty fish intake in humans effectively reduced weight gain in males

but not in females (Thorsdottir et al., 2007) suggesting a gender-dependent reaction. Interestingly however, other clinical studies report conflicting evidence regarding the efficacy of n-3 in reducing weight in females and males (Howe et al., 2014). Females have a greater capacity to convert ALA to EPA and DHA (Burdge and Wootton, 2002, Burdge et al., 2003), which may contribute to such observations in humans. Indeed female sex hormones interfere with the activity of the desaturase and elongase enzymes involved in PUFA metabolism (Decsi and Kennedy, 2011, Childs et al., 2008) and hence it has been suggested that gender should always be considered in studies regarding n-3 PUFA (Lohner et al., 2013). In the present study, there did not appear to be a difference between male and female n-6/n-3 tail fatty acid ratios prior to HFD. However, it has previously been shown that sex differences occur in adipogenesis, which are mediated by the effect of female sex hormones (Greenhill, 2016). These differences are removed in ovarectomized mice (Stubbins et al., 2012). In addition to differences in weight, there were subtle differences in glucose tolerance pre-HFD, which were eliminated following HFD feeding (Fig. S1). Insulin sensitivity appeared to be differentially regulated post-HFD with the *fat-1*/*fat-1* group displaying the lowest insulin area under the curve (AUC) following glucose administration (Fig. S2). Hence maternal n-3 status also appeared to subtly influence glucose metabolism.

Another mechanism by which the maternal fatty acid status may influence offspring weight is through immune regulation. It is possible that the lower n-6/n-3 ratio in *fat-1* mothers dampened maternal and placental inflammation, which induced an anti-inflammatory and anti-obesigenic environment in the offspring. This has been demonstrated previously using the same *fat-1* model in which maternal obesity induced maternal and placental inflammation, which was transmitted to the offspring resulting in a number of metabolic disruptions that were reversed in offspring of *fat-1* mothers (Heerwagen et al., 2013). Inflammation plays an important role in obesity and insulin sensitivity, as cytokine production and macrophage accumulation contribute to

adipogenesis (Greenberg and Obin, 2006). Interestingly however, circulating serum cytokines did not appear to differ between offspring groups (Fig. S6). This was also reflected in adipose tissue, where inflammatory gene expression in the offspring appeared to be mostly unchanged by maternal n-3 status. *TLR4* expression however, was significantly increased in WT/WT mice. This result is consistent with that for circulating LPS, which is an agonist of *TLR4*. A similar trend in these data suggests that the differences in serum LPS induced the elevation in *TLR4* expression, which may initiate low grade chronic inflammation and hence the observed increase in weight gain. However both of these increases were also observed in the *fat-1*/*fat-1* group.

Finally, the differing microbiota profiles between *fat-1* and WT mothers may have been passed somewhat onto the offspring thereby contributing to weight gain. The composition of an infant's microbiota is strongly influenced by that of the mother (Bäckhed et al., 2015). Therefore, the anti-inflammatory *fat-1* microbiota that has been described previously (Kaliannan et al., 2015) may have been partially passed on to the offspring thereby reducing the microbiota-associated weight gain. The gut microbiota have a well-established role in energy metabolism and obesity (Turnbaugh et al., 2009) by regulating energy harvest from macronutrients. The ratio of the two most dominant phyla, *Firmicutes* and Bacteroidetes, is considered a strong biomarker of weight gain (Turnbaugh et al., 2006). Firmicutes are known for more potent energy harvest capacity and are more abundant in states of obesity. This is thought to be due to their increased capacity to harvest energy from the diet. Interestingly, we found that *Firmicutes* were significantly higher in WT mothers than *fat-1* mothers. This was then transmitted to the offspring such that *fat-1*/WT and WT/WT displayed higher *Firmicutes* than both other groups. This increase in *Firmicutes* correlated significantly with both weight and n-6/n-3 status. One of the mechanisms by which *Firmicutes* contribute to obesity is through microbial fermentation of dietary polysaccharides that cannot be digested by mammalian enzymes. The end products of this fermentation process are typically short

chain fatty acids (SCFA) such as acetate and butyrate, which could contribute up to 10% of caloric intake (Bergman, 1990). Interestingly, the observed differences in weight gain were independent of dietary intake, which did not differ, suggesting that the WT/WT microbiota displayed an increased capactity for energy harvest. Indeed, a number of genera within the *Firmicutes* phyla that are involved in SCFA production were elevated in WT/WT including *Pseudobutyivibrio*, *Roseburia, Robinsoniella* and *Johnsonella*. Previously it has been shown that SCFA and such SCFA-producing species are elevated in states of obesity (Turnbaugh et al., 2006).

Erysipelotrichaceae, *Lachnospiraceae* and *Clostridia* were also all elevated in WT/WT and *fat-1*/WT and have all previously been associated with obesity through disrupted lipid metabolism and cholesterol homeostasis (Nadal et al., 2009, Zhang et al., 2009). Interestingly, the increase in *Lachnospiraceae* correlated positively with the observed increase in intestinal permeability in *fat-1*/WT and WT/WT animals. This correlation has been observed before in alcohol dependent subjects who display gut dysbiosis (Leclercq et al., 2014). *Lachnospiraceae* therefore, and the genera it encompasses, appear to be elevated by n-6 PUFA leading to increased gut permeability and weight gain. This effect was independent of changes to tight junction protein expression (Fig. S4). *Proteobacteria* and a number of associated genera appeared to be higher in *fat-1* mothers and offspring with *fat-1* foster mothers. This has been reported previously following fish oil feeding (Caesar et al., 2015). However, it has previously been reported that male *fat-1* mice display lower *Protebacteria* than WT, contradicting the results found in the present study in females (Kaliannan et al., 2015). Proteobacteria are divided into a number of classes including *Deltaproteobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* and *Epsilonproteobacteria*. Interestingly, the observed increase in Proteobacteria in *fat-1* mothers and their foster offspring were driven by *Deltaproteobacteria*, whereas *Gammproteobacteria* appeared to be similar or even reduced in these groups. *Deltaproteobacteria* consist of a number of sulphur-

reducing species whereas Gammproteobacteria (*enterobacteriaceae* family) contain a number of pathogenic endotoxin-producing species such as *Escherichia coli*, that contribute to low-grade chronic inflammation and are elevated in states of metabolic disruption. Hence, the different Proteobacteria classes may be differentially regulated by n-3 PUFA and subsequently exert differential effects on metabolic health. Previously, it was shown that *Bilophila wadsworthia*, a genus within the Deltaproteobacteria class, was significantly elevated in offspring of mothers fed fish oil in pharmacological excess (Gibson et al., 2015). Hence, Proteobacteria abundance may be more closely regulated by maternal n-3 PUFA status rather than direct dietary intake and further research is warranted to evaluate this effect on metabolic health. The overall contribution of *Desulfovibrionaceae* did not appear to contribute strongly to separation between WT and *fat-1* mothers in PCA loading (Fig. S11). *Verrucomicrobiaceae*, the family to which *Akkermansia* belong, appeared to contribute strongly to this separation, being more highly abundant in *fat-1* mothers. *Akkermansia* has garnered a lot of interest in recent times as a potent regulator of metabolic health during obesity in both animals and humans (Dao et al., 2016, Schneeberger et al., 2015).

Interestingly, as with the n-6/n-3 ratio, the gut microbiota of the offspring appeared to strongly match that of the lactating/foster mother rather than the biological mother. Therefore, WT/WT and *fat-1*/WT had similar microbiota composition, as did WT/*fat-1* and *fat-1*/*fat-1*. These microbiota outcomes correlated strongly with the fatty acid status, weight and other phenotypes. Indeed, it has been assumed that the fetus is sterile, however recent studies may suggest otherwise (Aagaard et al., 2014). The biological mother imprints a unique microbiota on the infant at birth (Bäckhed et al., 2015). The results reported here suggest that the 'birth microbiota' is quickly and comprehensively altered by the foster mother's microbiota, through differences in milk/dietary fatty acids following birth. These results would suggest that n-3 PUFA status in milk during lactation has a stronger impact on the infant microbiota than

maternal fatty acid status during gestation. Furthermore, despite the differences in n-3 and n-6 PUFA disappearing in adulthood after HFD, the observed differences in the microbiota persisted, suggesting that maternal fatty acid status and early neonatal feeding regime may have a permanent effect on the offspring microbiota throughout life.

This study has demonstrated for the first time using this unique transgenic model that maternal n-3 PUFA can significantly reduce weight gain in high fat fed offspring compared with maternal n-6 PUFA. This unique study design has demonstrated that the effect on weight is mediated by the gut microbiota, which is characterized during the post-natal period. Importantly, these effects on weight gain and gut microbiota composition induced by maternal n-3 status persist into adulthood even after the differences in fatty acid status disappear. These results have important implications for the current chronic disease epidemic. Obesity and related metabolic diseases partially originate *in utero* and during the neonatal period. Hence dietary interventions are now necessary during such early-life periods to prevent chronic disease risk. The gut microbiota is now appreciated as a root cause of chronic disease and hence a vital target for therapeutic dietary interventions. We have shown that a high maternal n-6/n-3 ratio can induce dysbiosis to the offspring microbiota, which persists throughout life. Re-balancing of this maternal ratio by elevating n-3 PUFA, primarily during the lactating period, has the potential to reduce excess weight gain during periods of high fat/calorie intake and the associated gut dysbiosis. Hence, maternal n-3 PUFA supplementation may stem the increase in global obesity and its related disorders through interaction with the gut microbiota.

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FIGURES

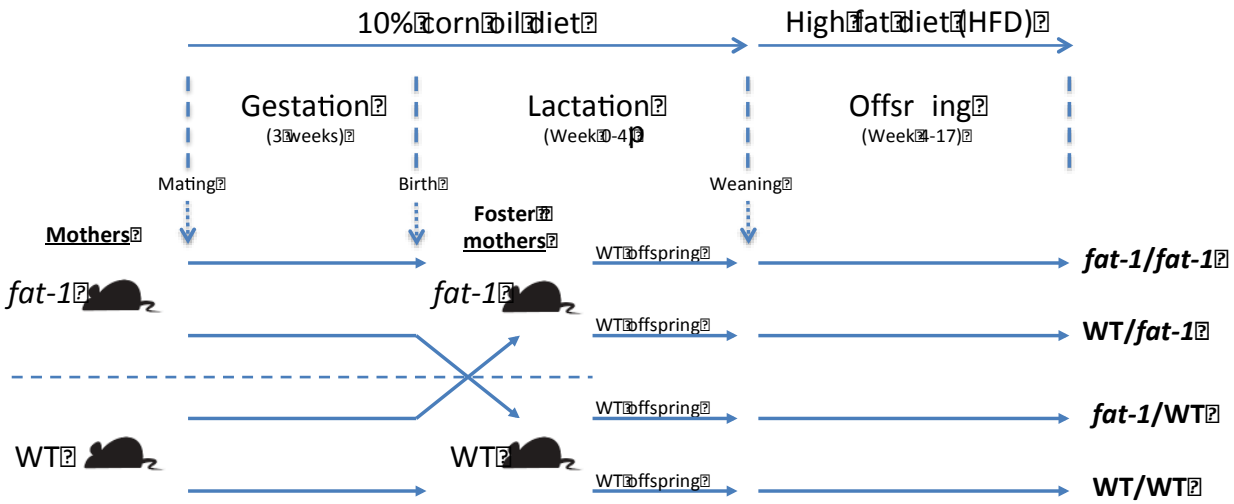


Figure 1. Experimental design. *fat-1* and WT mothers were mated whilst on a high n-6 PUFA diet (10% corn oil). At birth, WT offspring were cross-fostered to different mothers for the period of lactation (four weeks) to produce four experimental groups based on the mothers' genotype (biological mother's genotype/foster mother's genotype): *fat-1*/WT, WT/*fat-1*, *fat-1*/*fat-1*, WT/WT. During lactation, mothers were continued on a high n-6 PUFA diet. After 4 weeks of lactation, offspring were weaned onto a high fat diet (HFD) (60% kcal from fat) for 3 months during which body weights were assessed along with a number of other parameters.

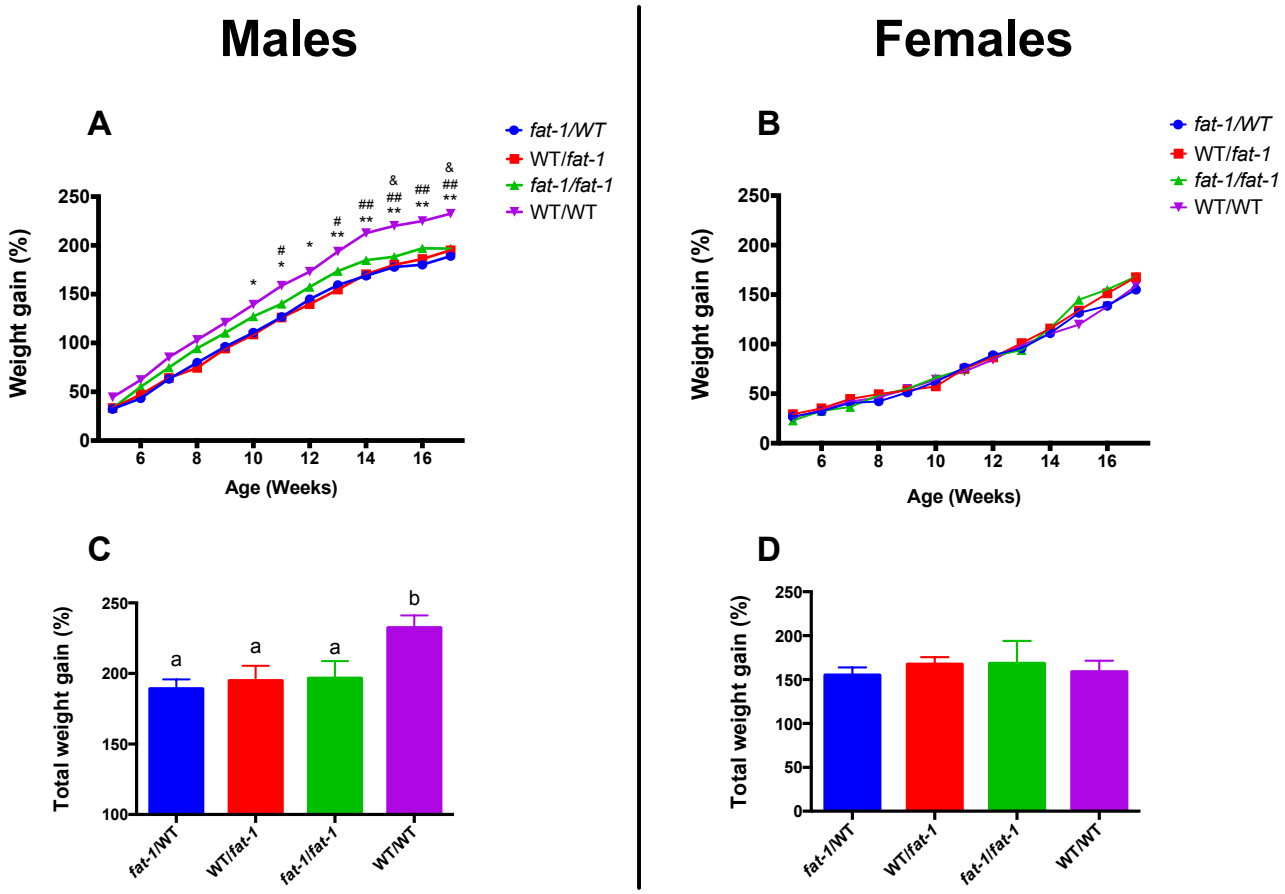


Figure 2. Weight gain on a HFD. WT/WT male offspring gained significantly more weight on a HFD than all other groups **(A and C)**. However there were no significant differences between female offspring groups following HFD feeding **(B and D)**. Data shown as mean ± SEM. n= 5-10 per group. *p < 0.05 and **p < 0.01, WT/WT vs. WT/*fat-1*; # p < 0.05 and ## p < 0.01 WT/WT vs *fat-1*/WT; $^{\&}$p<0.05, WT/WT vs. *fat-1*/*fat-1*.

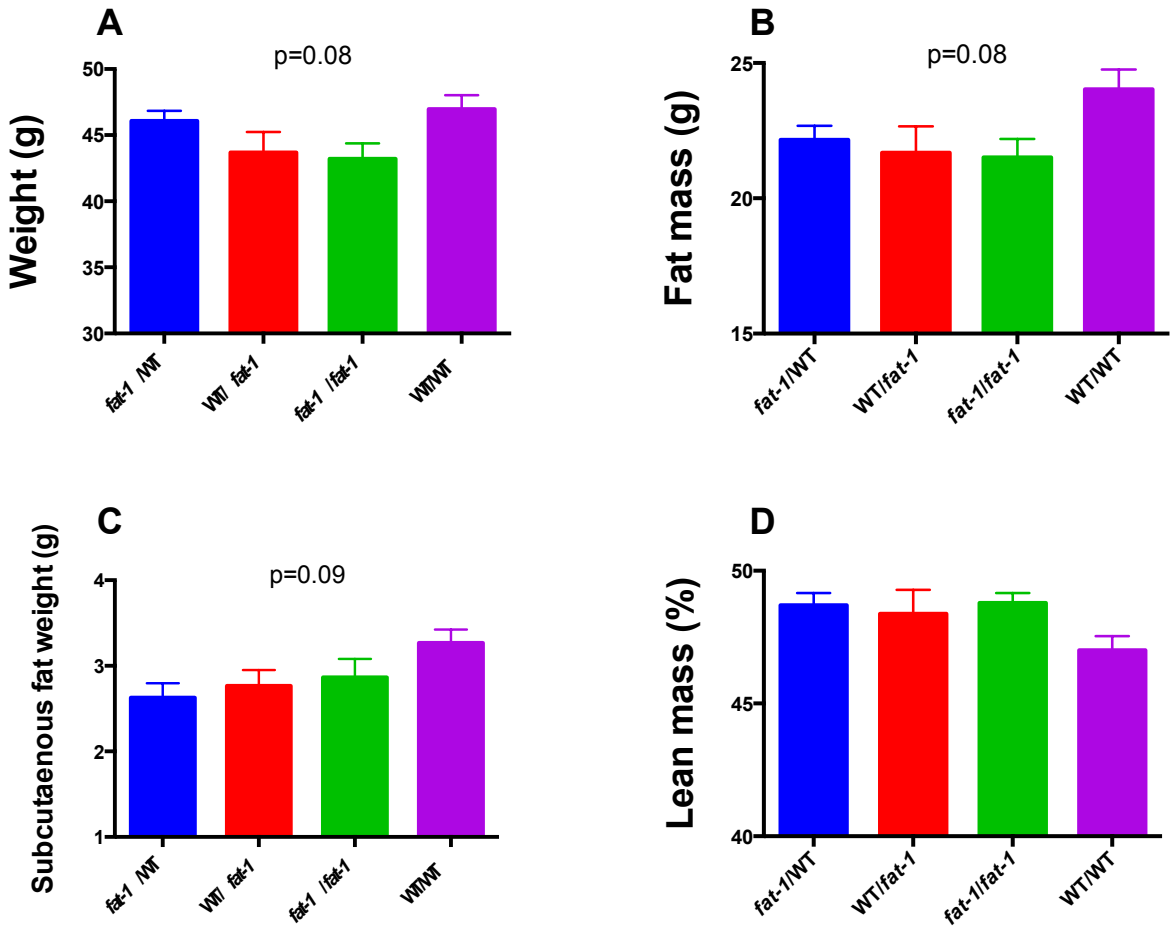


Figure 3. Male offspring body composition. WT/WT male offspring trended towards greater weight **(A)**, total fat mass **(B)**, subcutaneous fat mass **(C)** and lower lean mass **(D)** although not significantly. Data shown as mean ± SEM. n= 8-10 per group.

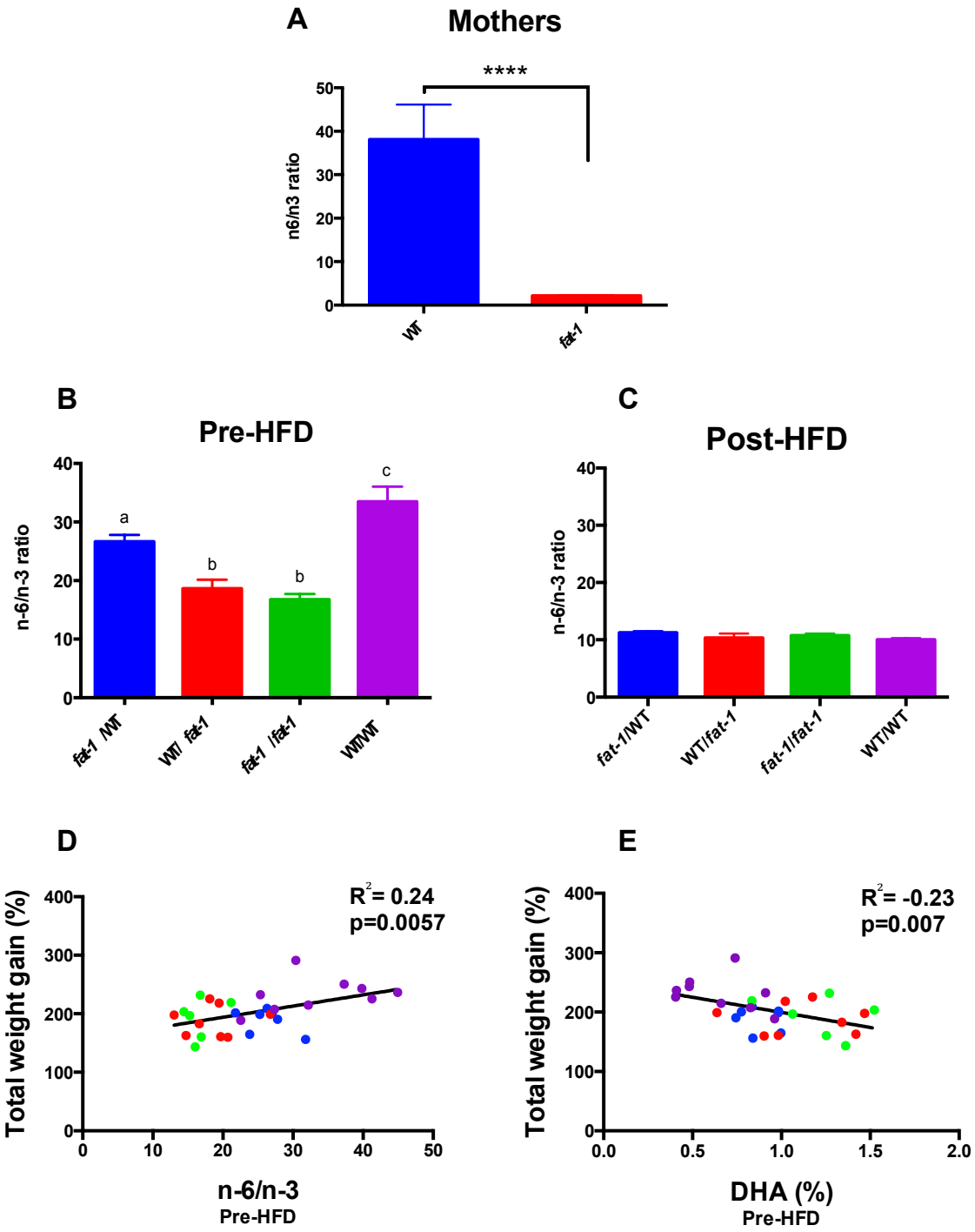


Figure 4. Tail n-6/n-3 fatty acid ratio. WT mothers displayed a significantly greater tail n-6/n-3 ratio compared with *fat-1* mothers **(A)**. Following lactation for four weeks and prior to HFD, WT/WT had significantly greater tail n-6/n-3 ratio compared with all other groups **(B)**. However, after 3 months on a HFD, differences in tail n-6/n-3 fatty acid ratio were eliminated and there were no significant differences between groups **(C)**. Total weight gain showed a significantly positive correlation with pre-HFD n-6/n-3 ratio **(D)** and a negative correlation with pre-HFD DHA **(E)**. Data shown as mean ± SEM. n= 8-15 per group. Bars with different letters are significantly different.

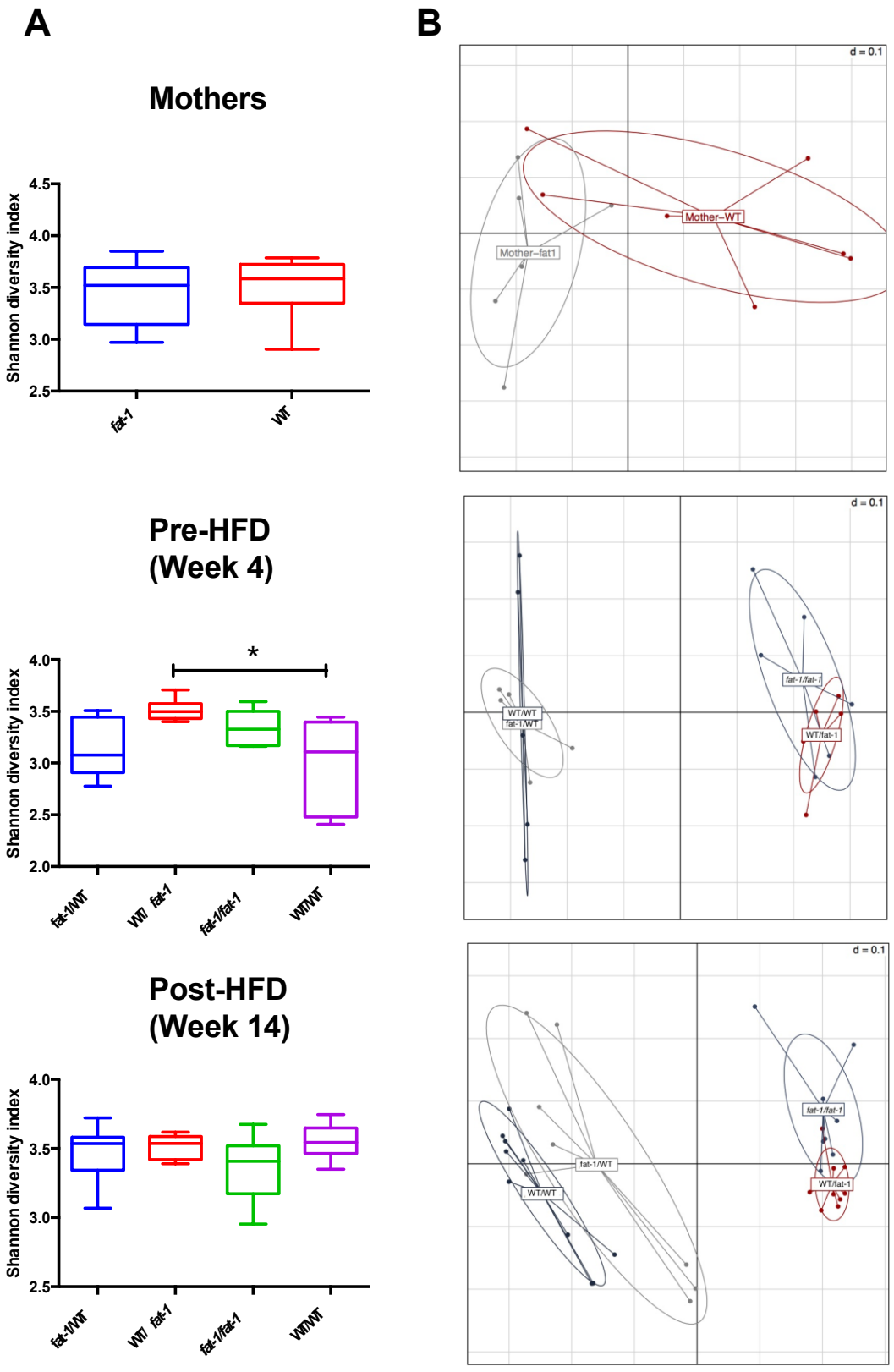


Figure 5. Microbiota diversity. Phylogenetic diversity as measured by the Shannon index did not differ between mothers or post-HFD groups, however appeared to be reduced in WT/WT compared with WT/*fat-1* before HFD feeding **(A)**. Principle coordinate analysis (PCoA) revealed clustering of groups based on foster mother genotype **(B)**. Data shown as mean ± SEM. n= 5-10 per group. *p<0.05.

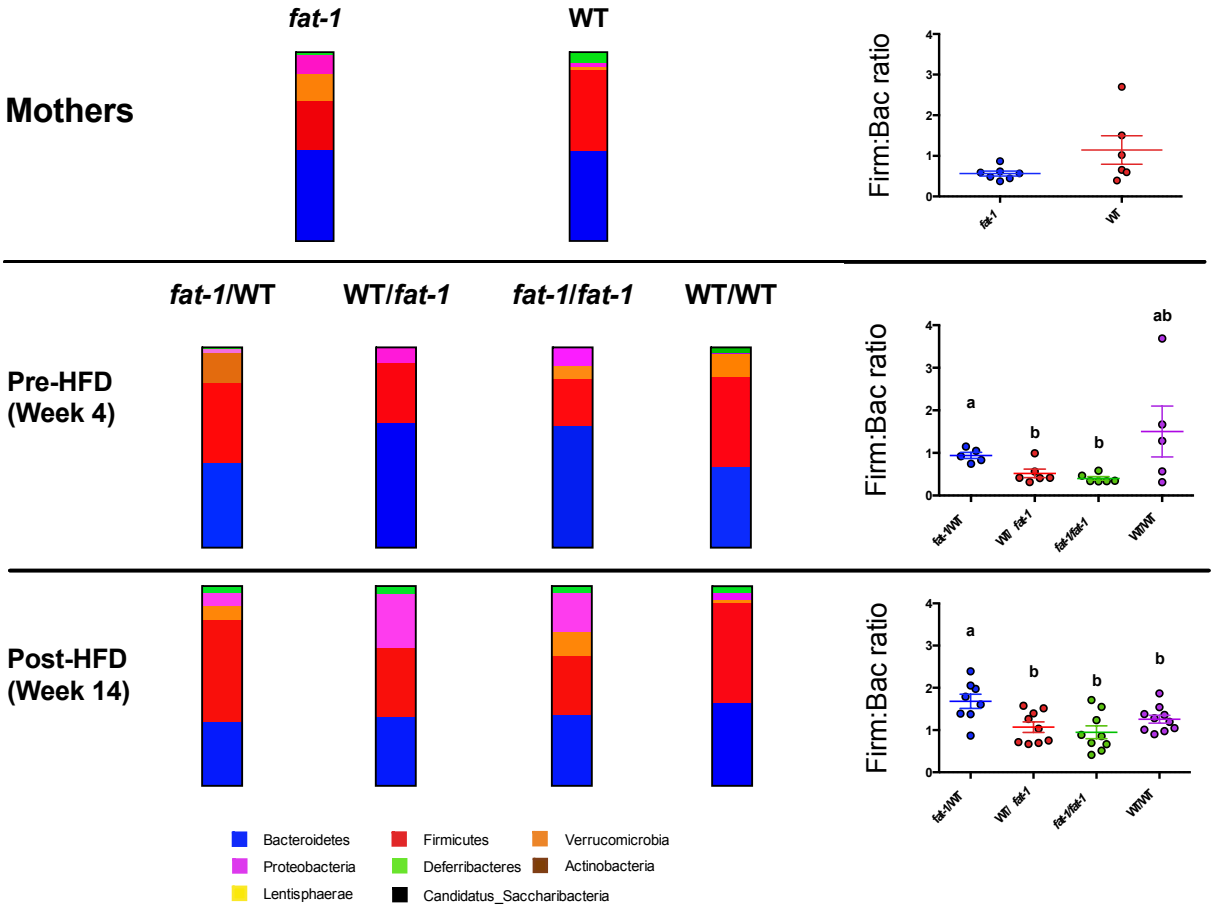


Figure 6. Phylum level distribution of fecal microbiota. Fecal microbiota composition differed significantly between groups at phylum level. Firmicutes appeared to be more abundant in WT mothers and offspring with WT foster mothers. This contributed to a significantly greater Firmicutes:Bacteroidetes ratio in *fat-1*/WT offspring. Data shown as mean ± SEM. n= 5-10 per group. Groups with different letters are significantly different.

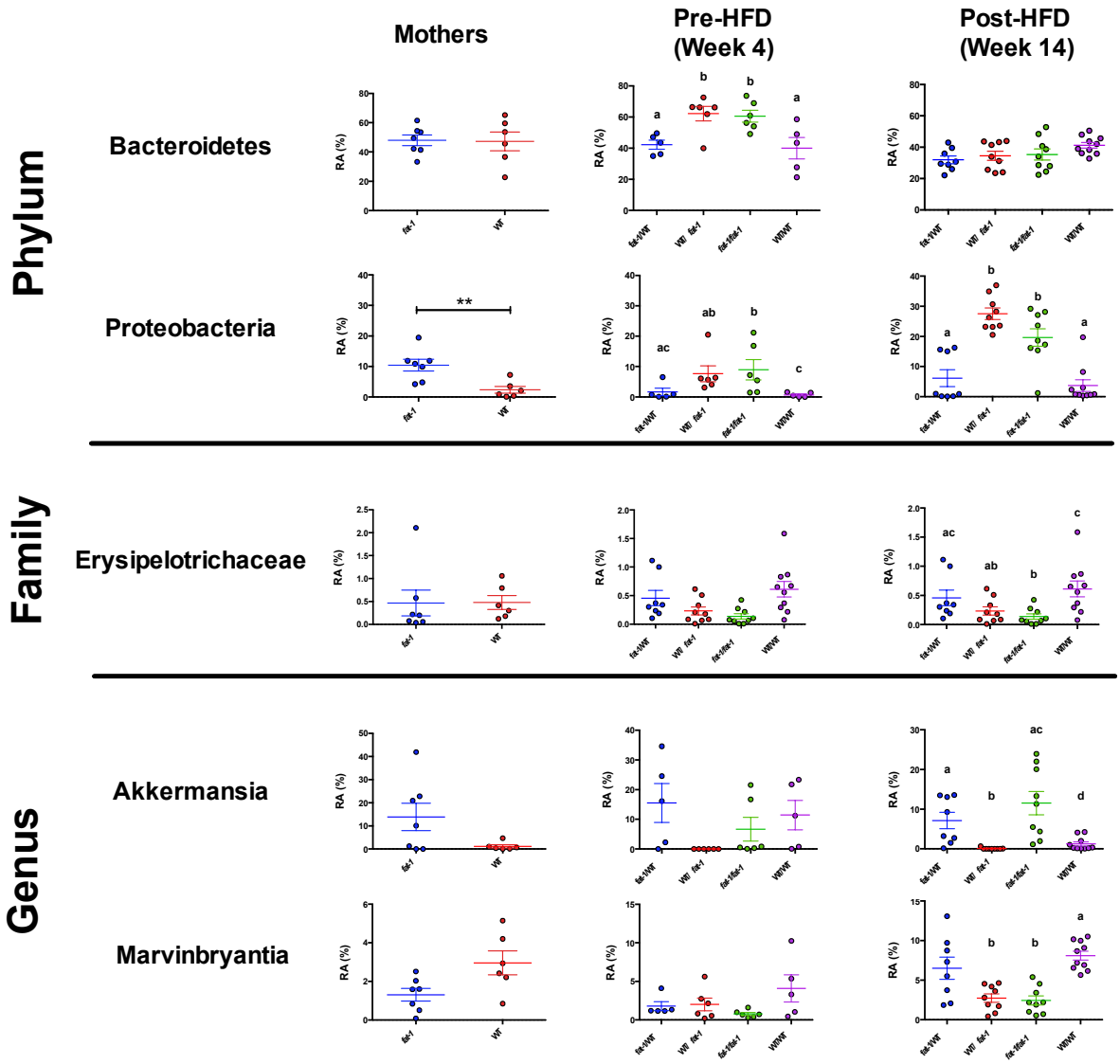


Figure 7. Phylum, family and genus level distribution of fecal microbiota. Fecal microbiota composition differed significantly between groups at different taxonomic levels. Trends observed in mothers appeared to be associated with those of foster offspring. RA (%), Relative Abundance. Data shown as mean ± SEM. n= 5-10 per group. Groups with different letters are significantly different.

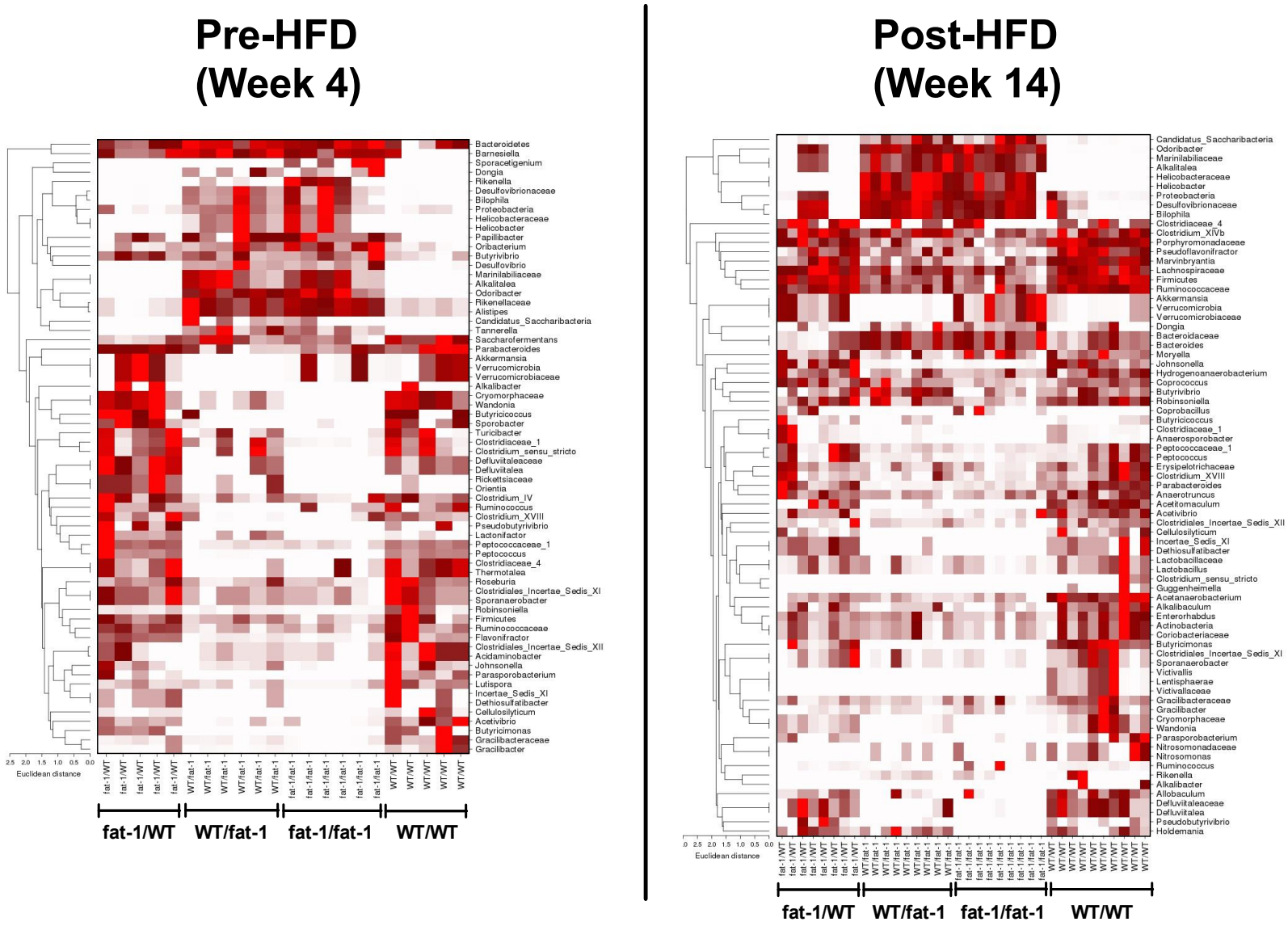


Figure 8. Heatmap of taxa of significantly different abundance between offspring groups. Normalized data of fecal microbiota abundance revealed distinct differences between offspring groups. Microbiota composition appeared similar in groups of the same foster mother genotype. Data normalized per taxonomic read. n= 5-10 per group.

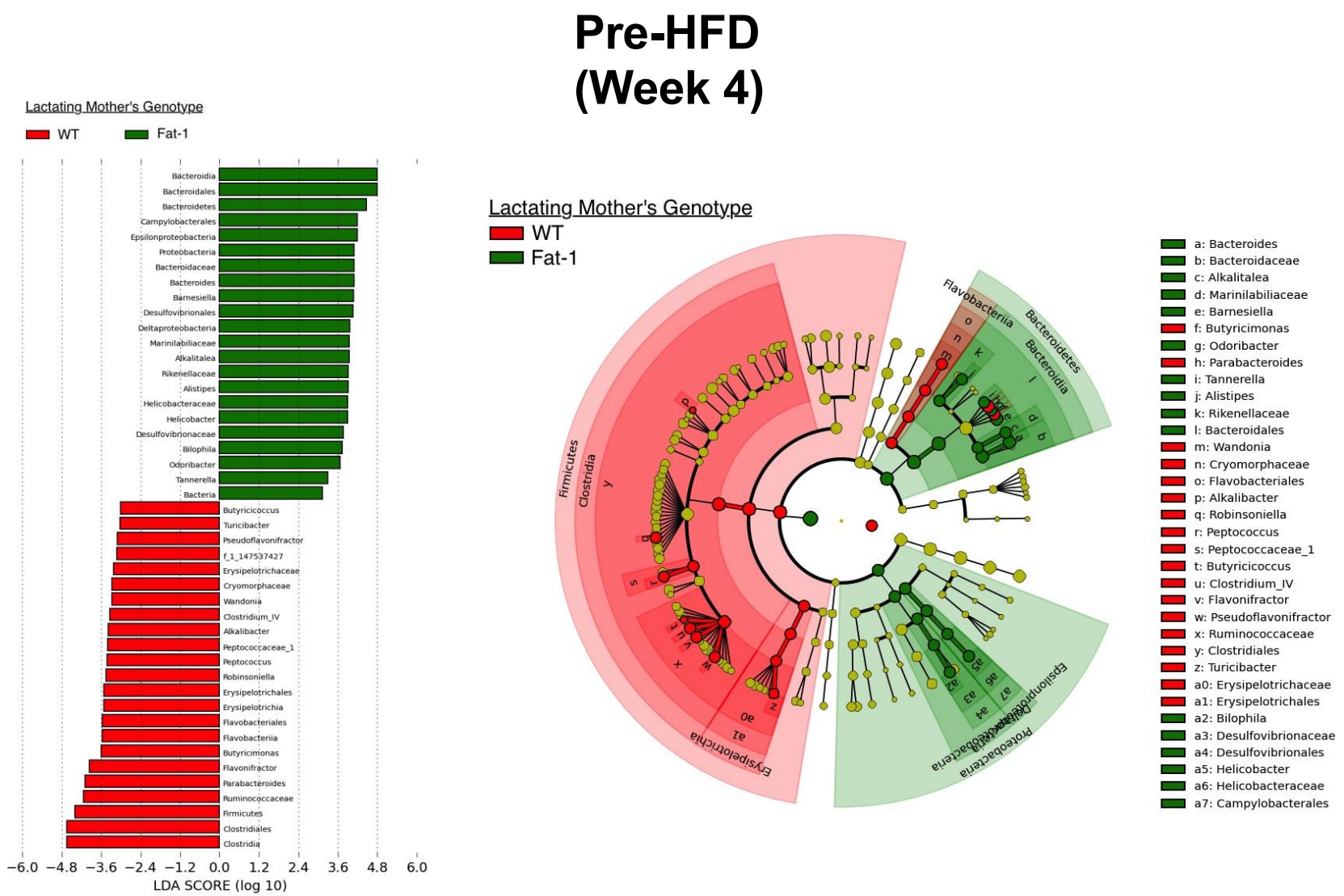


Figure 9. LEfSe analysis of pre-HFD differential taxa based on foster mother genotype. Least discriminant analysis of effect size (LEfSe) on clustered groups based on maternal genotype revealed that Firmicutes were more abundant in offspring of WT foster mothers whereas Bacteroidetes and Proteobacteria were more abundant in offspring of *fat-1* foster mothers.

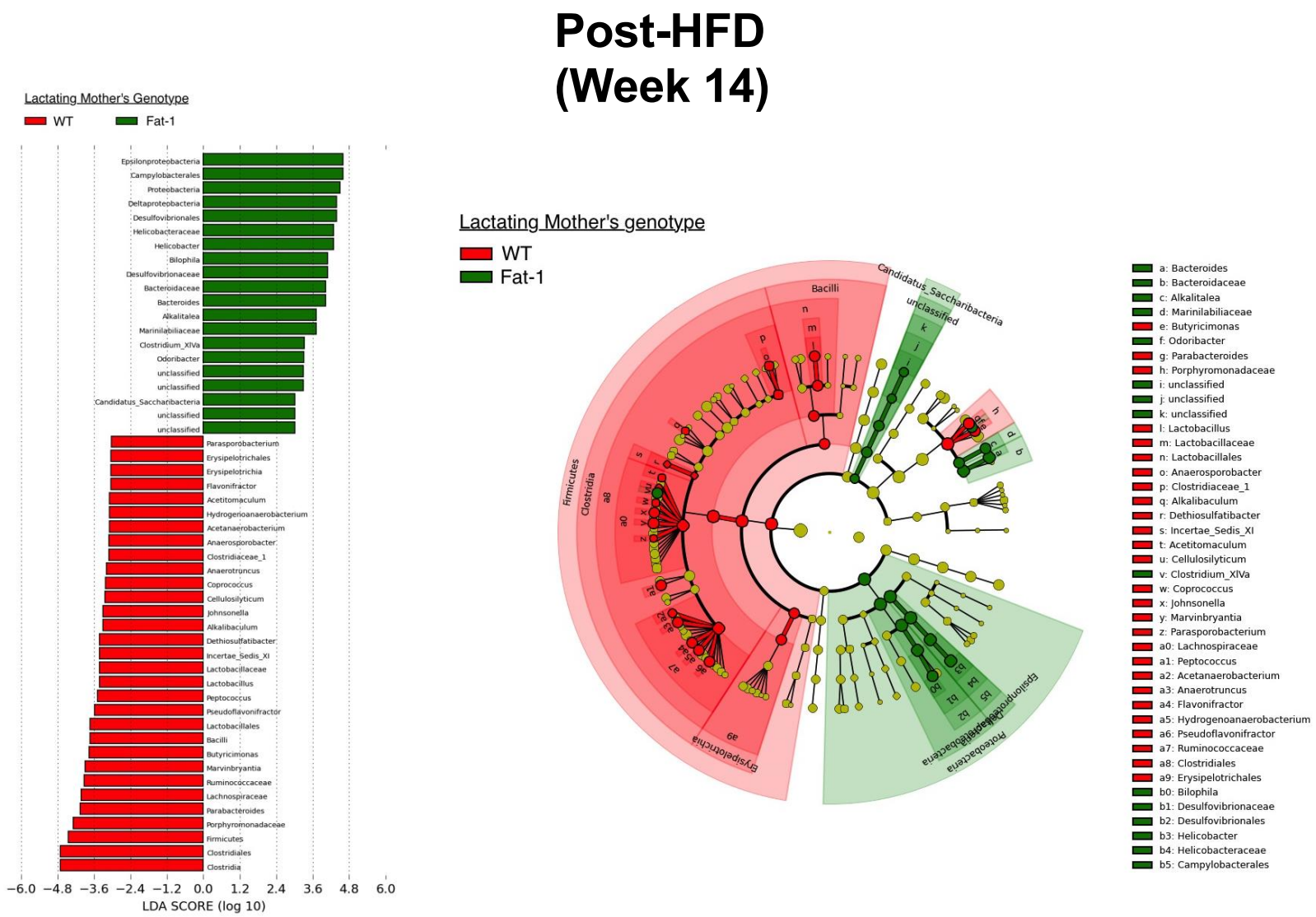


Figure 10. LEfSe analysis of post-HFD differential taxa based on foster mother genotype. Least discriminant analysis of effect size (LEfSe) on clustered groups based on maternal genotype revealed that Firmicutes were more abundant in offspring of WT foster mothers whereas Bacteroidetes and Proteobacteria were more abundant in offspring of *fat-1* foster mothers.

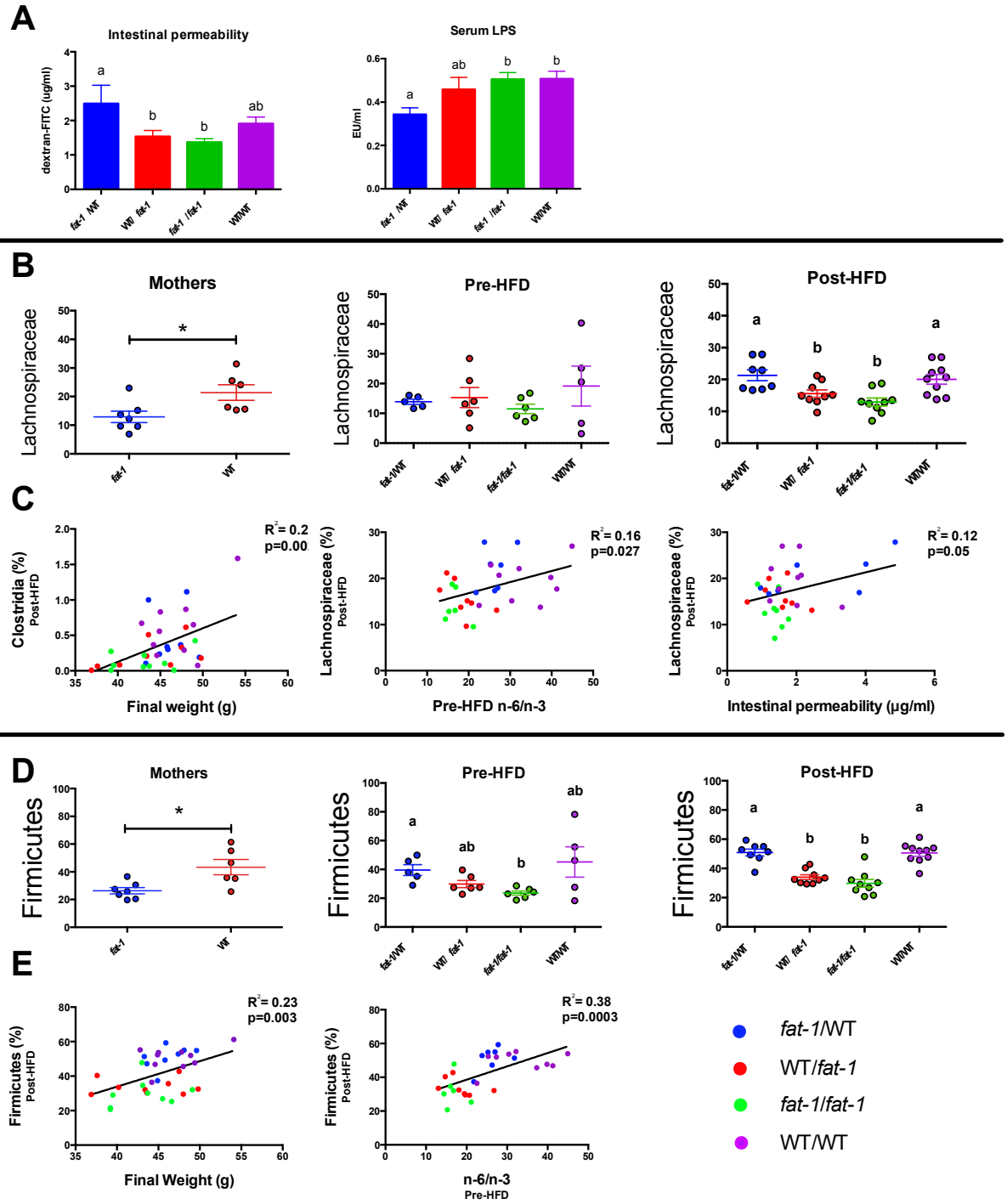


Figure 11. Fecal microbiota correlations with obese phenotypes. *fat-1*/*fat-1* offspring displayed the lowest gut permeability, whereas *fat*-1/WT displayed the lowest circulating LPS **(A)**. WT mothers, *fat-1*/WT and WT/WT offspring displayed significantly greater lachnospiraceae **(B)** and Firmicutes **(D)**. Clostridia, the class to which lachnospiraceae belongs, correlated positively with offspring weight while lachnospiraceae correlated positively with pre-HFD n-6/n-3 ratio and intestinal permeability **(C)**. Firmicutes abundance also positively correlated with weight and pre-HFD n-6/n-3 ratio **(E)**. Data shown as mean ± SEM. n= 5-10 per group. *p<0.05. Groups with different letters are significantly different.

SUPPLEMENTARY MATERIAL

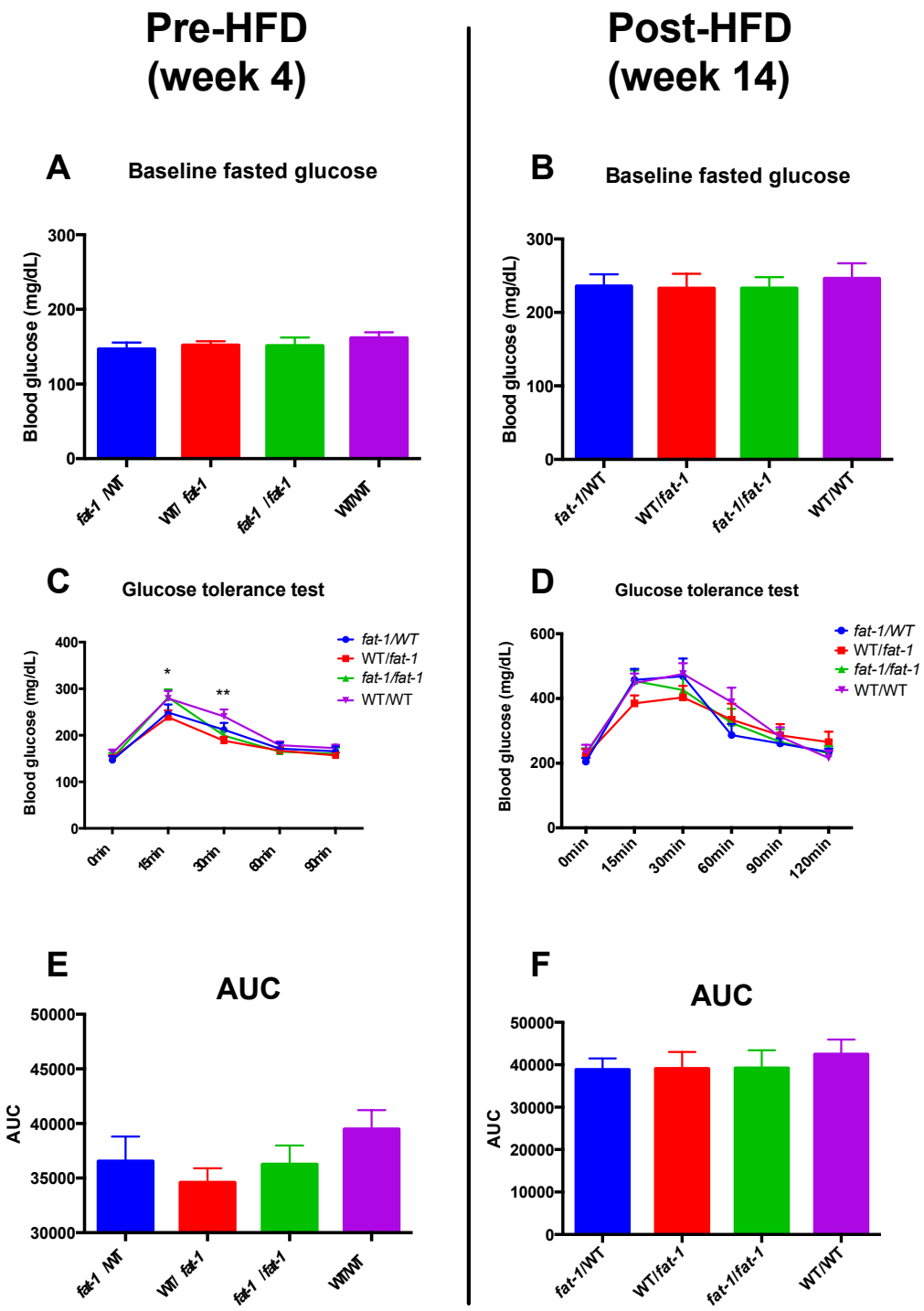


Figure S1. Glucose tolerance prior and post HFD. Maternal n-3 PUFA status did not significantly impact fasting glucose either prior to **(A)** or after 10 weeks **(B)** HFD feeding. WT/WT glucose was significantly higher than WT/*fat-1* at two time points during the pre-HFD GTT **(C)** however no significant differences were observed post-HFD. No significant differences were observed in the AUC of the GTT either prior to **(E)** or after **(F)** HFD feeding. Data shown as mean ± SEM. n= 7-12 per group. *p<0.05 **p<0.01, WT/WT vs. WT/*fat-1*.

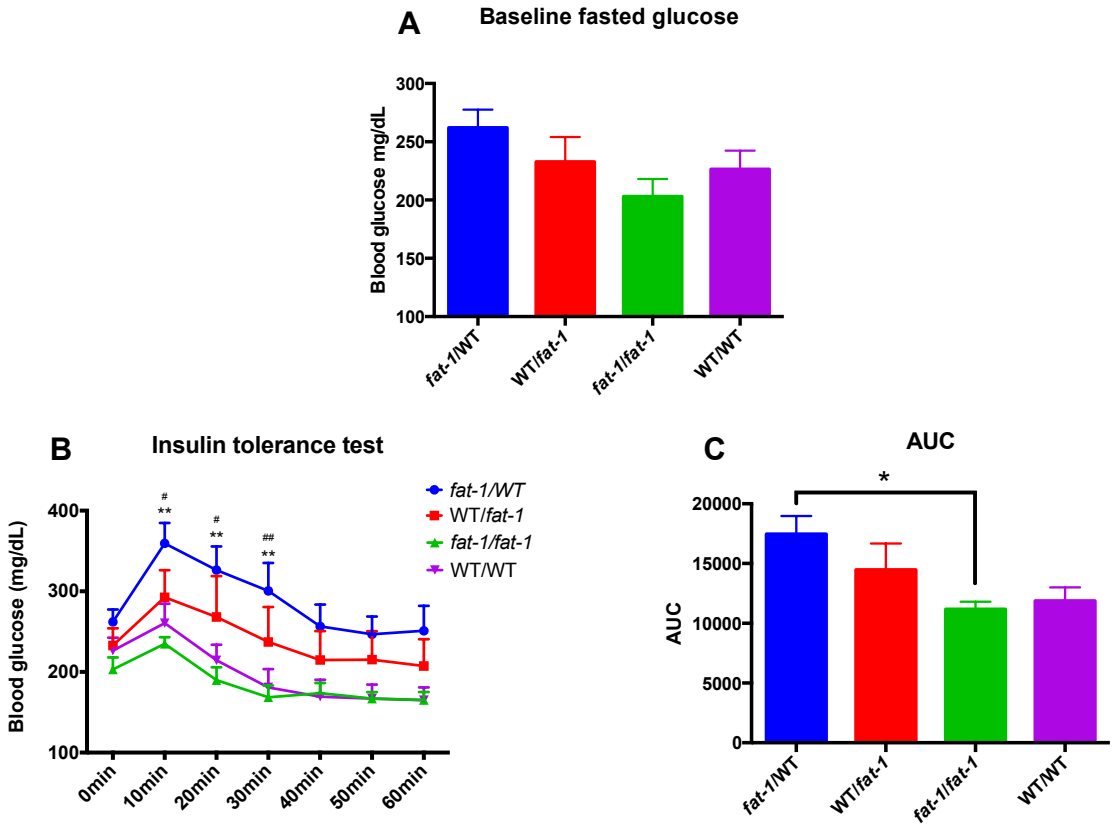


Figure S2. Insulin sensitivity. Maternal n-3 PUFA status did not significantly impact baseline fasting glucose after 12 weeks HFD feeding **(A)**. *fat*-1/*fat-1* and WT/WT glucose were significantly lower than *fat-1*/WT at three time points during the ITT **(B)**. *fat-1*/*fat-1* AUC during the ITT was significantly lower than *fat-1*/WT **(C)**. Data shown as mean ± SEM. n= 8-10 per group. **p<0.01, *fat-1*/WT vs. *fat-1*/*fat-1*; #p<0.05 ##p<0.01, *fat-1*/WT vs. WT/WT

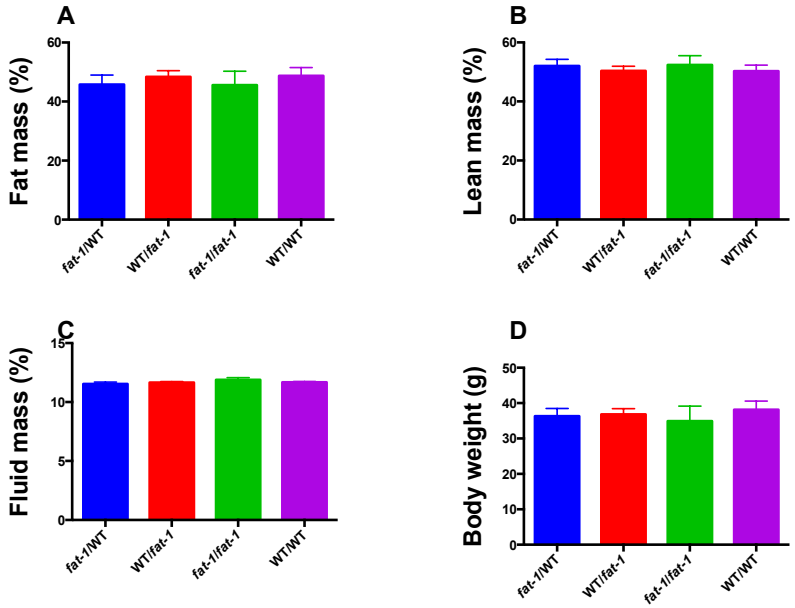


Figure S3. Female body comp. There were no differences between female groups in body composition.

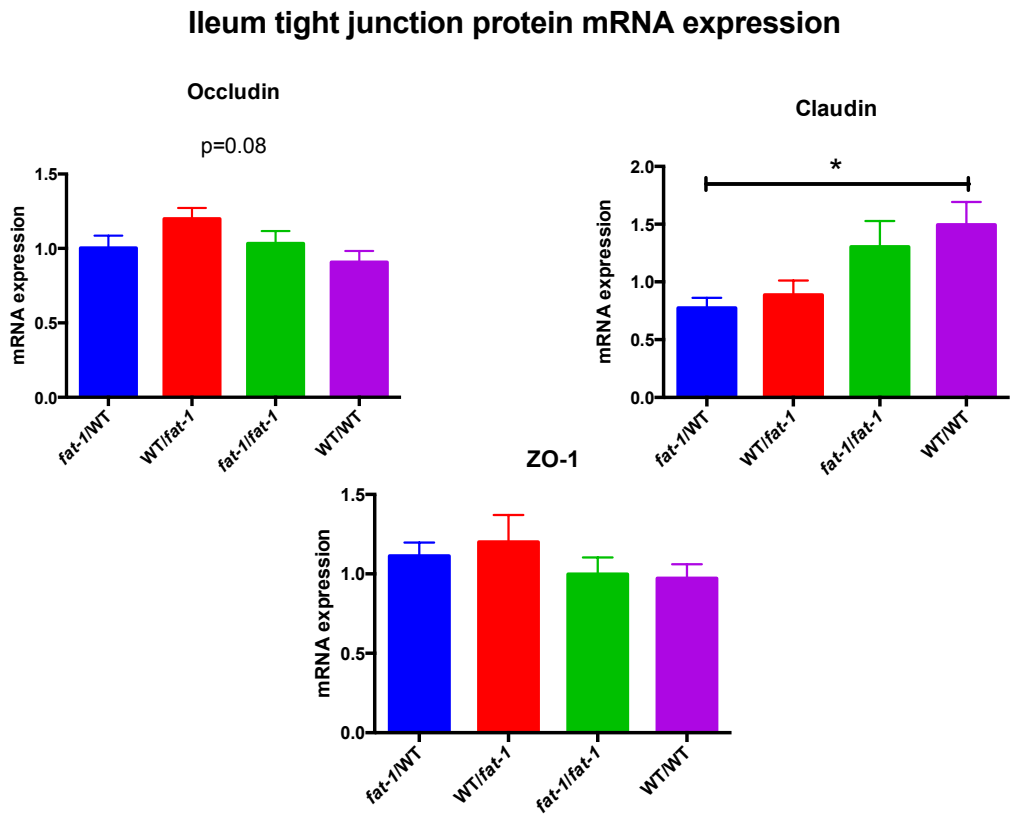


Figure S4. Ileal tight junction protein expression. WT/WT trended towards lower occluding expression (p=0.08) and had significantly greater claudin expression than *fat-1*/WT.

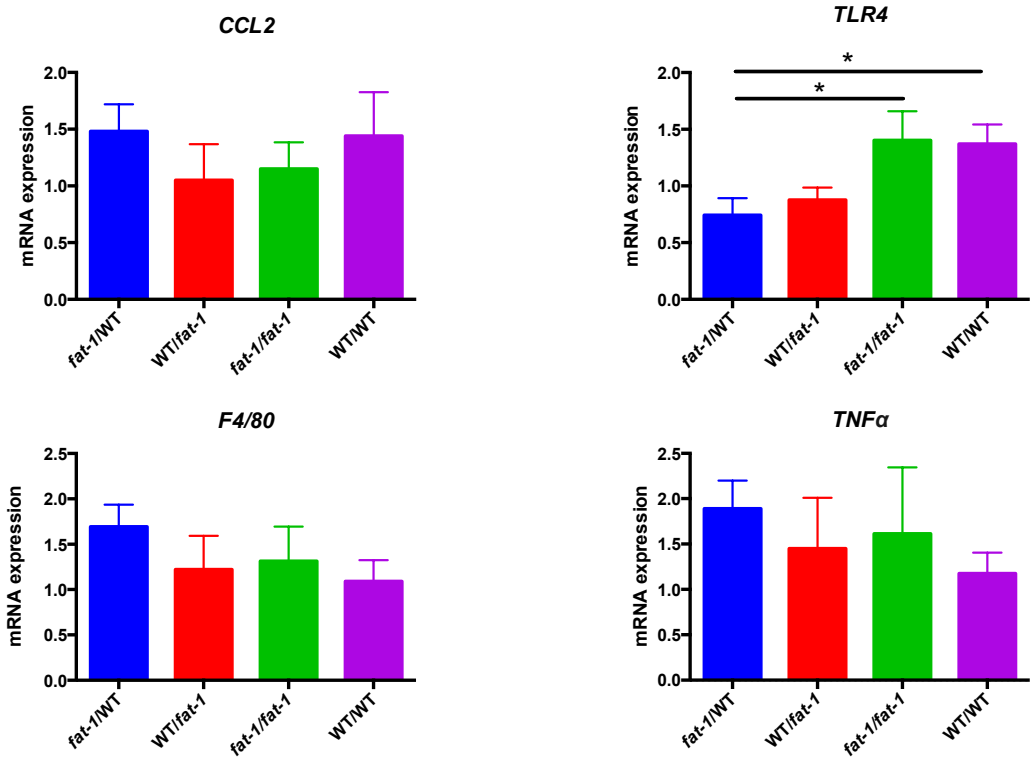


Figure S5. Subcutaneous adipose tissue inflammatory gene expression. There were no significant differences in subcutaneous adipose tissue expression of CCL2, F4/80 or TNFα. TLR4 expression was significantly lower in *fat-1*/WT offspring.

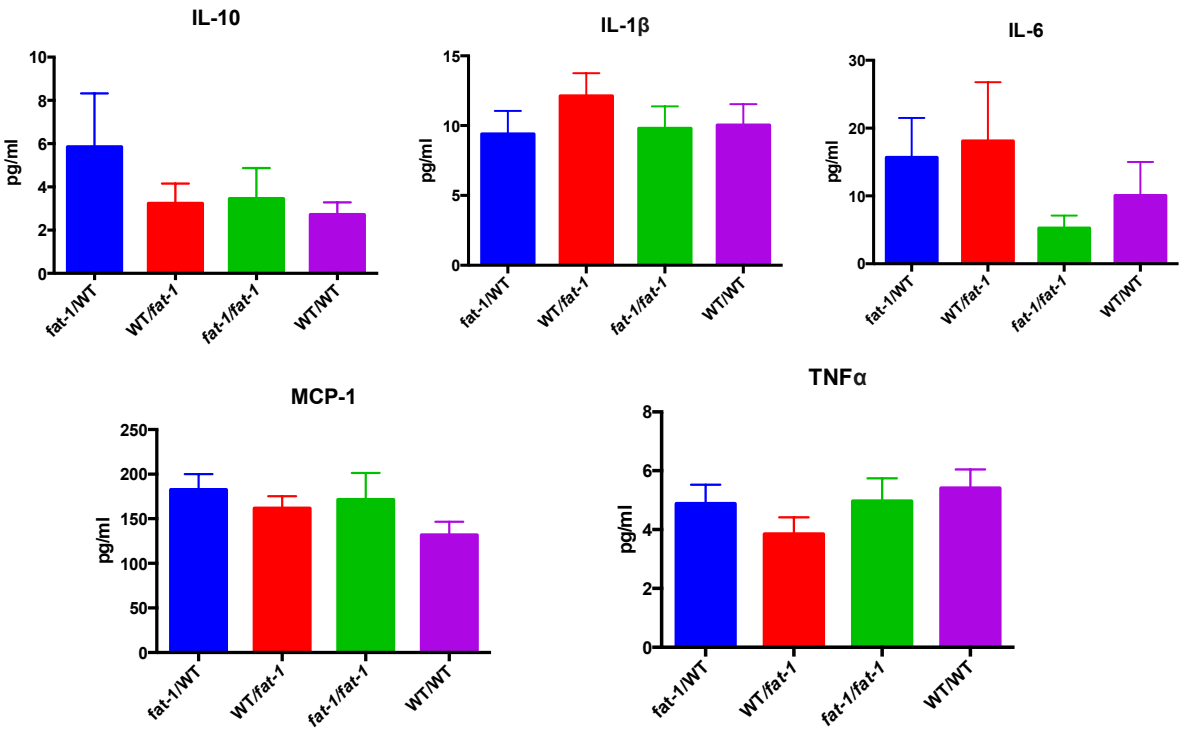


Figure S6. Circulating serum cytokines. There were no significant differences in male offspring circulating serum cytokines.

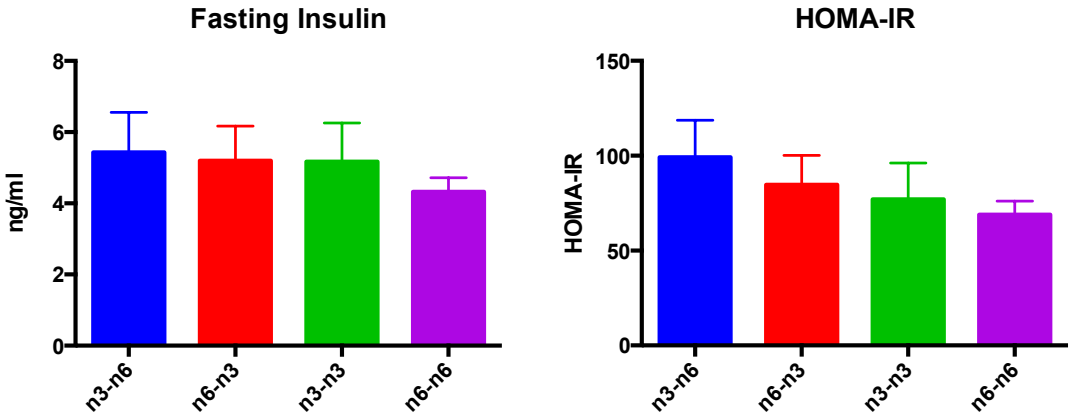


Figure S7. Fasting insulin. There were no significant differences in fasting insulin or homeostatic model assessment of insulin resistance (HOMA-IR) in adult male offspring after high-fat diet feeding.

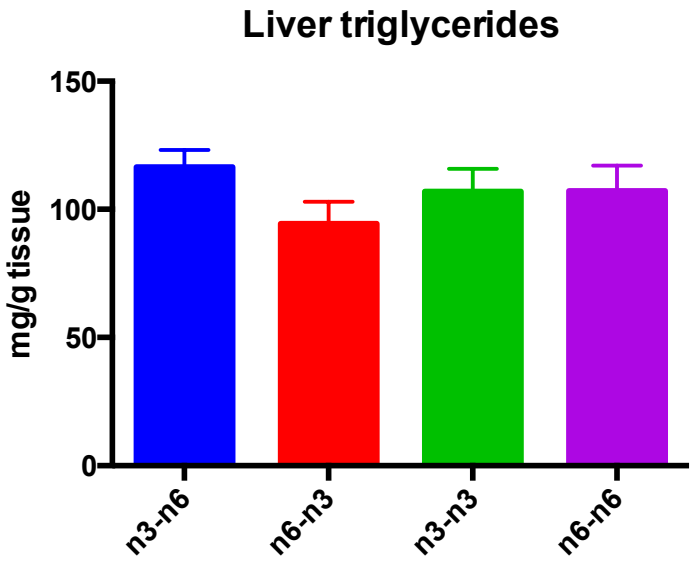


Figure S8. Liver triglycerides. There were no significant differences in liver triglycerides in adult male offspring after high-fat diet feeding.

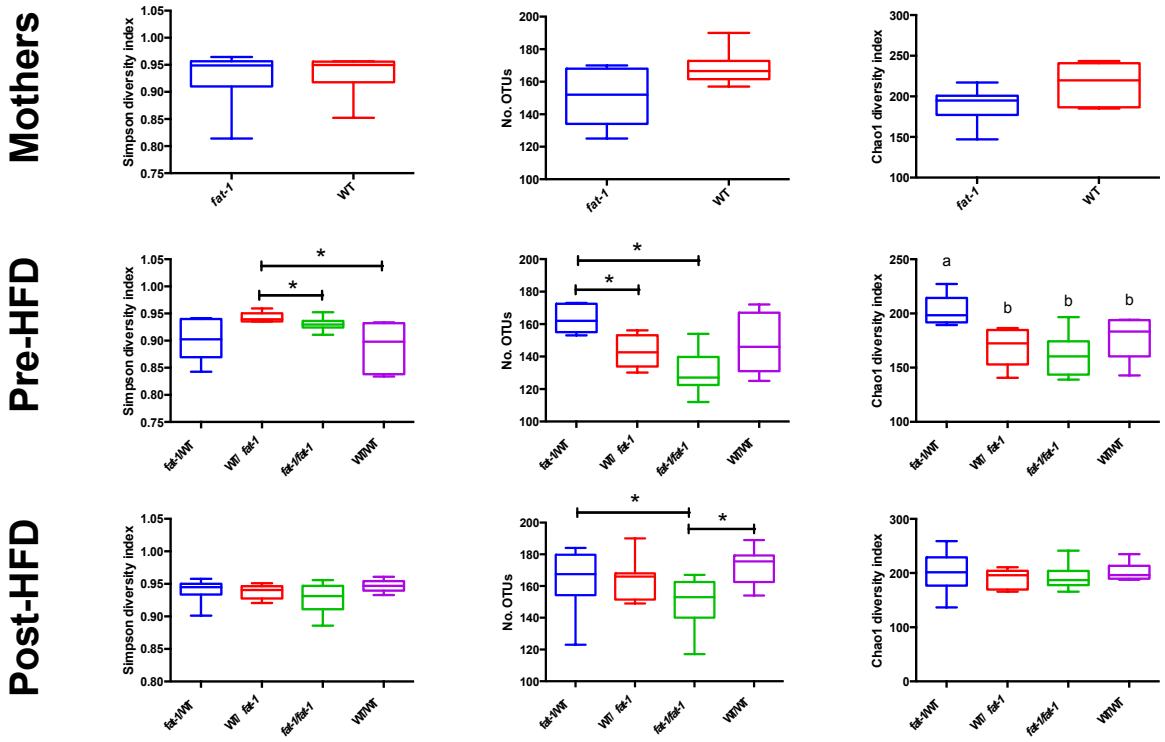


Figure S9. Alpha diversity of 16S microbiota. Phylogenetic diversity of mothers microbiota did not differ between groups. In pre-high fat diet (HFD) male offspring *fat-1*/WT diversity was higher than *fat-1*/*fat-1* and WT/*fat-1* offspring as assessed by number of operational taxonomic units (OTUs) and the Chao1 index. *Fat-1*/WT and WT/WT also displayed a greater number of OTUs than *fat-1*/*fat-1* in post-HFD offspring.

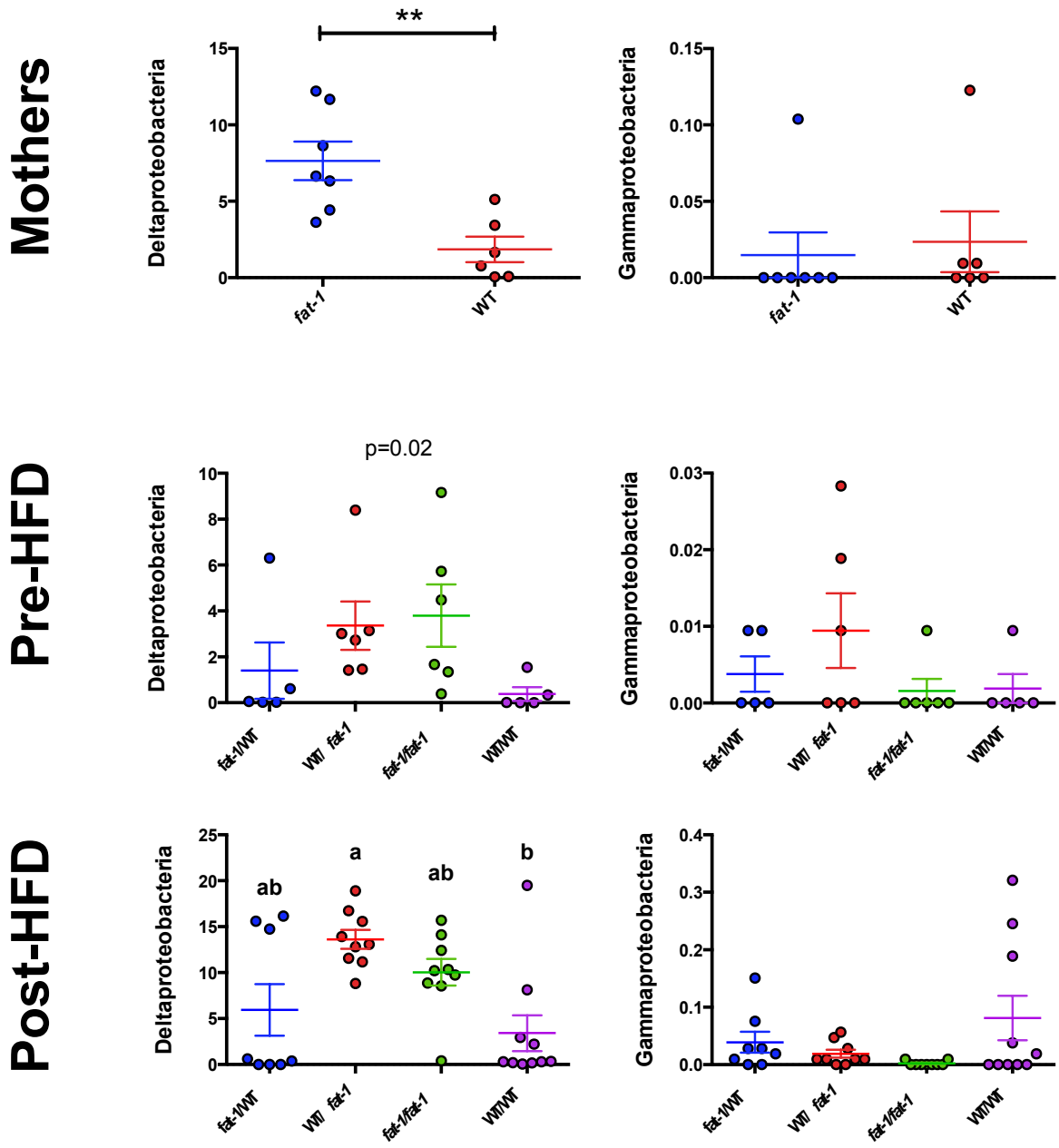


Figure S10. Proteobacteria abundance. Deltaproteobacteria abundance was lower in WT mothers and offspring with WT foster mothers. Gammaproteobacteria however was either not different between groups.

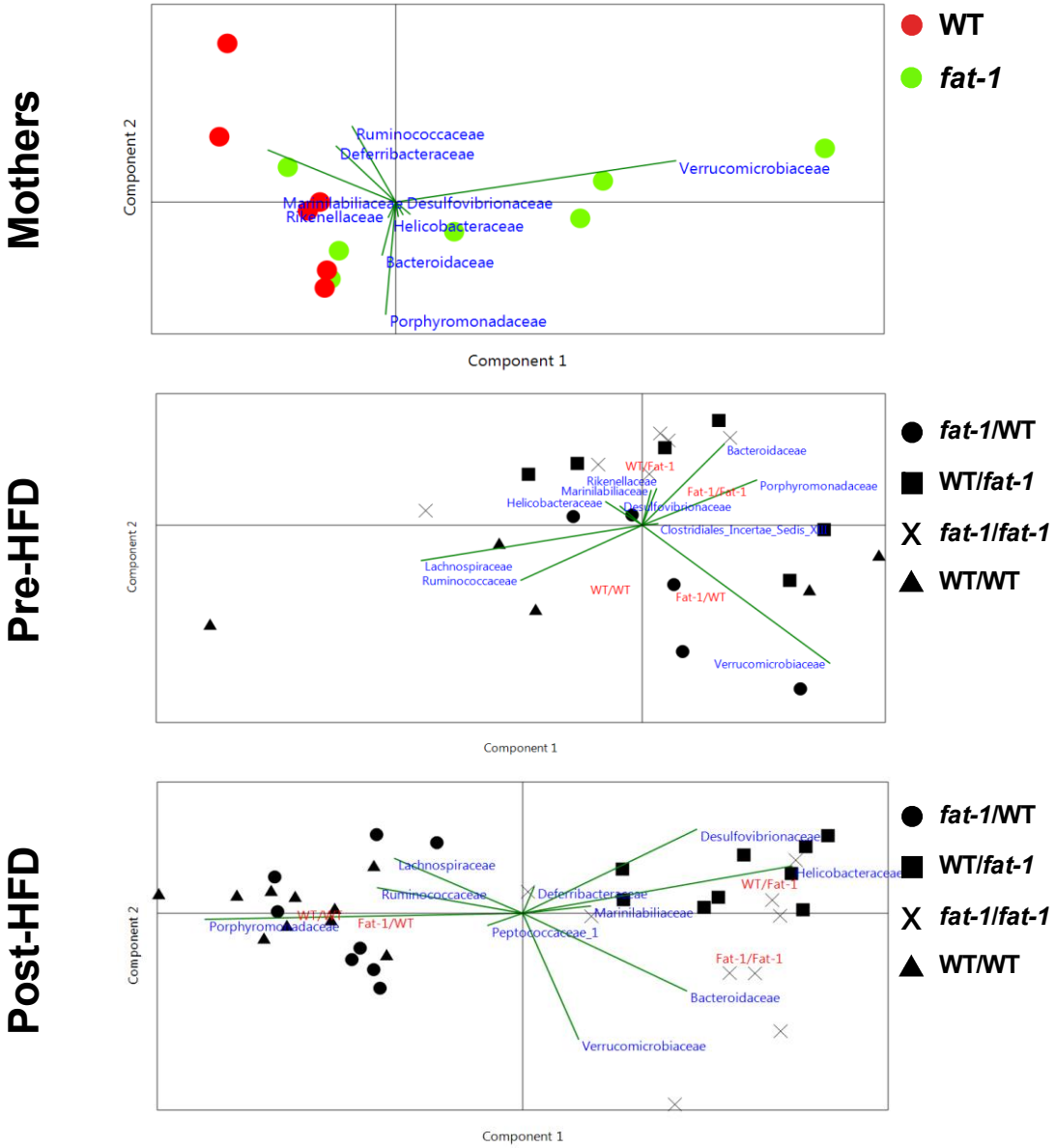


Figure S11. Family distribution. PCA clustering of top ten most abundant taxa at family level revealed that elevated *Verrucomicrobiaceae* in *fat-1* contributed strongly to separation between *fat-1* and WT mothers. Pre-HFD and Post-HFD separation was more evenly distributed among family taxa.

Table S1. Fatty acid profile of diet

Fatty acid	Diet	
	10% corn oil	**HFD**
C8:0	0.11	0
C10:0	0.27	0.04
C12:0	0.7	0.08
C14:0	0	1.1
C15	0	0.08
C16	0	19.6
C16:1	0.5	1.34
C17:0	0	0.35
C18:0	2.36	10.6
C18:1	28.2	34.0
C18:2 n-6	52.3	28.7
C18:3 n-3	1.1	2.04
C20:0	0	0.16
C20:1	0	0.59
C20:2 n-6	0	0.79
C20:3 n-6	0	0.12
C20:4 n-6	0	0.28
C20:5 n-3	0	0
C22:5 n-3	0	0.08
C22:6 n-3	0	0
SFA	18.2	32
MUFA	28.4	36
PUFA	53.4	32
n-6 PUFA	52.3	29.9
n-3 PUFA	1.1	2.1
n-6/n-3	47.6	14.1

SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids;

Table S2. Tail fatty acid profiles of mothers and offspring before and after high fat diet feeding

Tail fatty acid profile										
	Mothers		**Pre-HFD (week 4)**				**Post-HFD (week 17)**			
	WT	***fat-1***	***fat-1*/WT**	**WT/ *fat-1***	***fat-1 fat-1***	**WT/WT**	***fat-1*/WT**	**WT/ *fat-1***	***fat-1 fat-1***	**WT/WT**
SFA										
12:0	0.6 ± 0.1	2.1 ± 0.3	3 ± 0.2	3 ± 0.2	2.3 ± 0.1	3 ± 0.2	1.4 ± 0^{a}	1.2 ± 0^{b}	1.3 ± 0$^{a\ b}$	1.3 ± 0$^{a\ b}$
14:0	0.1 ± 0	0.4 ± 0.1	0.2 ± 0	0.2 ± 0	0.1 ± 0	0.2 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0
16:0	14.8 ± 0.3^{a}	18.3 ± 0.4^{b}	16.4 ± 0.2	16.9 ± 0.2	16.2 ± 0.1	16.4 ± 0.3	12.7 ± 0.1	13 ± 0.2	12.7 ± 0.2	13 ± 0.4
17:0	0.5 ± 0	0.5 ± 0	0.5 ± 0	0.5 ± 0	0.5 ± 0	0.5 ± 0	0.2 ± 0^{a}	0.2 ± 0$^{a\ b}$	0.2 ± 0$^{a\ b}$	0.2 ± 0^{b}
18:0	10.2 ± 0.3	11.6 ± 0.4	10.8 ± 0.3	10.9 ± 0.4	10.8 ± 0.4	10.4 ± 0.3	3.4 ± 0.1^{a}	4.4 ± 0.3$^{a\ b}$	5 ± 0.7^{b}	5 ± 0.4^{b}
20:0	1.5 ± 0.1	1 ± 0.1	1.1 ± 0.1	0.9 ± 0	1 ± 0.1	1.1 ± 0.1	0.4 ± 0^{a}	0.6 ± 0$^{a\ b}$	0.5 ± 0^{a}	0.6 ± 0.1^{b}
22:0	2.5 ± 0.2	1.6 ± 0.1	2.4 ± 0.4	1.9 ± 0.1	2.4 ± 0.2	1.9 ± 0.1	0.6 ± 0^{a}	0.6 ± 0^{a}	0.5 ± 0^{a}	0.9 ± 0.1^{b}
24:0	3.1 ± 0.2	2.8 ± 0.2	3.7 ± 0.2	3.4 ± 0.2	3.5 ± 0.2	3.6 ± 0.2	1.1 ± 0.1$^{a\ c}$	0.9 ± 0.1^{b}	0.8 ± 0.1^{b}	1.2 ± 0.1^{c}
Total SFA	29.8 ± 0.5	38.2 ± 0.8	39.4 ± 0.4	39 ± 0.6	38.2 ± 1.2	38.3 ± 0.7	20.4 ± 0.2^{a}	21.3 ± 0.4$^{a\ b}$	21.4 ± 0.7$^{a\ b}$	22.7 ± 0.7^{b}
MUFA										
14:1	0.4 ± 0	0.4 ± 0	0.4 ± 0	0.4 ± 0	0.4 ± 0	0.4 ± 0	0.2 ± 0^{a}	0.2 ± 0^{a}	0.2 ± 0$^{a\ b}$	0.2 ± 0^{b}
16:1	6 ± 0.7	5.4 ± 0.2	3.4 ± 0.1	3.6 ± 0.2	3.3 ± 0.2	3.5 ± 0.2	11.4 ± 0.3^{a}	10.2 ± 0.3^{b}	11.2 ± 0.2$^{a\ b}$	10.4 ± 0.3$^{a\ b}$
17:1	0.5 ± 0.1	0 ± 0	0.4 ± 0	0.4 ± 0	0.5 ± 0	0.4 ± 0	0.5 ± 0	0.5 ± 0	0.5 ± 0	0.5 ± 0
18:1	31.2 ± 1.4^{a}	30.1 ± 0.3^{b}	22.1 ± 0.4	22.7 ± 0.4	22.7 ± 0.5	22.8 ± 0.4	38.5 ± 0.3$^{a\ b}$	39.3 ± 0.7^{a}	37.9 ± 1.5$^{a\ b}$	35.7 ± 0.4^{b}
20:1	2.2 ± 0.1	2 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	2 ± 0.1	0.8 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.3 ± 0.1
22:1	1.6 ± 0.2	1.5 ± 0.1	1.2 ± 0.1	1 ± 0.1	1.5 ± 0.3	1.3 ± 0.1	0.3 ± 0^{a}	0.4 ± 0.1$^{a\ b}$	0.5 ± 0.1$^{a\ b}$	0.6 ± 0^{b}
24:1	1.6 ± 0^{a}	1.6 ± 0.1^{b}	1 ± 0.1	1 ± 0.1	1.3 ± 0.1	1.1 ± 0	0.3 ± 0^{a}	0.4 ± 0.1$^{a\ b}$	0.3 ± 0$^{a\ b}$	0.6 ± 0.1^{b}
MUFA	43.4 ± 1.9^{a}	40.9 ± 0.7^{b}	30.3 ± 0.7	30.8 ± 0.7	31.4 ± 0.8	31.6 ± 0.5	52.1 ± 0.3$^{a\ b}$	52.4 ± 0.8^{a}	51.9 ± 1.3$^{a\ b}$	49.3 ± 0.5^{b}
PUFA										
n-6 PUFA										
18:2 n-6	15.5 ± 1	12 ± 0.6	16.5 ± 0.4	16.9 ± 0.4	16.9 ± 0.7	17.4 ± 0.3	20.9 ± 0.3	19.6 ± 0.4	20.5 ± 0.4	20 ± 0.3
18:3 n-6	0 ± 0^{a}	0.2 ± 0^{b}	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0
20:2 n-6	1.2 ± 0.2	0.9 ± 0.1	1.2 ± 0.1	1.2 ± 0	1 ± 0	1.1 ± 0.1	0.7 ± 0	0.7 ± 0.1	0.8 ± 0	0.9 ± 0.1
20:3 n-6	0.3 ± 0.1	0.2 ± 0	0.7 ± 0	0.7 ± 0	0.5 ± 0.1	0.6 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.4 ± 0
20:4 n-6	4.7 ± 0.7^{a}	0.8 ± 0.1^{b}	9.1 ± 0.4	8.3 ± 0.4	8.8 ± 0.6	8.2 ± 0.4	2.7 ± 0.1^{a}	2.6 ± 0.2^{a}	2.3 ± 0.2^{a}	3.4 ± 0.1^{b}
22:4 n-6	0.7 ± 0.1	0 ± 0	1.7 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	0.5 ± 0$^{a\ b}$	0.5 ± 0.1$^{a\ b}$	0.5 ± 0^{a}	0.7 ± 0^{b}
Total n-6 PUFA	0.9 ± 0.2^{a}	14 ± 0.6^{b}	29.2 ± 0.9	28.6 ± 0.9	28.7 ± 1.5	29.2 ± 0.9	25.3 ± 0.3	23.8 ± 0.6	24.4 ± 0.6	25.4 ± 0.3
n-3 PUFA										
18:3 n-3	0.1 ± 0	2.2 ± 0.2	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	1.2 ± 0$^{a\ b}$	1 ± 0^{b}	1.3 ± 0.1^{a}	1.3 ± 0^{a}
20:3 n-3	0 ± 0^{a}	0.2 ± 0^{b}	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.2 ± 0
20:5 n-3	0 ± 0^{a}	1.7 ± 0.2^{b}	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0	1.2 ± 0.7	0 ± 0	0.1 ± 0
22:5 n-3	0 ± 0^{a}	1.7 ± 0.2^{b}	0.2 ± 0^{a}	0.4 ± 0.1^{b}	0.4 ± 0.1^{b}	0.2 ± 0^{a}	0.3 ± 0^{a}	0.2 ± 0$^{a\ b}$	0.2 ± 0^{b}	0.3 ± 0$^{a\ b}$
22:6 n-3	0.8 ± 0.2	1.3 ± 0.1	0.9 ± 0$^{a\ b}$	1.1 ± 0.1$^{b\ c}$	1.2 ± 0.1^{c}	0.7 ± 0.1^{a}	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1
Total n-3 PUFA	22.5 ± 1.8^{a}	7 ± 0.6^{b}	1.1 ± 0.1	1.6 ± 0.2	1.7 ± 0.1	0.9 ± 0.1	2.3 ± 0.1	2.5 ± 0.3	2.3 ± 0.1	2.5 ± 0.1
n-6/n-3	38.1 ± 8^{a}	2.1 ± 0.2^{b}	26.7 ± 1.1	18.6 ± 1.5	16.8 ± 1	33.5 ± 2.6	11.3 ± 0.3	10.3 ± 0.8	10.7 ± 0.4	10 ± 0.3

SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; HFD, High fat diet. Values with different superscript letters are significantly different between groups within timepoint. Data expressed as mean ± SEM. n=5-10 per group

Table S3. Liver fatty acid profiles of offspring after high fat diet feeding.

	Liver			
	***fat-1*/WT**	**WT/*fat-1***	***fat-1*/*fat-1***	**WT/WT**
SFA				
12:0	0.5 ± 0	0.5 ± 0	0.5 ± 0	0.5 ± 0
14:0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
16:0	26.8 ± 0.3	25.9 ± 0.6	26.8 ± 0.8	25.1 ± 0.8
17:0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0
18:0	3.7 ± 0.2	5.5 ± 0.6	5 ± 0.5	4 ± 0.4
20:0	0.1 ± 0[a]	0.2 ± 0[b]	0.2 ± 0[b] [c]	0.2 ± 0[a] [c]
22:0	0.1 ± 0	0.2 ± 0	0.2 ± 0	0.1 ± 0
24:0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Total SFA	31.4 ± 0.4	32.6 ± 0.8	32.9 ± 1.3	30 ± 1.1
MUFA				
14:1	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0
16:1	3.2 ± 0.3	2.6 ± 0.2	2.6 ± 0.2	2.9 ± 0.2
17:1	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0
18:1	42.2 ± 0.6	36.5 ± 1.6	38.2 ± 1.9	39.3 ± 1.6
20:1	1 ± 0.1	0.9 ± 0	0.9 ± 0.1	1 ± 0.1
22:1	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0
24:1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
MUFA	46.8 ± 0.9	40.4 ± 1.8	42 ± 2.2	43.6 ± 1.8
PUFA				
n-6 PUFA				
18:2 n-6	15.3 ± 0.4[a]	17.7 ± 0.6[a b]	17.4 ± 0.7[a b]	18 ± 0.5[b]
18:3 n-6	0.3 ± 0	0.3 ± 0	0.4 ± 0	0.4 ± 0
20:2 n-6	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.4 ± 0
20:3 n-6	0.8 ± 0[a]	0.9 ± 0[a b]	0.9 ± 0.1[a b]	1 ± 0[b]
20:4 n-6	2.7 ± 0.1	4.4 ± 0.7	3.5 ± 0.3	3.5 ± 0.3
22:4 n-6	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.4 ± 0
Total n-6 PUFA	19.7 ± 0.7[a]	24.1 ± 1.3[b]	22.7 ± 1.1[a b]	23.6 ± 0.7[a b]
n-3 PUFA				
18:3 n-3	0.4 ± 0	0.5 ± 0	0.5 ± 0	0.5 ± 0
20:3 n-3	0.1 ± 0	0 ± 0	0 ± 0	0 ± 0
20:5 n-3	0.1 ± 0	0.2 ± 0	0.1 ± 0	0.1 ± 0
22:5 n-3	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.3 ± 0
22:6 n-3	1.3 ± 0.1	2 ± 0.4	1.6 ± 0.2	1.8 ± 0.1
Total n-3 PUFA	2.1 ± 0.2	3 ± 0.5	2.4 ± 0.3	2.8 ± 0.2
n-6/n-3	9.7 ± 0.7	9.1 ± 0.8	10.1 ± 0.8	8.7 ± 0.4

SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids. Values with different superscript letters are significantly different between groups. Data expressed as mean ± SEM. n=5-10 per group

Table S4. Mothers taxa significantly different between groups

	Mothers			
Taxonomy	***fat-1***	**WT**	**P-value**	**FDR corrected**
Bacteroidetes,Bacteroidia,Bacteroidales,Porphyromonadaceae,Butyricimonas	0.003	3.452	0.013	0.204
Bacteroidetes,Bacteroidia,Bacteroidales,Porphyromonadaceae,Odoribacter	0.716	0.508	0.042	0.230
Bacteroidetes,Bacteroidia,Bacteroidales,Porphyromonadaceae,Tannerella	2.313	0.739	0.044	0.230
Bacteroidetes,Flavobacteriia,Flavobacteriales,Cryomorphaceae,Wandonia	0.011	0.390	0.024	0.230
Deferribacteres,Deferribacteres,Deferribacterales,Deferribacteraceae,Mucispirillum	1.298	5.810	0.015	0.206
Firmicutes,Clostridia,Clostridiales,Clostridiales_Incertae_Sedis_XI,Sporanaerobacter	0.005	0.038	0.045	0.230
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Acetitomaculum	0.050	0.851	0.02	0.230
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Clostridium_XlVb	0.449	0.846	0.003	0.165
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Johnsonella	0.009	0.371	0.009	0.198
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Marvinbryantia	1.309	2.958	0.032	0.230
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Robinsoniella	0.198	1.068	0.046	0.230
Firmicutes,Clostridia,Clostridiales,Peptococcaceae_1,Peptococcus	0.026	1.436	0.012	0.204
Firmicutes,Clostridia,Clostridiales,Peptostreptococcaceae,Clostridium_XI	0.155	0.442	0.045	0.230
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Acetanaerobacterium	0.031	0.109	0.005	0.183
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Flavonifractor	0.940	2.119	0.032	0.230
Proteobacteria,Alphaproteobacteria,Rhodospirillales,Rhodospirillaceae,Dongia	0.217	0.013	0.003	0.165
Proteobacteria,Deltaproteobacteria,Desulfovibrionales,Desulfovibrionaceae,Bilophila	5.919	0.772	0.007	0.193
Proteobacteria,Epsilonproteobacteria,Campylobacterales,Helicobacteraceae,Helicobacter	2.199	0.340	0.033	0.230

Table S5. Pre-HFD taxa significantly different between groups

	Pre-HFD					
Taxonomy	***fat-1*/ WT**	**WT/ *fat-1***	***fat-1*/ *fat-1***	**WT/ WT**	**P-value**	**FDR corrected**
Bacteroidetes,Bacteroidia,Bacteroidales,Marinilabiliaceae,Alkalitalea	0.006	6.365	4.904	0.002	0.001	0.012
Bacteroidetes,Bacteroidia,Bacteroidales,Porphyromonadaceae,Barnesiella	11.199	20.345	14.573	3.905	0.021	0.062
Bacteroidetes,Bacteroidia,Bacteroidales,Porphyromonadaceae,Butyricimonas	1.657	0.002	0.003	2.580	0.002	0.016
Bacteroidetes,Bacteroidia,Bacteroidales,Porphyromonadaceae,Odoribacter	0.004	2.721	3.002	0.004	0.001	0.012
Bacteroidetes,Bacteroidia,Bacteroidales,Porphyromonadaceae,Parabacteroides	15.235	6.274	8.647	18.275	0.014	0.051
Bacteroidetes,Bacteroidia,Bacteroidales,Porphyromonadaceae,Tannerella	0.000	1.622	0.785	0.000	0.001	0.012
Bacteroidetes,Bacteroidia,Bacteroidales,Rikenellaceae,Alistipes	0.604	5.917	6.241	1.006	0.001	0.012
Bacteroidetes,Bacteroidia,Bacteroidales,Rikenellaceae,Rikenella	0.000	0.013	0.171	0.000	0.026	0.072
Bacteroidetes,Flavobacteriia,Flavobacteriales,Cryomorphaceae,Wandonia	1.215	0.160	0.000	1.091	0.001	0.012
Firmicutes,Clostridia,Clostridiales,Clostridiaceae_1,Clostridium_sensu_stricto	0.430	0.226	0.005	0.394	0.021	0.062
Firmicutes,Clostridia,Clostridiales,Clostridiaceae_4,Thermotalea	0.051	0.003	0.011	0.066	0.004	0.024
Firmicutes,Clostridia,Clostridiales,Clostridiales_Incertae_Sedis_XI,Sporanaerobacter	0.123	0.039	0.028	0.128	0.014	0.051
Firmicutes,Clostridia,Clostridiales,Clostridiales_Incertae_Sedis_XII,Acidaminobacter	0.030	0.013	0.009	0.100	0.036	0.092
Firmicutes,Clostridia,Clostridiales,Defluviitaleaceae,Defluviitalea	0.379	0.057	0.000	0.136	0.002	0.016
Firmicutes,Clostridia,Clostridiales,Eubacteriaceae,Alkalibaculum	0.004	0.000	0.000	0.002	0.002	0.016
Firmicutes,Clostridia,Clostridiales,Gracilibacteraceae,Gracilibacter	0.117	0.017	0.005	0.762	0.005	0.028
Firmicutes,Clostridia,Clostridiales,Gracilibacteraceae,Lutispora	0.070	0.049	0.027	0.242	0.012	0.049
Firmicutes,Clostridia,Clostridiales,Incertae_Sedis_XI,Dethiosulfatibacter	0.009	0.002	0.000	0.015	0.050	0.119
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Butyrivibrio	0.085	0.068	0.091	0.019	0.020	0.062
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Cellulosilyticum	0.011	0.000	0.002	0.115	0.015	0.053
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Johnsonella	0.183	0.061	0.003	0.321	0.006	0.028
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Lactonifactor	1.070	0.344	0.002	0.072	0.017	0.055
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Oribacterium	0.081	0.275	0.522	0.036	0.002	0.016
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Parasporobacterium	0.026	0.000	0.000	0.060	0.001	0.012
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Pseudobutyrivibrio	0.302	0.000	0.000	0.128	0.012	0.049
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Robinsoniella	0.600	0.264	0.101	3.339	0.010	0.044

Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Roseburia	0.394	0.217	0.101	0.795	0.006	0.028
Firmicutes,Clostridia,Clostridiales,Peptococcaceae_1,Peptococcus	2.050	0.030	0.011	1.212	0.001	0.012
Firmicutes,Clostridia,Clostridiales,Peptostreptococcaceae,Sporacetigenium	0.000	0.000	0.013	0.000	0.006	0.028
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Acetivibrio	0.196	0.030	0.031	0.491	0.003	0.019
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Butyricicoccus	0.013	0.002	0.000	0.006	0.014	0.051
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Clostridium_IV	2.856	1.092	1.354	1.861	0.049	0.117
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Flavonifractor	5.213	0.574	0.434	6.796	0.001	0.012
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Papillibacter	0.008	0.017	0.017	0.002	0.031	0.081
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Ruminococcus	0.025	0.002	0.008	0.026	0.030	0.080
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Saccharofermentans	0.068	0.219	0.088	0.287	0.017	0.055
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Sporobacter	0.119	0.002	0.002	0.085	0.002	0.016
Firmicutes,Erysipelotrichia,Erysipelotrichales,Erysipelotrichaceae,Clostridium_XVIII	0.477	0.055	0.087	0.217	0.022	0.064
Firmicutes,Erysipelotrichia,Erysipelotrichales,Erysipelotrichaceae,Turicibacter	0.774	0.215	0.014	0.587	0.017	0.055
Proteobacteria,Alphaproteobacteria,Rhodospirillales,Rhodospirillaceae,Dongia	0.000	0.198	0.266	0.000	0.025	0.071
Proteobacteria,Alphaproteobacteria,Rickettsiales,Rickettsiaceae,Orientia	0.032	0.006	0.000	0.002	0.003	0.019
Proteobacteria,Deltaproteobacteria,Desulfovibrionales,Desulfovibrionaceae,Bilophila	0.021	2.800	3.334	0.009	0.003	0.019
Proteobacteria,Deltaproteobacteria,Desulfovibrionales,Desulfovibrionaceae,Desulfovibrio	0.000	0.220	0.149	0.002	0.001	0.012
Proteobacteria,Epsilonproteobacteria,Campylobacterales,Helicobacteraceae,Helicobacter	0.008	3.905	4.684	0.006	0.006	0.028
Verrucomicrobia,Verrucomicrobiae,Verrucomicrobiales,Verrucomicrobiaceae,Akkermansia	15.501	0.009	6.658	11.433	0.006	0.028

Table S6. Post-HFD taxa significantly different between groups

	Post-HFD					
Taxonomy	***fat-1*/ WT**	**WT/ *fat-1***	***fat-1*/ *fat-1***	**WT/ WT**	**P-value**	**FDR corrected**
Actinobacteria,Actinobacteria,Coriobacteriales,Coriobacteriaceae,Enterorhabdus	0.042	0.040	0.023	0.085	0.017	0.053
Bacteroidetes,Bacteroidia,Bacteroidales,Bacteroidaceae,Bacteroides	4.488	13.269	14.115	7.227	0.003	0.013
Bacteroidetes,Bacteroidia,Bacteroidales,Marinilabiliaceae,Alkalitalea	1.026	4.100	3.535	0.004	<0.001	<0.001
Bacteroidetes,Bacteroidia,Bacteroidales,Porphyromonadaceae,Butyricimonas	2.452	0.010	0.000	4.236	<0.001	<0.001
Bacteroidetes,Bacteroidia,Bacteroidales,Porphyromonadaceae,Odoribacter	0.376	1.295	1.396	0.004	<0.001	<0.001
Bacteroidetes,Bacteroidia,Bacteroidales,Porphyromonadaceae,Parabacteroides	9.759	1.050	2.154	8.437	<0.001	<0.001
Bacteroidetes,Bacteroidia,Bacteroidales,Rikenellaceae,Rikenella	0.000	0.065	0.040	0.199	0.010	0.032
Bacteroidetes,Flavobacteriia,Flavobacteriales,Cryomorphaceae,Wandonia	0.140	0.015	0.000	0.377	<0.001	<0.001
Firmicutes,Bacilli,Lactobacillales,Lactobacillaceae,Lactobacillus	1.932	1.055	0.731	3.017	0.018	0.055
Firmicutes,Clostridia,Clostridiales,Clostridiaceae_1,Anaerosporobacter	1.594	0.043	0.006	0.302	<0.001	<0.001
Firmicutes,Clostridia,Clostridiales,Clostridiaceae_1,Clostridium_sensu_stricto	0.000	0.000	0.000	0.014	0.041	0.098
Firmicutes,Clostridia,Clostridiales,Clostridiales_Incertae_Sedis_XI,Sporanaerobacter	0.080	0.022	0.009	0.102	0.007	0.025
Firmicutes,Clostridia,Clostridiales,Clostridiales_Incertae_Sedis_XII,Guggenheimella	0.000	0.000	0.000	0.007	0.041	0.098
Firmicutes,Clostridia,Clostridiales,Defluviitaleaceae,Defluviitalea	0.152	0.026	0.001	0.162	<0.001	<0.001
Firmicutes,Clostridia,Clostridiales,Eubacteriaceae,Alkalibaculum	0.000	0.000	0.000	0.003	0.004	0.016
Firmicutes,Clostridia,Clostridiales,Gracilibacteraceae,Gracilibacter	0.013	0.020	0.030	0.115	0.026	0.070
Firmicutes,Clostridia,Clostridiales,Incertae_Sedis_XI,Dethiosulfatibacter	0.017	0.001	0.000	0.017	<0.001	<0.001
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Acetitomaculum	0.066	0.002	0.000	0.078	<0.001	<0.001
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Butyrivibrio	0.033	0.075	0.014	0.035	0.002	0.009
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Cellulosilyticum	0.015	0.000	0.000	0.033	<0.001	<0.001
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Clostridium_XlVb	1.721	1.600	0.950	1.475	0.032	0.080
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Coprococcus	0.077	0.066	0.017	0.055	0.001	0.005
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Johnsonella	1.471	0.044	0.022	1.011	<0.001	<0.001
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Marvinbryantia	6.508	2.741	2.439	8.098	<0.001	<0.001
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Moryella	0.068	0.028	0.101	0.083	0.032	0.080
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Parasporobacterium	0.021	0.000	0.001	0.052	0.010	0.032

Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Pseudobutyrivibrio	0.642	0.012	0.000	0.198	0.001	0.005
Firmicutes,Clostridia,Clostridiales,Lachnospiraceae,Robinsoniella	0.913	0.751	0.246	0.950	0.002	0.009
Firmicutes,Clostridia,Clostridiales,Peptococcaceae_1,Peptococcus	2.944	0.304	0.190	1.801	0.004	0.016
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Acetanaerobacterium	0.042	0.028	0.024	0.160	<0.001	<0.001
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Acetivibrio	0.458	0.047	0.273	0.718	<0.001	<0.001
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Anaerotruncus	2.483	1.225	1.301	2.241	0.005	0.019
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Butyricicoccus	0.028	0.006	0.000	0.005	0.050	0.119
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Hydrogenoanaerobacterium	2.497	1.630	1.581	2.352	0.023	0.065
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Pseudoflavonifractor	5.418	4.643	3.152	6.997	0.001	0.005
Firmicutes,Clostridia,Clostridiales,Ruminococcaceae,Ruminococcus	0.057	0.016	0.523	0.077	0.032	0.080
Firmicutes,Erysipelotrichia,Erysipelotrichales,Erysipelotrichaceae,Allobaculum	0.067	0.034	0.045	0.152	0.001	0.016
Firmicutes,Erysipelotrichia,Erysipelotrichales,Erysipelotrichaceae,Clostridium_XVIII	0.329	0.139	0.074	0.421	0.023	0.065
Firmicutes,Erysipelotrichia,Erysipelotrichales,Erysipelotrichaceae,Coprobacillus	0.013	0.000	0.017	0.000	0.025	0.069
Firmicutes,Erysipelotrichia,Erysipelotrichales,Erysipelotrichaceae,Holdemania	0.047	0.051	0.000	0.037	0.003	0.013
Lentisphaerae,Lentisphaeria,Victivallales,Victivallaceae,Victivallis	0.000	0.000	0.000	0.423	<0.001	<0.001
Proteobacteria,Alphaproteobacteria,Rhodospirillales,Rhodospirillaceae,Dongia	0.044	0.191	0.193	0.090	0.006	0.022
Proteobacteria,Betaproteobacteria,Nitrosomonadales,Nitrosomonadaceae,Nitrosomonas	0.000	0.003	0.003	0.018	0.010	0.032
Proteobacteria,Deltaproteobacteria,Desulfovibrionales,Desulfovibrionaceae,Bilophila	5.532	12.961	9.579	2.844	0.022	0.065
Proteobacteria,Epsilonproteobacteria,Campylobacterales,Helicobacteraceae,Helicobacter	0.006	13.610	9.318	0.008	<0.001	<0.001
Verrucomicrobia,Verrucomicrobiae,Verrucomicrobiales,Verrucomicrobiaceae,Akkermansia	7.123	0.090	11.512	1.239	<0.001	<0.001

Table S7. Primer sequences for qPCR

Primers for qPCR		
Gene	**Forward**	**Reverse**
TNFa	TGGGACAGTGACCTGGACTGT	TTCGGAAAGCCCATTTGAGT
F4/80	TGACAACCAGACGGCTTGTG	GCAGGCGAGGAAAAG ATAGTGT
CCL2	AGGTCCCTGTCATGCTTCTGG	CTGCTGCTGGTGATCCTCTTG
B-actin	GACCCAGATCATGTTTGAGA	GAGCATAGCCCTCGTAGAT
TLR4	ACCAGGAAGCTTGAATCCCT	TCCAGCCACTGAAGTTCTGA

SUPPLEMENTARY METHODS

Glucose tolerance test

Glucose tolerance test (GTT) was performed as previously described (Kaliannan et al., 2013) at weaning (PND 28) and following 13-15 weeks on a HFD. Due to the age and size of the animals at weaning, a number of small modifications were made to the GTT procedure compared with that performed at adulthood. Briefly, fasting blood glucose was measured using a glucometer following 5-6 h fasting. Glucose [20% solution in ddH_20 (weaning); 10% solution in phosphate buffered saline (post-HFD)] was administered at 1g/kg body weight (weaning) or 2g/kg body weight (post-HFD) by i.p. injection (weaning) or gavage (post-HFD). A small incision was made in the tail and blood samples were collected at 15, 30, 60, 90 and 120 min post glucose administration to measure glucose levels. Glucose tolerance was assessed by calculating the incremental area under the curve (AUC) of the GTT.

Insulin tolerance test

Insulin tolerance test (ITT) was performed as previously described (Kaliannan et al., 2013) following 13-15 weeks on a HFD. Briefly, fasting blood glucose was measured using a glucometer following 6 h fasting. Insulin (solution in PBS) was administered at 0.75 units/kg body weight by i.p. injection. A small incision was made in the tail and

blood samples were collected at 10, 20, 30, 40, 50 and 60 min post insulin administration to measure glucose levels. Insulin tolerance was assessed by calculating the incremental area under the curve (AUC) of the ITT. The homeostasis model assessment of insulin resistance [HOMA-IR = glucose (mg/dL) × insulin (mU/L)/405] was calculated as an insulin sensitivity index.

Cytokines

Serum cytokine levels (TNF-α, IL-1β, IL-6, MCP-1, and IL-10) were assessed using a Bio-Plex immunoassay kit (Bio-Rad) according to manufacturers instructions. Data acquisition and analysis was performed using Xponent softward (Luminex, Austin, TX).

Liver triglycerides

Liver triglycerides were quantified using commercial TAG reagent (Sigma, Aldrich) according to manufacturers instructions following lipid extraction using chloroform:methanol (2:1).

Chapter 5

The Anti-Inflammatory Effect of Omega-3-rich Algae-Derived Lipid Extracts on Lipopolysaccharide-Stimulated Human THP-1 Macrophages

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ABSTRACT

Algae contain a number of anti-inflammatory bioactive compounds such as omega-3 polyunsaturated fatty acids (*n*-3 PUFA) and chlorophyll *a*, hence as dietary ingredients, their extracts may be effective in chronic inflammation-linked metabolic diseases such as cardiovascular disease. In this study, anti-inflammatory potential of lipid extracts from three red seaweeds (*Porphyra dioica*, *Palmaria palmata* and *Chondrus crispus*) and one microalga (*Pavlova lutheri*) were assessed in lipopolysaccharide (LPS)-stimulated human THP-1 macrophages. Extracts contained 34%–42% total fatty acids as *n*-3 PUFA and 5%–7% crude extract as pigments, including chlorophyll *a*, β-carotene and fucoxanthin. Pretreatment of the THP-1 cells with lipid extract from *P. palmata* inhibited production of the pro-inflammatory cytokines interleukin (IL)-6 ($p < 0.05$) and IL-8 ($p < 0.05$) while that of *P. lutheri* inhibited IL-6 ($p < 0.01$) production. Quantitative gene expression analysis of a panel of 92 genes linked to inflammatory signaling pathway revealed down-regulation of the expression of 14 pro-inflammatory genes (*TLR1*, *TLR2*, *TLR4*, *TLR8*, *TRAF5*, *TRAF6*, *TNFSF18*, *IL6R*, *IL23*, *CCR1*, *CCR4*, *CCL17*, *STAT3*, *MAP3K1*) by the lipid extracts. The lipid extracts effectively inhibited the LPS-induced pro-inflammatory signaling pathways mediated via toll-like receptors, chemokines and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling molecules. These results suggest that lipid extracts from *P. lutheri*, *P. palmata*, *P. dioica* and *C. crispus* can inhibit LPS-induced inflammatory pathways in human macrophages. Therefore, algal lipid extracts should be further explored as anti-inflammatory ingredients for chronic inflammation-linked metabolic diseases.

1. INTRODUCTION

Inflammation is a complex physiological process involving activation of the immune system. It occurs following physical injury or invasion by pathogenic bacteria, viruses or tumor cells in the host (Calder, 2006). Inflammation is essential to identify and destroy invading pathogens and is vital for host health in acute disease states. However, in many cases, inflammation occurs at a chronic, subclinical level and if this process becomes unregulated and prolonged, the activated immune system can begin to damage host tissues and supplement chronic disease states such as cardiovascular disease (CVD) (Hansson, 2005), inflammatory bowel disease (IBD) (Fiocchi, 1998), obesity (Choy and Panayi, 2001, Gregor and Hotamisligil, 2011) and Alzheimer's disease (AD) (Akiyama et al., 2000). Dietary interventions to control chronic inflammation may provide useful strategies for the management of such diseases (Rallidis et al., 2003, Calder et al., 2009, Esposito et al., 2004, Ruiz-Núñez et al., 2013), therefore the discovery of novel, effective, anti-inflammatory bioactives is warranted to identify long-term dietary therapies for inflammatory metabolic disorders.

The use of synthetic pharmacological agents to inhibit inflammation has shown promise in metabolic diseases such as type 2 diabetes and CVD (Esser et al., 2014); however, chronic use of such drugs is often associated with adverse gastrointestinal side effects (Sostres et al., 2010). The application of natural anti-inflammatory compounds is desirable as a long-term strategy to suppress chronic inflammation and alleviate disease-associated symptoms. A number of dietary components have been shown to exhibit potent anti-inflammatory activity, and evidence suggests certain dietary changes may be key as long-term alternatives to pharmaceutical compounds (Calder et al., 2009). Long-chain *n*-3 polyunsaturated fatty acids (*n*-3 LC-PUFA), for example, have been demonstrated to reduce inflammation in both *in vitro* and *in vivo* studies (Calder, 2006, Calder, 2012, Calder, 2013a). *n*-3 LC-PUFA act as precursors to potent anti-

inflammatory mediators, termed eicosanoids, which inhibit inflammatory cytokine production and inflammatory gene expression (Wall et al., 2010). Indeed, the *n*-3 LC-PUFA eicosapentaenoic acid (EPA, 20:5 *n*-3) and docosahexaenoic acid (DHA, 22:6 *n*-3) inhibit interleukin-6 (IL-6) and interleukin-1β (IL-1β) production in human macrophages (Weldon et al., 2007, Mullen et al., 2010)]. It has been suggested that *n*-3 LC-PUFA may play a role as nutritional therapeutic ingredients for the prevention and treatment of inflammatory diseases (Calder, 2013b). Certain polyphenols, pigments, and other plant bioactives have also demonstrated anti-inflammatory activity both *in vitro* and *in vivo* (González et al., 2011, Peng et al., 2011, Bai et al., 2005) suggesting potential for dietary ingredients to ameliorate symptoms of chronic inflammatory diseases.

Marine and freshwater algae produce a number of bioactive compounds including peptides, carbohydrates, phenols, pigments, and lipids with potential health benefits (Lordan et al., 2011, Robertson et al., 2013). For example, marine macroalgae (seaweeds) typically contain around 2%–5% lipid on a dry weight basis, where up to 70% of the total fatty acids can be PUFA (Dawczynski, 2007, Schmid et al., 2014). Some microalgae are reported to contain high protein, lipid (up to 60% of dry weight) and PUFA contents (up to 60% of total fatty acids), and have been exploited for their protein and lipid content for a number of nutritional and industrial purposes (Ratledge, 2004, Ward and Singh, 2005). Both micro- and macroalgae may thus act as sources of essential vitamins, minerals, and bioactive pigments.

Algae potentially represent an abundant and underexplored resource of health promoting functional food ingredients, which may be particularly useful in the Western diet, which is otherwise rich in meat products. Additionally, fish stocks are facing global decline alongside increasing toxin accumulation due to pollution (FAO Fisheries and Aquaculture Department, 2010, Branch et al., 2011, Kris-Etherton et al., 2002)], hence algae may constitute a more economical and sustainable resource for dietary *n*-3

LC-PUFA and other bioactive marine ingredients (Adarme-Vega et al., 2012). Indeed, a number of whole algae-extracts and algae-derived compounds have exhibited anti-inflammatory activity through the inhibition of pro-inflammatory cytokine and eicosanoid production, and reduction of expression of pro-inflammatory genes (Hwang et al., 2011, Jo et al., 2010, Khan et al., 2007, Vo et al., 2011, Park et al., 2008, Lee et al., 2013). Yang *et al*. found that extracts from five Korean seaweeds (*Laurencia okamurae*, *Grateloupia elliptica*, *Sargassum thunbergii*, *Gloiopeltis furcata*, and *Hizikia fusiformis*) reduced inflammation in the mouse macrophage cell line RAW 264.7 through inhibition of the pro-inflammatory eicosanoid prostaglandin E2 (PGE2) and the pro-inflammatory cytokines IL-6 and tumor-necrosis factor α (TNFα) (Yang et al., 2010). Furthermore, Nauroth *et al*. demonstrated the anti-inflammatory activity of microalgal oil when fed to rats (Nauroth et al., 2010). In addition, Banskota *et al*. reported anti-inflammatory effects of lipid extracts of *Tetraselmis chuii*, *Chlorella sorokiniana* and *Chondrus crispus* in RAW 264.7 macrophages (Banskota et al., 2013a, Banskota et al., 2013b, Banskota et al., 2014). Accordingly, there is evidence to suggest that algal extracts may be beneficial as functional food ingredients to control inflammation.

The aim of this study was to evaluate the anti-inflammatory activity of three algal species from the Irish coast and a microalga and to identify their potential as anti-inflammatory functional food ingredients. To this end, lipid extracts of the edible and commercially valuable red macroalgae *Porphyra dioica*, *Palmaria palmata*, *Chondrus crispus* (all Rhodophyta) and *Pavlova lutheri* (Haptophyta) were characterized for their fatty acid, pigment, lipid and LC-PUFA partitioning profiles. Subsequently the anti-inflammatory bioactivities of these extracts were assessed through their potential to influence cytokine production and inflammatory gene expression in human THP-1 macrophages.

2. METHODS

2.1. Materials

All chemicals and reagents were purchased from Sigma-Aldrich Ireland Ltd. (Arklow, Republic of Ireland) unless otherwise stated. Tissue culture plastics were purchased from Sarstedt (Wexford, Republic of Ireland). The human THP-1 monocytic cell line was purchased from the American Tissue Culture Collection (ATCC, LGC Standards, Middlesex, UK). Working solutions of bacterial lipopolysaccharide (LPS; *Escherichia coli* 055:B5) and phorbol-12-myristate-13-acetate (PMA) were stocked in RPMI 1640 medium.

2.2. Collection of Macroalgae and Biomass Preparation

Samples of the three red algae *Chondrus crispus*, Stackhouse (Gigartinales,), *Palmaria palmata* (L.) F. Weber & D. Mohr (Palmariales) and *Porphyra dioica*, J. Brodie & L. M. Irvine (Bangiales) were collected in Galway Bay (Spiddal 53°14′22″ N, 9°18′52″ W) during March 2013. Algae were placed on ice for approximately 2–3 hours during transportation to the laboratory, rinsed with fresh water and cleaned of all visible epiphytes, if present, and frozen at −20 °C. Algal material was lyophilised using Labconco Freezone® (Kansas City, MO, USA) 6 freeze-dryer system and ground to fine powder for lipid extraction.

Algal species were identified and extracts supplied by the Algal Biosciences Research Group of Dagmar Stengel (Botany and Plant Science, School of Natural Sciences, Ryan Institute for Environment, Marine and Energy, National University of Ireland Galway).

2.3. Microalgal Strain, Culture Conditions and Biomass Preparation

An axenic strain of *Pavlova lutheri* CCAP 931/6, formerly renamed *Diacronema lutheri* (Bendif et al., 2011), was obtained from the Culture Collection of Algae and Protozoa at the Scottish Marine Institute (SAMS Research Services Ltd., Oban, Scotland, UK) and cultivated in the laboratory at the National University of Ireland Galway.

P. *lutheri* were cultivated under batch conditions on F/2-RSE medium in three 15 L polycarbonate Nalgene® culture vessels at 15 °C and under continuous illumination (100 µmol photons $m^{-2}\cdot s^{-1}$), provided by lumilux cool daylight fluorescent lamps (OSRAM L18W/865,Munich,Germany). F/2-RSE medium, a modified version of Guillard's (1975) F/2 medium (Guillard, 1975) where filtered seawater is substituted by ReefSalt (H2Ocean, Pro+, Essex, IG6 3UT, England, UK), was used and complemented, as described by Guihéneuf *et al*. (Guihéneuf and Stengel, 2013)], with few modifications, a higher initial $NaNO_3$ concentration (350 $mg\cdot L^{-1}$) and $NaHCO_3$ supplementation (1 $g\cdot L^{-1}$). Cultures were agitated by air-bubbling (~0.03%–0.04% CO_2).

P. *lutheri* cells were harvested after 50 days (late-exponential growth phase), just after nitrate-depletion was reached on day 40 in order to allow maximum accumulation of oil containing *n*-3 LC-PUFA as described by Guihéneuf *et al*. (Guihéneuf and Stengel, 2013), by centrifuging using a modified Alistar milk cream separator

(80 $L\cdot h^{-1}$). Prior to lipid extraction, the fresh algal paste collected was frozen at −20 °C and freeze-dried as previously described.

2.4. Lipid Extraction

Total lipids were extracted by solid-liquid procedure using a modified version of Bligh and Dyer's method (Bligh and Dyer, 1959). Briefly, freeze-dried ground macroalgal

biomass or freeze-dried microalgal powder was extracted with methanol:chloroform 1:1 (*v*/*v*) in a glass bottle closed under nitrogen gas (N_2) to limit oxidation, and stirred overnight at 4 °C. The first extract obtained was filtered and the remaining biomass re-extracted 2–3 times similarly. All the filtered-extracts were then combined and Milli-Q water added to give a final chloroform:methanol:water ratio of 1:1:0.9 (*v*/*v*/*v*). After phase separation, the organic phase containing lipids (lower phase) was collected, and the extraction was repeated 2–3 times by adding chloroform to the remaining methanol:water phase. The combined organic phases containing the total crude lipid extract were then evaporated to dryness using a rotavapor (BÜCHI R-210 equipped with a Heating Bath B-491 and a Vacuum Pump V700, BÜCHI Labortechnik AG, 9230 Flawil, Switzerland) under reduced pressure at 40 °C.

2.5. Lipid Class Separation

The lipid class separation procedure combined the method described by Sukenik *et al*. (Sukenik et al., 1989) using Sep-Pak silica cartridges to separate the neutral and polar lipids, and thin-layer chromatography procedures adapted from Williams (Williams, 1978), Christie (Christie, 1982), and Henderson and Tocher (Henderson and Tocher, 1992) to separate individual lipid classes.

2.5.1. Lipid Separation by Silica Cartridge Chromatography

A known amount of total crude lipid extract dissolved in chloroform was fractionated on reversed phase silica gel columns (Bond Elut™ JR-SI 500 mg, Agilent Technologies, Santa Clara, CA, USA) after an activation step with 5 mL of methanol followed by 5 mL of chloroform. Neutral lipids were eluted using 20 mL of chloroform, while polar lipids were eluted with 20 mL of methanol (Sukenik et al., 1989). The

neutral and polar fractions obtained were evaporated to dryness using a rotary evaporator under reduced pressure at 40 °C and dissolved in a small volume of chloroform or methanol, respectively.

2.5.2. Neutral and Polar Lipid Fractionation by Thin-Layer Chromatography (TLC)

Both neutral and polar lipids were subjected to one-dimensional thin-layer chromatography (TLC) for lipid class separation and identification, using TLC plates coated with silica gel 60 (Silica Gel 60, 20 × 20 cm, 0.25 mm thickness, Merck, Darmstadt, Germany). Neutral lipids were eluted using petroleum ether:diethyl ether:acetic acid 80:20:1 (*v*/*v*/*v*). Polar lipids were eluted into individual lipids using a five-component mixture of chloroform:acetone:methanol:acetic acid:water 50:20:10:10:5 (*v*/*v*).

Two-dimensional TLC was later performed to confirm the tentative identification of polar lipid classes, using chloroform/methanol/water 65:25:4 (*v*/*v*) as first solvent, and a mixture of chloroform/acetone/methanol/acetic acid/water 50:20:10:10:4 (*v*/*v*) as second solvent. A brief exposure to iodine vapor was used as general staining to visualize the different lipid spots subsequently recovered from the TLC plates. Each individual class was then subjected to gas chromatography (GC) for quantification of its fatty acid profile, after direct-transmethylation. Individual lipid classes were identified by comparison of R_f values to R_f values provided in the literature (Christie, 1982), by using specific lipid charring reagents as described by Mereiles *et al*. (Meireles et al., 2003), and/or by running commercial lipid standards (Avanti® Polar Lipids, Inc., Alabaster, AL, USA) along with the samples.

2.6. Fatty Acid Analysis

Fatty acid methyl esters (FAME) were obtained by transmethylation of an aliquot of total lipid extract, or by direct-transmethylation of the silica powder containing the different lipid class fractions and/or the freeze-dried algal powder, with dry methanol containing 2% (*v*/*v*) H_2SO_4 and heating at 80 °C for 1.5 h with continuous stirring under a nitrogen atmosphere as described by Schmid *et al*. (Schmid et al., 2014).

Gas chromatographic analysis of FAME was performed on an Agilent 7890A GC (Agilent Technologies) equipped with a flame ionization detector and a fused silica capillary column (DB-WAXETR, 0.25 mm × 30 m × 0.25 µm, Agilent Technologies, Santa Clara, CA, USA). Hydrogen was used as a carrier gas. The injector and detector temperatures were 250 °C and 300 °C, respectively. The temperature was programmed at 140 °C for 1 min, raised from 140 to 200 °C at a rate of 15 °C·min^{-1}, and then from 200 to 250 °C at a rate of 2 °C·min^{-1}.

Identification of FAME was obtained by co-chromatography with authentic commercially available FAME standards (Supelco™ 37 Component FAME Mix, Supelco, Bellefonte, PA, USA) and FAME of fish oil (Menhaden Oil, Supelco). Fatty acid contents were quantified by comparison with a known amount of added pentadecanoic acid 15:0 (Pentadecanoic acid, 99%, Catalog No. A14664-09, Alfa Aesar, Heysham, England, UK) as internal standard. Results are expressed as the mean values of two replicates (n = 2) obtained from two analytical repetitions for each extract.

2.7. Pigment Analysis

Pigments were analyzed according to the method described by Wright *et al*. (Wright et al., 1991), modified by Bidigare *et al*. (Bidigare et al., 2005). A known amount of total crude lipid extract was re-suspended in a known volume of cold 90% aqueous acetone

(HPLC grade, Fischer Scientific, Loughborough LE11 5RG, England UK). After overnight at 4 °C to allow full re-dissolution of algal pigments, the samples were filtered before High Performance Liquid Chromatography (HPLC) analysis.

Pigments were separated and quantified on a Agilent 1200 series HPLC (Agilent Technologies) equipped with a Diode Array Detector (DAD) and a Fluorescence Detector (FLD), and separated using a C18 column (150 mm × 4.6 mm inner diameter, Eclipse XDB-C18, Agilent Technologies). The gradient mobile phase consisted of 80:20 (*v*/*v*) methanol/0.5 M ammonium acetate (pH = 7.2, with 0.1% (*w*/*v*) added butylated hydroxytoluene (BHT)), 87.5:12.5 (*v*/*v*) acetonitrile/Milli-Q water (0.1% (*w*/*v*) added BHT) and ethyl-acetate. Identification of algal pigments was obtained by comparison of peak retention times and spectra with commercial standards (DHI, Hørsholm, Denmark; Sigma-Aldrich, St. Louis, MO, USA). Pigment concentrations were calculated using standard curves obtained by injection of precisely quantified amounts of commercial pigment standards. Results are expressed as the mean values of two replicates ($n = 2$) obtained from two analytical repetitions for each extract.

2.8. Cell Culture

Human THP-1 monocytes were cultured in RPMI 1640 medium supplemented with 10% (*v*/*v*) heat inactivated fetal bovine serum (FBS), L-glutamine and 0.5% (*w*/*v*) penicillin/streptomycin, and incubated at 37 °C in a 5% CO_2 atmosphere. For monocyte-macrophage differentiation, cells were seeded at a density of 2×10^5 cell·mL^{-1} in 12-well plates or 5×10^4 cell·mL^{-1} in 96-well plates, and differentiation was induced by exposing the cells to 10 ng·mL^{-1} phorbol-12-myristate-13-acetate (PMA) in 2.5% (*v*/*v*) FBS supplemented media for 72 h. Macrophages were subsequently treated with PMA-free media with 2.5% FBS for a further 24 h. Lipid extracts from *P. lutheri*, *P. palmata*, *P. dioica* and *C. crispus* were prepared in dimethyl

sulfoxide (DMSO) and added to fresh culture medium at a concentration of 3 μg·mL^{-1} total fatty acids and incubated for 24 h. Control cells were treated with DMSO alone at a concentration of 0.1% (*v*/*v*).

2.9. Cytotoxicity Assay

Cytotoxicity of the extracts was measured by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay using the CellTiter 96® AQ_{ueous} One Solution Assay (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. Briefly, cells were seeded in a 96-well plate for 24 h and then treated with the algal lipid extracts at different concentrations. Subsequently, 20 μL of MTS reagent was added into each well. Following incubation at 37 °C and 5% CO_2 for 4 h, the absorbance at 490 nm with reference wavelength of 610 nm was read using a microplate reader. For subsequent analysis, cells were treated with a concentration of extract that exhibited no significant reduction in cell viability.

2.10. Cytokine Analysis

For cytokine analysis, differentiated THP-1 macrophage cells were first pretreated with algal lipid extracts for 24 h followed by induction of pro-inflammatory response by treating with 0.1 μg·mL^{-1} LPS for 24 h. Following the LPS stimulation, cell culture supernatants were collected, centrifuged at 1000× *g* for 10 min and stored at −80 °C until further analysis. Abundance of cytokine (TNFα, IL-6, IL-8, IL-1β, IL-12p70, IFNγ, IL-10) in the cell culture supernatants was performed using the MSD Human proinflammatory 7-plex Ultra-sensitive electrochemiluminescent (ECL) assay (Meso Scale Discovery (MSD), Gaithersburg, MD, USA). Briefly, 25 μL of supernatant samples were added to wells of a 96-well plate precoated with primary capture

antibodies. The plate was then sealed and incubated for 1 h at room temperature with vigorous shaking (300 rpm). Detection antibody (25 μL) was then added to the wells and the plate was sealed and incubated for 1 h at room temperature with continuous shaking (300 rpm). The plate was then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 prior to the addition of 150 μL read buffer. The plate was then read in a SECTOR Imager (Meso Scale Discovery) and quantification of individual cytokines was performed following the supplier's guidelines.

2.11. Preparation of RNA and cDNA Synthesis

Total RNA was extracted from THP-1 macrophages using a Qiagen RNeasy Mini Kit (Qiagen, Manchester, UK) according to the manufacturer's protocol. RNA was dissolved in 20 μL of nuclease-free water and then subjected to deoxyribonuclease I (DNase I) treatment to eliminate the genomic DNA contamination. RNA yield was assessed by measuring absorbance at 260 nm using a Nano-drop ND-1000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). Sufficient RNA purity was determined by a 260:280 nm absorbance ratio of ≥1.8 and by visualizing on a 1.5% (*w*/*v*) RNA agarose gel. Total RNA (1 μg) was reverse-transcribed to cDNA using a RevertAid H Minus First Strand cDNA synthesis Kit (Fermentas GmbH, St. Leon-Rot, Germany) according to the manufacturer's protocol.

2.12. Quantitative Real-Time PCR Analysis

The quantitative expression of a panel of 96 genes (list of genes provided in supplementary Table S1) involved in the inflammatory signaling cascade in human was evaluated using a PCR array in a LightCycler 480 instrument (Roche Diagnostics

GmbH, Mannheim, Germany). This is a customized array designed by Sigma-Aldrich Corp. that enables screening of 92 different genes involve in inflammatory signaling pathways and 4 internal reference genes in human. For the PCR array experiment, 25 µL cDNA (after 1:5 dilution) from each individual wells within a single treatment group were pooled. Real-time reverse transcriptase PCR was performed on a 20 µL reaction mixture per well containing 1 µL pooled cDNA, 9 µL water and 10 µL SYBR green master mix (Applied Biosystems, Foster City, CA, USA). The thermal cycle conditions were 94 °C for 30 s followed by 60 °C for 1 min, for 40 cycles. The mRNA abundances were expressed in crossing point (Cp) values, the number of PCR cycles after which the PCR product crosses a threshold value. In this experiment, a Cp value of 35 was considered as the cut-off limit.

Normalization of the expression of 92 target genes included in the PCR array was performed based on three reference genes (beta actin (*ACTB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and beta glucuronidase (*GUSB*)) and following $2^{-\Delta\Delta Cp}$ method. Briefly, average ΔCp was calculated as the difference of Cp values of any target gene minus average of the Cp value of the three reference genes. Then, fold change was calculated as $2^{(-\text{average } \Delta Cp \text{ target gene})}/2^{(-\text{average } \Delta Cp \text{ reference gene})}$. A fold difference cut-off point was set at ≥2.5.

Validation of the PCR array data was performed through evaluation of the quantitative expression of three target genes (*TLR8*, *TLR1* and *TRAF5*) and one reference gene (*GAPDH*) in each of the replicate samples on the LightCycler 480 instrument (Roche Diagnostics GmbH). The PCR reaction (10 µL) contained 1 µL cDNA (following 1:5 dilution), 3 µL nuclease free water, 1 µL forward and reverse primer and 5 µL SYBR green master mix (Roche Diagnostics GmbH). The thermal cycle conditions were as follows: 94 °C for 10 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Samples were analyzed in triplicate and Cp

values <35 were used for analysis. The mRNA abundance of three target genes (*TLR8*, *TLR1* and *TRAF5*) were normalized against the expression of *GAPDH*.

2.13. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.0 for Windows (GraphPad Software). Extract composition results are expressed as the mean values of two replicates ($n = 2$) ± standard deviation (SD) obtained from two analytical repetitions for each extract. Cytokine production and qPCR results are presented as means ± standard error of the mean (SEM) of at least three independent experiments. To assess whether differences between the exposures were significant, multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test or Fisher's least significant difference test. A p-value of ≤ 0.05 was considered statistically significant.

3. RESULTS

3.1. Fatty Acid Composition of Algal Lipid Extracts

The fatty acid composition, expressed as % of total fatty acids of the algal lipid extracts are shown in Table 1. Each extract contained a broad spectrum of medium to long-chain saturated and unsaturated fatty acids. Total saturated fatty acid (SFA) content was similar across all four algal species, ranging from 28% to 32.4% of total saturated fatty acids. SFA content was dominated by palmitic acid (16:0) in the three seaweed species (*P. palmata*, *P. dioica* and *C. crispus*) ranging from 22.3% to 28.1%, whereas the dominant SFA in the *P. lutheri* (microalgal) extract was myristic acid (14:0) at 14.3%, followed by 12.8% of 16:0.

Total monounsaturated fatty acid (MUFA) content ranged from 6.1%–8.8% in the three red seaweed species whereas *P. lutheri* displayed 19.5% of total fatty acids as MUFA, of which palmitoleic acid (16:1 *n*-7) was most abundant with 14.4%. Oleic acid (18:1 *n*-9) was another MUFA present at high levels, ranging from 1.7% in *P. dioica* to 5.5% in *C. crispus*.

Polyunsaturated fatty acids (PUFA) were more dominant than SFA and MUFA in all extracts, ranging from 51.9% to 64.6% of total fatty acids. Of these PUFA, *n*-3 fatty acids were present in greater quantities than *n*-6 PUFA in all extracts. EPA was the most abundant PUFA and the most abundant fatty acid in all extracts, ranging from 27.7% in *P. lutheri* to 57.9% in *P. palmata*. DHA accounted for 10.5% of total fatty acids in *P. lutheri* and was present in undetectable or very low quantities in all other extracts. Of note, *P. lutheri* contained 5% stearidonic acid (SDA, 18:4 *n*-3). Additionally the *n*-6 LC-PUFA arachidonic acid (ARA, 20:4 *n*-6) was present in very high quantities in *C. crispus* at 19.9% of total fatty acids. The ratio of *n*-6/*n*-3 fatty acids was <0.2 in *P. lutheri*, *P. palmata* and *P. dioica* (0.09, 0.04, and 0.17, respectively) whereas *C. crispus* had an *n*-6/*n*-3 ratio of 0.66 due to its higher proportion of *n*-6 ARA.

3.2. Pigments

Pigment contents of the different extracts are detailed in Table 2. Individual pigment values are expressed as % molar and total pigment values are expressed as % of dry weight crude extract. Pigments concentrations were relatively low but substantial in all extracts, ranging from 4.9% to 6.8% of the crude extract. Chlorophyll *a* and its degradation products were most abundant in all extracts accounting for 41.1%–53% of total pigments. β-carotene and zeaxanthin were the only other pigments detected in the red seaweed extracts ranging from 19.2%–30.2% and 22.8%–27.5%, respectively. Fucoxanthin and its degradation products, as well as diadinoxanthin and diatoxanthin

and their degradation products, were also present in relatively high proportions (26.9% and 16.1%, respectively) in *P. lutheri*, alongside a number of other pigments (*i.e.*, β-carotene, *cys*-fucoxanthin, and chlorophyll *c*).

3.3. Lipid Class Composition and LC-PUFA Partitioning

The lipid class composition of each lipid extract and the distribution of the major LC-PUFA (SDA, ARA, EPA and DHA) within the different lipid fractions are detailed in Figure 1.

In *P. lutheri*, the triacylglycerol (TAG) and glycolipid families represent together 67.1% of total fatty acids. The neutral lipid fraction, composed by TAG (24.1%), diacylglycerols (DAG, 0.8%), monoacylglycerols (MAG, 1.3%), and free fatty acids (FFA, 8.4%) accounted for 34.5% of total fatty acids. The polar lipid fraction was mainly composed of monogalactosyldiacylglycerols (MGDG, 15.2%), digalactosyldiacylglycerols (DGDG, 18.0%) and sulfoquinovosyldiacylglycerols (SQDG, 9.8%), which represent a total glycolipid proportion of 43% of total fatty acids. Other unidentified lipid classes accounting together for 12.2% of total fatty acids were also found in the polar lipid fraction.

In the red seaweeds (*P. palmata*, *P. dioica*, *C. crispus*), TAG and FFA represent the main neutral lipid classes with 11.1%–13.4% and 4.3%–8.1% of total fatty acids, respectively. The polar fraction was essentially composed of MGDG (17.0%–23.1%), DGDG (17.4%–21.7%) and SQDG (16.8%–25.4%), representing 71.6%–76.9% of total fatty acids. Phospholipids (11.1%–16.4%) such as diphosphatidylacylglycerols (DPG), phosphatidylethanolamines (PE), phosphatidylglycerols (PG), phosphatidylcholines (PC) and phosphatidylinositols (PI) were also found in the polar fraction of the red macroalgae.

Despite small differences between species, LC-PUFA were mainly partitioned into the glycolipid (MGDG, DGDG and SQDG) and TAG fractions. In *P*. *lutheri*, DHA was also substantially incorporated into the other unidentified lipid classes (11.3% of the total DHA); EPA was also incorporated into the PG of *P*. *dioica* (10.1% of the total EPA).

3.4. Inhibition of Inflammatory Cytokine Production in Lipopolysaccharide (LPS)-Stimulated THP-1 Macrophages

The effects of THP-1 macrophage exposure to the four algal lipid extracts on lipopolysaccharide (LPS)-stimulated IL-6, IL-8 and TNFα production are shown in Figure 2. Following 24 h-exposure to *P*. *lutheri* lipid extract, the production of the pro-inflammatory cytokine IL-6 ($p < 0.01$) was significantly downregulated in comparison to the carrier control. Incubation of the THP-1 cells with *P*. *palmata* lipid extract significantly reduced production of IL-6 ($p < 0.05$) and IL-8 ($p < 0.05$), whereas incubation with *C*. *crispus* lipid extract led to significantly increased production of TNFα relative to the carrier control ($p < 0.05$). *P*. *dioica* did not appear to significantly influence the production of any of the examined cytokines. There were no significant responses in IFNγ, IL-12p70, IL-10 or IL-1β production by THP-1 cells following exposure to any of the algal lipid extracts.

3.5. Inhibition of Inflammatory Gene Expression by Algal Lipid Extracts

In order to assess the anti-inflammatory effects of the algal lipid extracts in the THP-1 macrophages, quantitative expression of a panel of 92 inflammatory marker genes was evaluated. Of the 92 target genes analyzed, 16 genes were differentially expressed following exposure to the algal lipid extracts as shown in Table 3. mRNA abundances

of toll-like receptor 1 (*TLR1*) and toll-like receptor 8 (*TLR8*) and TNF receptor associated factor 5 (*TRAF5*) were consistently downregulated by the lipid extracts from all four algal species. In addition, *P*. *dioica* extract downregulated nine other pro-inflammatory genes including pro-inflammatory markers toll-like receptor (*TLR4*) and signal transducer and activator of transcription 3 (*STAT3*). Other genes that were downregulated by algal lipid extracts predominantly represent pro-inflammatory pathways such as those sharing toll-like receptors, TNF receptor associated factors, chemokine, cytokines and their receptors. While the lipid extracts from all four algal species predominantly indicated an inhibitory effect on the pro-inflammatory genes, the up-regulation of *NOS2* by *P*. *palmata* and *PTGER1* by *P*. *lutheri* and *P*. *dioica* indicated activation of a pro-inflammatory response which may be due to the fact that the crude lipid extracts may contain certain compounds with pro-inflammatory activities.

3.6. Validation of PCR Array Results through Quantitative PCR (qPCR)

For the validation purpose, three genes (*TLR1*, *TLR8* and *TRAF5*) were selected for qPCR analysis given that these genes were consistently downregulated by all four algal lipid extracts as evident in the PCR array data. The oligonucleotide sequences for the primers used for quantitative PCR for these genes are outlined in Table 4. The effects of the four algal lipid extracts on *TLR1*, *TLR8* and *TRAF5* gene expression in LPS-stimulated macrophages are shown in Figure 3. Compared to the carrier control, exposure of the THP-1 cells to the lipid extracts from *P*. *lutheri*, *P*. *palmata* and *P*. *dioica* resulted in inhibition of *TLR1*, *TLR8*, and *TRAF5* mRNA abundances indicating consistency of the PCR array data which were generated through gene expression analysis in the pooled cDNA samples. For the *C*. *crispus* lipid extract, a reduction in the expression of these genes was least pronounced due to a high standard deviation (SD) because of one individual replicate.

4. DISCUSSION

In this study we investigated the anti-inflammatory activity of lipid extracts of four algal species in human THP-1 macrophages. The fatty acid, pigment and lipid class, and LC-PUFA partitioning profiles of three red seaweeds (*Palmaria palmata*, *Porphyra dioica*, *Chondrus crispus*) and one microalga (*Pavlova lutheri*) were identified, which contained high proportions of *n*-3 PUFA (34%–42% of total fatty acids) and 5%–7% pigments including chlorophyll *a*, β-carotene, and fucoxanthin. When exposed to human THP-1 macrophages for 24 h, lipid extracts of *P. lutheri* ($p < 0.01$) and *P. palmata* significantly ($p < 0.05$) suppressed LPS-induced production of the pro-inflammatory cytokine IL-6, compared with the untreated control. Similarly, *P. palmata* ($p < 0.05$) lipid extract significantly reduced production of the pro-inflammatory cytokine IL-8 compared with the control. All extracts inhibited the expression of a number of inflammatory genes in THP-1 macrophages, including those involved in toll-like receptor activity and chemokine and cytokine-linked signaling pathways. These results suggest that the algal lipid extracts examined have potential to suppress inflammation through inhibition of inflammatory cytokine production and down-regulation of genes involved in a number of inflammatory signaling pathways.

The results provide additional compositional data of lipid extracts from these four algal species based on fatty acid, pigment, and lipid profiles. In accordance with previous reports, this study demonstrated algal LC-PUFA are mainly partitioned into complex polar lipids constituting the membranes and in a less extent into TAG of some species with the ability to accumulate TAG containing LC-PUFA. Each algal species contained high proportions of *n*-3 PUFA (34%–62% total fatty acids), with EPA being the most abundant fatty acid in all extracts, ranging from 28% to 58% of total fatty acids. Indeed, *P. lutheri* has been widely examined for the ability to produce high concentrations of both EPA and DHA, and optimized culture conditions have been

studied to maximize *n*-3 LC-PUFA production (Guedes et al., 2010, Tatsuzawa and Takizawa, 1995, Meireles et al., 2003, Volkman et al., 1989). Similarly, red seaweeds, including those examined in this study, have also been identified as rich sources of EPA and desirable *n*-6:*n*-3 PUFA ratios (Schmid et al., 2014). Macroalgal lipid composition, however, is susceptible to seasonal variation and it has been shown that pigment and LC-PUFA levels are optimized under low light and temperature conditions as observed, for example, in winter (Schmid et al., 2014).

Indeed, *n*-3 LC-PUFA within algae are primarily incorporated into complex lipid classes such as MGDG, DGDG, and SQDG (Guihéneuf et al., 2010, Sanina et al., 2004, Robertson et al., 2013). Banskota *et al*. have previously demonstrated that polar lipids isolated from red algae demonstrate stronger anti-inflammatory activity than pure EPA isolated from the same species (Banskota et al., 2014), suggesting that the entire polar lipid structure contributes to anti-inflammatory activity. Hence, the polar lipid structures, into which the fatty acids are incorporated in the species examined in this study, may contribute to the observed inhibition of cytokine production and inflammatory gene expression. Interestingly however, the *C*. *crispus* extract significantly increased production of TNFα, a proinflammatory cytokine. The reason for this may be due to the synergistic effect of a number of bioactive pigments and fatty acids in the extract including ARA, which was present in high quantities in the *C*. *crispus* extract and which is recognized as a pro-inflammatory fatty acid (Kuehl and Egan, 1980). Chlorophyll *a*, β-carotene, fucoxanthin, and zeaxanthin have all previously demonstrated anti-inflammatory activity (Park et al., 2014, Bai et al., 2005, Heo et al., 2010, Nidhi et al., 2015) and hence may have contributed to the anti-inflammatory activity observed in this study. Chlorophyll *a* is of particular interest as it was present from 2.1 to 3.1 g/100 g crude extract. However, due to the extremely diverse fatty acid and pigment composition of the extracts, it is not possible to attribute the observed biological effects to any individual fatty acids or pigments. It is likely that there are

synergistic effects between many bioactives including fatty acids and pigments, which contribute to the observed anti-inflammatory effects in a number of pathways.

The results presented here expand on previous findings showing that algal extracts inhibit inflammation *in vitro*. Yang *et al*. reported anti-inflammatory activity in five Korean seaweed extracts in mouse RAW 264.7 macrophages, in which IL-6, TNFα and prostaglandin E2 (PGE2) production were inhibited (Yang et al., 2010). Hwang *et al*. reported similar results for an extract from *Sargassum hemiphyllum* in the same cell line (Hwang et al., 2011). In addition, both Khan *et al*. and Ishihara *et al*. isolated pure PUFA from *Undaria pinnatifida* extracts and also demonstrated their anti-inflammatory activity (Khan et al., 2007, Ishihara et al., 1998). Our data provide novel evidence for the anti-inflammatory activity of algal extracts and show for the first time that *P. lutheri*, *P. palmata*, *P. dioica* and *C. crispus* lipids can inhibit induced inflammatory response in human macrophages. Hence these results suggest that these algal species, as dietary ingredients, may have the potential to suppress chronic inflammation *in vivo*; however further work is warranted to assess if the *in vitro* activity is translated to an *in vivo* model.

It is likely that the crude mix of fatty acids, polar lipids and pigments in the extracts influenced a number of inflammatory pathways in the cell, as seen by the wide range of genes altered in the gene expression array. LPS is an agonist of toll-like receptor 4 (TLR4), a membrane-bound receptor that activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway that leads to a pro-inflammatory response (Chow et al., 1999)]. In the present study, treatment of the macrophages with *P. dioica* extract downregulated expression of *TLR4* by more than four folds, indicating inhibition of NF-κB mediated pro-inflammatory pathway, however *TLR4* expression was not altered by any of the other extracts. In the present study, a number of genes involved in NF-κB signaling were significantly downregulated by the algal lipid extracts. Expression of *TLR1*, a toll-like receptor involved in

pathogen-associated molecular pattern (PAMP) recognition and NF-κB activation, was downregulated by all extracts and has previously been shown to be altered by unsaturated and saturated fatty acids (Lee et al., 2004). STAT3 and MAP3K1 are signaling molecules also involved in NF-κB activation through the JAK-STAT (Janus kinase-signal transducer and activator of transcription) and JNK kinase pathways respectively, which have been shown to be susceptible to change in expression by PUFA and a carotenoid extract of *Dunaliella salina* (Zeyda et al., 2003, Li et al., 2005, Yang et al., 2013). These genes were significantly downregulated by lipid extracts of *P*. *dioica* and *P*. *lutheri*, further elucidating a role that such extracts play in the downregulation of the NF-κB pathway. Moreover, a lipid extract from the cyanophyte *Nostoc commun*e which contained *n*-3 PUFA, β-carotene and a number of other bioactives has previously been demonstrated to inhibit inflammatory response in macrophages through inhibition of NF-κB signaling.

In this study, we also evaluated the effect of algae-derived lipid extract on the expression of *NOS2* gene in LPS-stimulated THP-1 cells. While the mRNA levels of *NOS2* gene were not affected by the extracts from *P*. *lutheri*, *P*. *dioica* and *C*. *crispus*, the expression was increased by the *P*. *palmata* lipid extracts. A higher *NOS* gene expression is likely to increase the level of inducible nitric oxide (iNOS) that mediates pro-inflammatory response. LPS was reported to stimulate iNOS production in THP-1 cells (Wang et al., 2013). In the present experiment, the high *NOS2* gene expression by *P*. *palmata* lipid extracts could be due to the failure of this extract to suppress the LPS induced *NOS2* gene expression and/or exaggerations of the iNOS mediated pro-inflammatory response in THP-1 cells. Due to the presence of crude and complex mixture of fatty acids and pigments in the extract, it was difficult to speculate the precise bioactive compound/s that drives such an increase in the *NOS2* gene expression or whether such a response is translated into higher iNOS leading to a pro-inflammatory response. It is likely that certain bioactive pigments such as β-carotene, and fatty acids,

such as arachidonic acid, which have previously demonstrated immune enhancing activities (Chew and Park, 2004, Cranmer-Byng et al., 2015), and that are present in the crude lipid extracts of *P*. *palmata* contributed to an increase in *NOS2* expression, as was previously reported for an extract of *Ulva rigida* in murine RAW 264.7 macrophages (Leiro et al., 2007). Hence, isolation and purification of individual bioactive compounds from the algal crude lipid extract and understanding their molecular mode of action will provide further details of the immune modulatory potential of these novel bioactives.

The present study explored the anti-inflammatory bioactivity of algal lipid extracts containing a crude mixture of fatty acids, pigments, and polar lipids, each of which may affect a number of related and unrelated gene expression and cytokine production, and the identification of individual anti-inflammatory pathways affected by the bioactive component; their precise molecular mode of action warrants further study. Of the species studied, lipid extract from *C*. *crispus* appeared to display the lowest anti-inflammatory potential. This may be due to its high content of *n*-6 ARA, which has been associated with pro-inflammatory properties. Extracts from *P*. *lutheri* and *P*. *palmata* were the most potent extracts in inhibiting inflammatory cytokine production, whereas those from *P*. *dioica* inhibited the expression of more inflammatory genes than any of the other species tested. As microalgae tend to have much higher lipid contents than macroalgae, are more efficient producers of *n*-3 LC-PUFA and contain more diverse pigment profiles, as observed here, *P*. *lutheri* may pose the best potential as an anti-inflammatory functional food ingredient.

In conclusion, we demonstrated for the first time that lipid extracts from *P*. *lutheri*, *P*. *palmata*, *P*. *dioica* and *C*. *crispus* downregulated LPS-induced pro-inflammatory responses in human macrophages through inhibition of IL-6 and IL-8 production. This anti-inflammatory mechanism of algal lipid extract is most likely due to alteration in the expression of genes that encode signaling molecules linked to the NF-κB pathway. We hypothesize that the potent anti-inflammatory effect shown by

these extracts is attributable to a synergistic effect of bioactive compounds such as pigments and *n*-3 LC-PUFA incorporated into complex lipid structures which have previously been shown to exert similar anti-inflammatory effects. However further work is warranted to identify which compound exert the strongest biological effects. The bioactivity of these novel algal-lipid extracts as anti-inflammatory functional food ingredients in chronic inflammation-linked metabolic diseases should be further explored.

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TABLES

Table 1. Fatty acid composition of the four algal lipid extracts.

Extracts	***Pavlova lutheri***		***Palmaria palmata***		***Porphyra dioica***		***Chondrus crispus***	
(% Total fatty acids)	Average	SD	Average	SD	Average	SD	Average	SD
Saturated fatty acids (SFA)								
12:0	0.19	0.14	0.16	0.05	0.32	0.13	0.42	0.15
14:0	14.26	0.47	4.51	0.35	0.53	0.10	1.98	0.39
16:0	12.84	0.19	22.27	0.40	28.12	1.19	27.33	0.50
18:0	0.68	0.02	1.69	0.07	2.16	0.11	2.71	0.05
Sum of SFAs	27.97	0.61	28.63	0.63	31.14	1.51	32.44	0.90
Monounsaturated fatty acids (MUFA)								
14:1	0.55	0.01	0.51	0.07	0.54	0.12	0.48	0.11
15:1	1.66	0.09	1.53	0.14	1.51	0.02	1.69	0.27
16:1 *n*-7	14.36	0.45	1.17	0.20	2.37	0.09	0.58	0.08
18:1 *n*-9	2.60	0.07	2.18	0.02	1.72	0.02	5.47	0.02
18:1 *n*-7	0.31	0.05	0.73	0.01	0.68	0.00	0.59	0.01
20:1 *n*-9	n.d.		n.d.		1.62	0.04	n.d.	
Sum of MUFAs	19.49	0.54	6.12	0.11	8.44	0.06	8.82	0.24
Polyunsaturated fatty acids (PUFA)								
16:2 *n*-6	0.85	0.22	n.d.		n.d.		n.d.	
16:2 *n*-4	1.68	0.11	n.d.		n.d.		n.d.	
16:4 *n*-3	0.62	0.13	n.d.		n.d.		n.d.	
18:2 *n*-6	0.59	0.02	0.65	0.01	1.64	0.01	1.58	0.05
18:3 *n*-6	2.09	0.04	0.26	0.01	0.56	0.01	0.49	0.01
18:3 *n*-3	1.13	0.01	0.65	0.01	1.34	0.02	0.23	0.00
18:4 *n*-3	4.98	0.08	2.16	0.04	1.58	0.01	0.25	0.01
20:2 *n*-6	n.d.		1.06	0.15	0.87	0.02	0.37	0.05
20:3 *n*-6	n.d.		n.d.		2.21	0.35	0.19	0.16
20:4 *n*-6	0.60	0.02	0.67	0.01	3.03	0.07	19.85	0.82
20:5 *n*-3	27.67	0.37	57.94	0.89	46.35	0.91	33.47	0.21
22:5 *n*-3	1.17	0.20	n.d.		n.d.		n.d.	
22:6 *n*-3	10.47	0.38	1.15	0.24	n.d.		n.d.	
Sum of PUFAs	51.85	0.84	64.55	0.87	57.57	1.35	56.42	1.29
n-3	46.04	1.05	61.91	0.71	49.26	0.93	33.94	0.20
n-6	4.13	0.18	2.64	0.17	8.31	0.44	22.47	1.10
Ratio *n*-6/*n*-3	0.09	0.01	0.04	0.00	0.17	0.01	0.66	0.03
Others	0.69	0.30	0.70	0.12	2.85	0.14	2.32	0.61

Data are expressed as % of total fatty acid methyl esters (FAME) and are presented as mean values ± standard deviation (SD); n.d., not detected.

Table 2. Pigment composition of four algal lipid extracts.

Extracts	***Pavlova lutheri***		***Palmaria palmata***		***Porphyra dioica***		***Chondrus crispus***	
Pigments (% Molar)	Average	SD	Average	SD	Average	SD	Average	SD
chlorophyll *a* + deg.	41.10	0.05	52.03	0.05	53.02	0.37	43.95	0.30
β-carotene	7.05	0.09	25.17	0.34	19.15	0.27	30.22	0.09
zeaxanthin	n.d.		22.80	0.38	27.53	0.59	25.84	0.40
diadinoxanthin + diatoxanthin + deg.	16.11	0.03	n.d.		n.d.		n.d.	
cys-fucoxanthin	5.51	0.01	n.d.		n.d.		n.d.	
chlorophyll *c* + deg.	3.36	0.08	n.d.		n.d.		n.d.	
fucoxanthin + deg.	26.87	0.07	n.d.		n.d.		n.d.	
Pigments (% crude extract)	6.80	0.05	6.03	0.41	5.13	0.06	4.87	0.04

Data are expressed as % molar and are presented as mean values ± standard deviation (SD); Total pigments are expressed as % of dry weight of crude extract; n.d., not detected; deg., degradation products.

Table 3. Effect of algal lipid extracts treatment followed by lipopolysaccharide (LPS) exposure on the differential expression of a panel of inflammatory genes in THP-1 macrophages.

Extract	Gene Symbol	Name	Fold change	Gene description and function
P. lutheri	*TLR8*	Toll-like receptor 8	−3.33	PAMP recognition and activation of innate immunity. Mediates cytokine production through activation of NF-κB
	TLR1	Toll-like receptor 1	−4.16	Interacts with TLR2 for immune activation through PAMP recognition.
	TRAF5	TNF receptor-associated factor 5	−2.69	Mediates signal transduction of the TNF receptor family. Mediates NF-κB and JNK activation
	MAP3K1	Mitogen-activated protein kinase 1	−2.63	Activates protein kinase signal transduction cascade such as the ERK and JNK kinase pathways and NF-κB pathway.
	PTGER1	Prostaglandin E receptor 1	+2.58	Encodes a receptor for PGE2. Down-regulates COX2 and hence resolves inflammation.
P. palmata	*TLR8*	Toll-like receptor 8	−2.91	PAMP recognition and activation of innate immunity. Mediates cytokine production through activation of NF-κB
	TLR1	Toll-like receptor 1	−3.99	Interacts with TLR2 for immune activation through PAMP recognition.
	TRAF5	TNF receptor-associated factor 5	−2.73	Mediates signal transduction of the TNF receptor family. Mediates NF-κB and JNK activation
	NOS2	Nitric oxide synthase 2	+2.83	Encodes nitric oxide synthase which mediates tumoricidal and bactericidal actions in macrophages
P. dioica	*CCR1*	Chemokine (C-C motif) receptor 1	−3.46	Acts as a receptor for chemokines such as MIP-1α and MCP-3 which assist in immune cell recruitment
	TLR8	Toll-like receptor 8	−3.29	PAMP recognition and activation of innate immunity. Mediates cytokine production through activation of NF-κB
	TLR2	Toll-like receptor 2	−4.25	Interacts with TLR1 for PAMP recognition leading to NF-κB activation and cytokine production
	TLR1	Toll-like receptor 1	−2.5	Interacts with TLR2 for immune activation through PAMP recognition.
	TRAF5	TNF receptor-associated factor 5	−3.37	Mediates signal transduction of the TNF receptor family. Mediates NF-κB and JNK activation
	TNFSF18	TNF (ligand) superfamily, member 18	−2.93	Regulates T-cell activities by lowering the threshold for T-cell activation.
	TRAF6	TNF receptor-associated factor 6	−2.99	Mediates signal transduction from TNF receptors and Toll/IL-1 receptors.
	MAP3K1	Mitogen-activated protein 3 kinase 1	−3.63	Activates protein kinase signal transduction cascade such as the ERK and JNK kinase pathways and NF-κB pathway.
	STAT3	Signal transducer and activator of transcription 3	−3.56	Activated by cytokines to create transcription factors that form part of JAK-STAT signaling cascade.
	CCR5	Chemokine (C-C motif) receptor 5	−2.72	Chemokine receptor, expressed in macrophages involved in immune cell recruitment.
	TLR4	Toll-like receptor 4	−4.44	PAMP recognition and activation of inflammatory cascade. Specifically recognises LPS
	IL6R	Interleukin 6 receptor	−2.54	Binds with low affinity to the inflammatory cytokine IL-6 regulating immune response and acute phase reactions.
	PTGER1	Prostaglandin E receptor 1	+2.7	Encodes a receptor for PGE2. Down-regulates COX2 and hence resolves inflammation.
C. crispus	*IL23*	Interleukin 23	−4.19	Activates STAT4 and stimulates production of IFNγ Associated with autoimmune inflammation and tumorigenesis.
	TLR8	Toll-like receptor 8	−2.59	PAMP recognition and activation of innate immunity. Mediates cytokine production through activation of NF-κB
	CCL17	Chemokine (C-C motif) ligand 17	−3.33	Encodes a cytokine that is a chemotactic factor for T-lymphocytes. Recruitment and activation of mature T-cells
	TLR1	Toll-like receptor 1	−3.25	Interacts with TLR2 for immune activation through PAMP recognition.
	TRAF5	TNF receptor-associated factor 5	−2.76	Mediates signal transduction of the TNF receptor family. Mediates NF-κB and JNK activation
	TRAF6	TNF receptor-associated factor 6	−3.04	Mediates signal transduction from TNF receptors and Toll/IL-1 receptors.

Values are expressed as fold change in gene expression relative to the dimethyl sulfoxide (DMSO) vehicle control. Negative values represent gene down-regulation and positive values represent gene up-regulation. A fold difference cut-off point was set at ≥2.5. PAMP: pathogen-associated molecular pattern; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; TNF: tumor necrosis factor; JNK: c-Jun *N*-terminal kinase; ERK: extracellular signal-regulated kinase; PGE2: prostaglandin E2; COX2: cyclooxygenase 2; STAT4: signal transducer and activator of transcription 4; MIP-1α: macrophage inflammatory proteins-1α; MCP-3: monocyte-specific chemokine-3; JAK: Janus kinase; IFNγ: interferon gamma.

Table 4. Oligonucleotide sequences of forward and reverse primers for quantitative real-time PCR (qPCR).

Gene name	Primers	Nucleotide sequence (5′ to 3′)	T_m (°C)
GAPDH	Forward	ACAGTTGCCATGTAGACC	55.7
	Reverse	TTTTTGGTTGAGCACAGG	59.9
TLR1	Forward	CCCTACAAAAGGAATCTGTATC	58.2
	Reverse	TGCTAGTCATTTTGGAACAC	57.8
TLR8	Forward	TGGAAAACATGTTCCTTCAG	60.1
	Reverse	TGCTTTTTCTCATCACAAGG	60.4
TRAF5	Forward	GGAATGGCTTATTCAGAAGAG	59.4
	Reverse	CCACAAACTGGTACTCTATAC	52.8

T_m: Melting temperature.

FIGURES

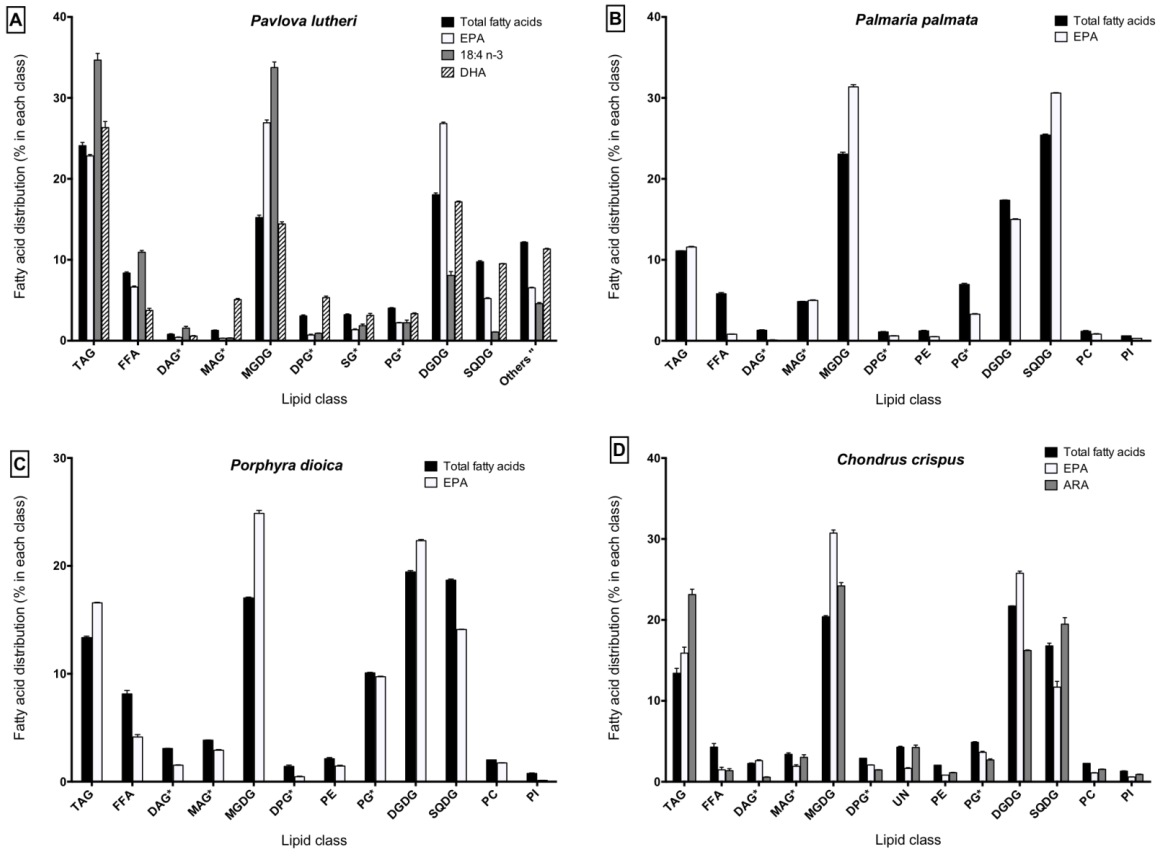


Figure 1. Distribution of fatty acids within lipid classes in (**A**) *Pavlova lutheri*, (**B**) *Palmaria palmata*, (**C**) *Porphyra dioica*, (**D**) *Chondrus crispus*. * Identified using R_f values from the literature using similar migration solvent systems without using standards; " Sum of other unidentified polar lipids, *i.e.*, phospholipids and betaine lipids; No symbol: Identified using R_f values from the literature and standards; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; TAG: triacylglycerol; FFA: free fatty acids; DAG: diacylglycerols; MAG: monoacylglycerols; MGDG: monogalactosyldiacylglycerols; DPG: diphosphatidylacylglycerols; SG: acylated sterol glycosides; PE: phosphatidylethanolamines; PG: phosphatidylglycerols; DGDG: digalactosyldiacylglycerols; SQDG: sulfoquinovosyldiacylglycerols; PC: phosphatidylcholines; PI: phosphatidylinositols; UN: unidentified.

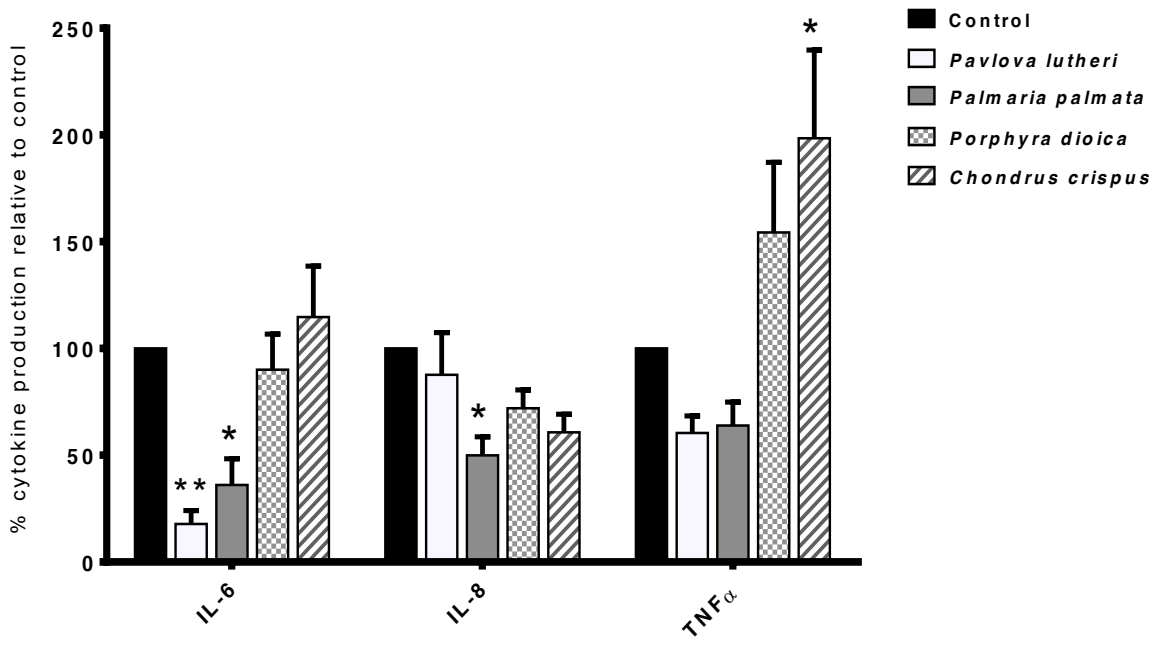


Figure 2. Effects of algal lipid extracts exposure on interleukin-6 (IL-6), IL-8 production and tumor necrosis factor α (TNFα) production in lipopolysaccharide (LPS)-stimulated THP-1 macrophages. THP-1 macrophages were exposed to the respective algae-lipid extracts or the vehicle control (dimethyl sulfoxide, DMSO) for 24 h and incubated with 100 ng·mL^{-1} LPS for a further 24 h. Values represent mean ± standard error (SE) normalized to DMSO control. * $p \leq 0.05$; ** $p \leq 0.01$.

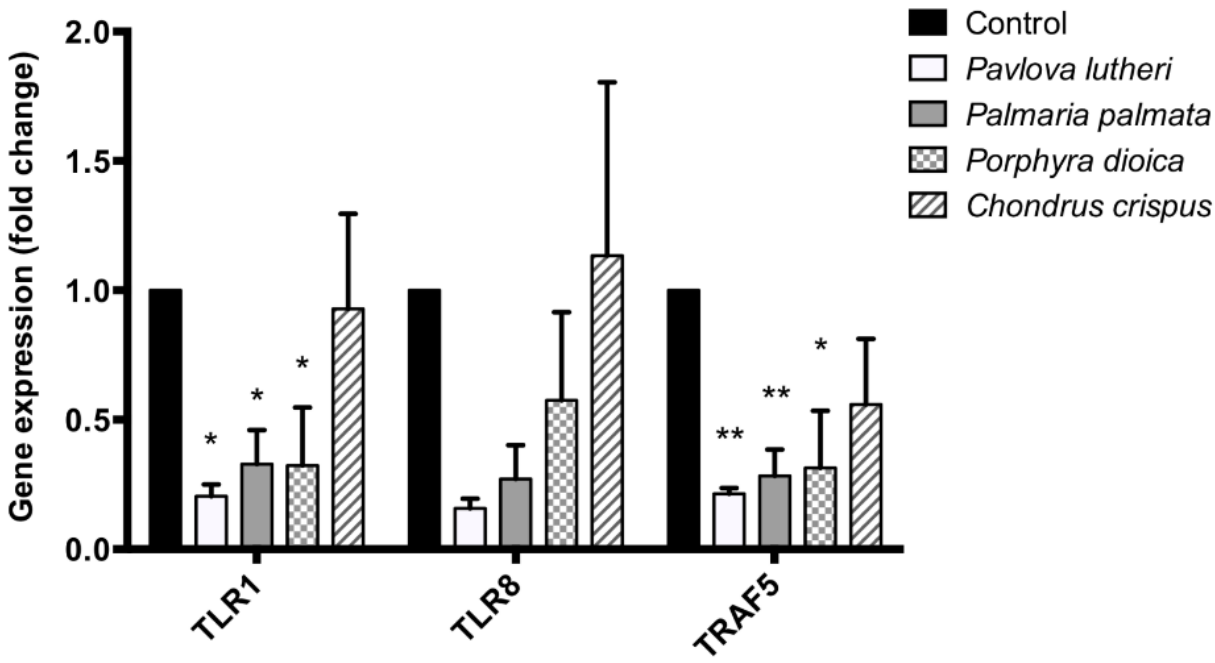


Figure 3. Validation of PCR array results through quantitative PCR. Effects of algal lipid extracts exposure on expression of *TLR1*, *TLR8*, and *TRAF5* genes in lipopolysaccharide (LPS)-stimulated THP-1 macrophages. * $p \leq 0.05$. ** $p \leq 0.01$.

Chapter 6

An assessment of the techno-functional and sensory properties of yoghurt fortified with an omega-3-rich lipid extract from the microalga *Pavlova lutheri*

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ABSTRACT

Marine microalgae are potential sources of functional bioactive compounds such as omega-3 polyunsaturated fatty acids (n−3 PUFA), pigments and minerals. In this study, plain yoghurt was manufactured with or without a food grade lipid extract of the microalgae species *Pavlova lutheri* (0.25% and 0.5% w/v). Techno-functional properties, fatty acid analysis and sensory characteristics were evaluated over 28 days storage to assess the suitability of yoghurt as a vehicle for the microalgal extract. Inclusion of the extract at 0.25% and 0.5% w/v had no significant impact on yoghurt composition, viscosity, pH, starter culture viability (for 21 days) or whey separation. Addition of the extract dose-dependently increased concentrations of total n−3 PUFA ($p < 0.0001$) in the yoghurt. Sensory analysis revealed that supplemented yoghurts were not well accepted by the panellists compared with the control yoghurt. These results suggest that techno-functional properties of yoghurt are unaffected following manufacture with a lipid extract of *P. lutheri*. In addition, incorporation of this extract into yoghurt is a suitable method to increase n−3 PUFA concentration, however further research is warranted to improve the perceived negative sensory attributes associated with the presence of the extract in yoghurt.

1. INTRODUCTION

One of the biggest changes in the modern 'Western diet' has been in the quantity and quality of fat consumption (Simopoulos, 1999). Despite much focus being placed on saturated fatty acid (SFA) consumption in recent times (Siri-Tarino et al., 2010), the dietary ratio of omega-6 (n-6) to omega-3 (n-3) polyunsaturated fatty acids (PUFA) has changed drastically. n-3 and n-6 PUFA are primary constituents of mammalian cell membranes and are essential for a number of structural and functional cellular mechanisms. Long chain n-3 PUFA (n-3 LC-PUFA) in particular, are essential for foetal neurodevelopment (Innis, 2008) and have also shown consistent cardioprotective (Adkins and Kelley, 2010), neuroprotective (Bazinet and Layé, 2014), anti-obesigenic (Buckley and Howe, 2009) and anti- inflammatory (Calder, 2006) properties. n-6 PUFA however, typically have opposing physiological effects to n-3 PUFA, particularly with regard to inflammation (Kaliannan et al., 2015), and increased consumption has been associated with a number of chronic inflammatory diseases (Patterson et al., 2012). Historically, dietary ratios of n-6/n-3 PUFA consumption were 1:1 however this has increased rapidly to approximately 15:1 in recent times in the western diet leading to relative n−3 deficiency (Simopoulos, 2002).

Oily fish such as salmon, mackerel and anchovies are potent sources of the n-3 PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and increased consumption has been associated with reduced risk of cardiovascular disease (Kromhout et al., 1985), certain cancers (Fernandez et al., 1999) and mental illness (Hibbeln, 1998). However declining global fish stocks and pollutant accumulation pose significant threats to n-3 PUFA consumption from fish sources (FAO Fisheries and Aquaculture Department, 2010). Algae, particularly marine microalgae, are also potent sources of n-3 PUFA and hence pose potential as novel alternative sources of EPA, DHA and other essential n-3 fatty acids (Adarme-Vega et al., 2012). EPA and DHA production from

microalgae can be optimized based on culture conditions (Guihéneuf and Stengel, 2013) and hence much research has now begun to examine the most suitable algal species for the industrial production of n−3 PUFA (Robertson et al., 2013).

n−3 PUFA production from microalgae has many advantages over other marine sources including selective fatty acid accumulation, sustainability, and increased bioavailability (Kagan et al., 2015). However, the sensory properties of marine n-3 PUFA-rich oils are limited by characteristic 'fishy-tastes' which are viewed negatively by the consumer (Van Elswyk, 1997). Fortification of carrier foods with marine n−3 PUFA-rich oils has the potential to mask some of the negative sensory properties of marine oils and may allow easier incorporation of n-3 PUFA into habitual diets (Lovegrove et al., 1997). Dairy products have previously proven successful carriers for bioactive nutraceuticals (Let et al., 2007). O′Sullivan et al. examined the potential for milk to act as a carrier for algal extracts (O'Sullivan et al., 2014). Yoghurt, in particular, has been widely studied as a vehicle for the delivery of functional ingredients including probiotics, prebiotics and antioxidants. Previously, yoghurt was proven a suitable carrier for fish oil to increase plasma n-3 PUFA status (McCowen et al., 2010). However, there are limited data on the incorporation of n−3 PUFA from algae into vehicle food products.

We previously reported the high n−3 PUFA and bioactive pigment contents in lipid extracts from a number of algal species and subsequently demonstrated the anti-inflammatory effects of these extracts in human THP-1 macrophages (Robertson et al., 2015). In this study, we investigate the potential for yoghurt to act as a carrier food for one of these extracts, from the microalgal species Pavlova lutheri, and assess fatty acid profile, techno-functional (pH, composition, starter culture viability, rheology, syneresis, colorimetry) and sensory properties following fortification.

2. MATERIALS AND METHODS

2.1. Microalgal strain, culture conditions, biomass preparations

An axenic strain of Haptophyte *P*. *lutheri* CCAP 931/6, formerly renamed *Diacronema lutheri* (Bendif et al., 2011), was obtained from the Culture Collection of Algae and Protozoa at the Scottish Marine Institute (SAMS Research Services Ltd., Oban, Scotland, UK) and cultivated in the laboratory at the National University of Ireland Galway.

P. *lutheri* was pre-cultivated under batch conditions on a modified F/ 2 medium (Guillard, 1975) in 15 L polycarbonate Nalgene® culture vessel at 15 °C and under continuous illumination (100–120 μmol photons $m^{-2} s^{-1}$), provided by lumilux cool daylight fluorescent lamps (OSRAM L18W/865, Germany). F/2 medium was composed and complemented as described previously (Guihéneuf and Stengel, 2013) with few modifications; i.e. using natural filtered seawater, an initial $NaNO_3$ concentration of 200 mg L^{-1} and no $NaHCO_3$ supplementation. The medium was sterilized by autoclaving for 20 min at 120 °C, and vitamins were filtered and added when the medium cooled to room temperature. Cultures were agitated by air-bubbling.

After 8 days of batch-cultivation in polycarbonate Nalgene® culture vessel, the 12 L of *P*. *lutheri* pre-culture was transferred to a custom-made 75 L low-cost flat panel photobioreactor (FP-PBR 120 × 95 × 7 cm, adapted and similar to one previously patented (Boussiba and Zarka, 2005)), containing 60 L of fresh F/2 medium supplemented with 1 g L^{-1} $NaHCO_3$. Growth conditions were similar to those used for pre-cultivation (i.e. continuous light of 100–120 μmol photons $m^{-2} s^{-1}$ and temperature of 15 °C). Light was provided on one side of the FP-PBR which consisted of a plastic bag placed between two frames. Compressed-air was bubbled at a rate of 50 L min^{-1} at the base of the panels through a perforated plastic tube, for mixing and gas

exchange. *P*. *lutheri* was cultivated using a semi-continuous mode during a total period of 60 days. 60 L of culture was harvested at 20 day intervals. After each harvesting event, the remaining culture was topped-up with 60 L of fresh F/2 medium. For each cultivation cycle, nitrate was depleted after approximately 16 days and kept for 4 additional days. This process allows the accumulation of lipid and triacylglycerols containing n-3 LC-PUFA triggered by nitrogen limitation and inorganic carbon ($NaHCO_3$) availability as described previously (Guihéneuf and Stengel, 2013). Microalgal cells were harvested by centrifugation using a modified Alistar milk cream separator 80 L h^{-1}, and the fresh algal paste collected were frozen at −20 °C and freeze-dried using Labconco Freezone® (Kansas City, MO, USA) 6 freeze-dryer system.

2.2. Lipid extraction

Total lipids were extracted by solid–liquid procedure using the microalgal food grade lipid extraction method as previously described (Ryckebosch, 2011). Briefly, 50 g of freeze-dried microalgal biomass was extracted with 250 mL of hexane/isopropanol (3:2, v/v) in a glass bottle closed under nitrogen gas (N_2) to limit oxidation, sonicated for 1 h and stirred overnight at 4 °C. The first extract obtained was filtered and the remaining biomass re-extracted 2 times similarly. All the filtered extracts were combined and the total crude lipid extract was evaporated to dryness using a rotavapor (BÜCHI R-210 equipped with a Heating Bath B-491 and a Vacuum Pump V700, BÜCHI Labortechnik AG, 9230 Flawil, Switzerland) under reduced pressure at 40 °C.

Since the food grade extract was not washed with water as in the traditional lipid extraction method of Bligh and Dyer (Bligh and Dyer, 1959) using a chloroform/methanol/water mixture, it may have contained not only lipids but also more polar compounds such as carbohydrates and proteins. To determine the lipid fraction of

the extract, the non-lipid polar components were eliminated by dissolving an aliquot of the extract in 8 mL of chloroform/methanol (1:1) and washed with 2 mL of water. The upper layer containing the non-lipid constituents was removed, while the bottom layer containing the lipids was dried under N_2 flow and weighed. The non-lipid part of the extract was estimated at approximately 8–10% of the total food grade lipid extract.

2.3. Pigment analysis

Pigment analysis was carried out by high performance liquid chromatography (HPLC) following the method by Wright et al. (Wright et al., 1991) with modifications by Bidigare et al (Bidigare et al., 2005) as previously described (Robertson et al., 2015).

2.4. Yoghurt manufacture

Yoghurt production and analyses were performed in triplicate batches. All techno-functional analyses were carried out at 7 day intervals. Prior to yoghurt production, P. lutheri extract was added to milk (Avonmore low fat (1.5%) milk, Glanbia Consumer Foods, Dublin Ireland) at 0.25% and 0.5% w/v and mixed for 5 min by mechanical homogenizing at 1000 rpm (Silverson L4RT, East Longmeadow, MA, USA). Control and enriched milk samples were then heated in a waterbath at 63 °C for 10 min before homogenization at 500 bar (APV 1000, SPX Flow, Crawley, West Sussex, UK) to aid dissolution of the algal extract. Milk samples were then heated in a waterbath at 93 °C for 15 min. Samples were subsequently cooled to 42 °C before addition of starter culture (YC-380 — 0.03% w/v, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, Chr. Hansen, Denmark) as per manufacturer's instructions. Samples were placed in an incubator at 42 °C and agitated for 15 min to achieve a homogenous culture. Incubation was continued at 42 °C until a pH of 4.3 was reached. Yoghurt samples (100 g) were stirred and packed aseptically into sterile containers and stored for

up to 28 days at 4 °C.

2.5. Compositional analysis

Yoghurt pH was measured every 7 days using a standard pH meter (Mettler Toledo MP220; British Standards Institution, 1976). Moisture and fat contents were assessed using the SMART Trac rapid moisture/fat analyser as per manufacturer's protocol (CEM Corporation, North Carolina, USA). Ash content was assessed using a muffle furnace by heating the samples at 550 °C for 12 h according to the method of the Association of Official Analytical Chemists (AOAC, 1995). Protein nitrogen content was assessed by Kjeldahl method (International Dairy Federation, 2001) and values were multiplied by 6.38 to obtain total protein values. Carbohydrate content was calculated by difference.

2.6. Bacterial enumeration

Enumeration of starter cultures was carried out in duplicate via standard bacterial culture. Briefly, 10 g of yoghurt was added to 90 mL sterile maximum recovery diluent (MRD, Oxoid, Fannin Healthcare, Dublin, Ireland) in a sterile stomacher bag and stomached for 30 s (Stomacher, Lab-Blender 400, Seward, Thetford, Norfolk, UK). Ten-fold serial dilutions of the diluted yoghurt were then performed in sterile MRD. Streptococcus thermophilus was cultured and enumerated on M-17 agar (Difco laboratories, Detroit MI, USA) supplemented with 0.5% sterile lactose solution (Oxoid, Fannin Healthcare, Dublin, Ireland) under aerobic conditions at 42 °C. L. delbrueckii subsp. bulgaricus (L.bulgaricus) was cultured and enumerated on MRS agar (Difco) under anaerobic conditions at 37 °C. Results are expressed as $\log_{10}$CFU (colony forming units)/g yoghurt.

2.7. Syneresis

The extent of syneresis/whey separation was examined in duplicate according to Keogh and O′Kennedy (Keogh and O'Kennedy, 1998). Briefly, 20 g yoghurt samples were centrifuged at 3000 g for 10 min at 4 °C. Supernatants were removed, weighed and expressed as a percentage of original yoghurt weight.

2.8. Rheology

Rheology was carried out using an AR-2000ex rheometer (TA Instruments, Crawley, UK) using parallel plate geometry (60 mm) at 20 °C according to the method of Kearney et al (Kearney et al., 2011). Prior to rheological analysis, samples were stirred 20 times. Briefly, a pre-shear was carried out at 200 s^{-1} for 1 min followed by equilibration for 1 min. The sample was then sheared from 0.01 to 200 s^{-1}, held at 200 s^{-1} for 1 min, then sheared from 200 to 0.01 s^{-1} over 2 min. Results are expressed as mean mPa values at 200 s^{-1} shear rate.

2.9. Colorimetry

Colorimetry was carried out using a standard colorimeter in duplicate (Minolta Chroma-Meter CR-400, Mason Technology Ltd., Dublin 8, Ireland). Calibration was carried out using a white tile (C: Y = 93.6, x = 0.3130, y = 0.3193). Yoghurt samples (2 mL) were placed in transparent cuvettes, colour was measured and Yxy values were converted under the L*a*b* colour space, where ‘L*’ represents lightness, ‘a*’ represents greenness and ‘b*’ represents yellowness.

2.10. Fatty acid analysis

Transmethylation of P. lutheri extract fatty acids and analysis by gas chromatography

was carried out as previously described (Robertson et al., 2015).

Fatty acid analysis of yoghurt was performed as follows: Lipids were extracted from 2 g yoghurt samples using 2:1 v/v chloroform:methanol. Methylation was subsequently carried out by the addition of 10 mL 0.5 N NaOH and by heating at 90 °C for 10 min, followed by 10 mL 14% BF_3 in methanol and further heating at 90 °C for 10 min (Park and Goins, 1994). Fatty acid methyl esters (FAMEs) were recovered in hexane and dried over anhydrous sodium sulphate for 1 h followed by storage at −20 °C until analysis.

Fatty acid analysis was performed by gas chromatography-flame ionization detection (GC-FID). FAMEs were separated using GC (Agilent 7890B, Agilent Technologies, Santa Clara, CA, USA) fitted with a flame ionization detector, using a Chrompack CP Sil 88 column (Chrompack, JVA Analytical, 100 m × 0.25 mm internal diameter, 0.25 μm film thickness) and helium as the carrier gas. The column oven was held at 80 °C for 8 min and increased by 8.5 °C/min to a final column temperature of 200 °C. Automatic sample injection (CTC Autosampler, Model 80) was performed on a Multimode Inlet on-column temperature programmable injector, with an injection volume of 0.5 μl. Peaks were integrated using the Agilent OpenLAB Chemstation software (Version A.01.06.111). Identification of peaks was carried out by comparison of retention times with pure FAME standards (Nu-Chek Prep, Elysian, MN, USA).

2.11. Sensory analysis

Sensory analysis was performed in the University College Cork Food Sensory Laboratory on day 7 (n = 20 assessors) and day 28 (n = 14 assessors) using naïve untrained assessors (aged 20 to 50 yr). Both hedonic (colour, flavour, texture, acceptability) and intensity (thickness, odour, off-odour, sour taste, yoghurt flavour, off-flavour, fishy taste) descriptors were assessed.

Analysis was performed in panel booths conforming to international standards (ISO 8589:2007). Coded yoghurt samples were presented to assessors in random order to prevent first order and flavour carryover effects. Water was provided to assessors to cleanse their palate in between tastings. Assessors were provided with a 10 cm line scale and required to indicate degree of liking on the scale from 0 (extremely dislike) to 10 (extremely like).

2.12. Statistics

Data represent results from triplicate trials. Statistical analysis was carried out using GraphPad Prism (6.0, San Diego, CA, USA). Results were subject to one-way or two-way ANOVA (analyses of variances) followed by Tukey's multiple comparison tests. Sensory data were subject to ANOVA-Partial Least Squares Regression (APLSR) using Unscrambler Software, version 9.8 (CAMO ASA, Trondheim, Norway). Statistical significance for the relationships determined in the quantitative APLSR was derived by analysis of jack-knifing of regression coefficients, which is based on cross-validation and stability plots (Martens and Martens, 1999, Martens and Martens, 2001). Results are expressed as mean ± standard error of the mean (SEM). Results were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Extract fatty acid and pigment profile

Table 1 shows the fatty acid profile of the food grade *P*. *lutheri* extract prior to inclusion in yoghurt. As reported previously (Guihéneuf et al., 2010, Robertson et al., 2015), *P*. *lutheri* is a potent producer of n−3 PUFA, particularly EPA, DHA and relatively large amounts of stearidonic acid (SDA, 18:4 n−3). Here, these results demonstrate that more than 50% of fatty acids within the extract are n−3 PUFA, with EPA being the most abundant fatty acid in the extract at 9g/100g crude extract. SDA and DHA make up 2.2% and 3.5% of the extract respectively. In addition, n−6 PUFA content within the extract is relatively low, at 1.5%, which contributes to a low n−6/n−3 ratio of 0.1. These data demonstrate that *P*. *lutheri* lipid extracts are good sources of n-3 PUFA and hence offer potential as functional nutraceuticals. More importantly, these results highlight that food grade lipid extraction using hexane/isopropanol achieves similar fatty profiles compared to conventional extractions using other organic solvents (Robertson et al., 2015). In addition to n−3 PUFA, HPLC analysis identified 11% of the *P*. *lutheri* extract to be composed of pigments including fucoxanthin (26% of total pigments), chlorophyll a (28%) and β-carotene (13%), similar to previous reports (Robertson et al., 2015), and as outlined in Table 2. Fucoxanthin, chlorophyll a and β-carotene have previously demonstrated bioactive functionalities, especially with regard to resolving inflammation (Peng et al., 2011, Bai et al., 2005) and hence offer additional functional benefits to n-3 PUFA. Indeed, it is possible that the extract contains a number of additional unidentified lipid soluble compounds not identified here including carotenoids, proteins and sterols The lipid partitioning profile of this extract, however, has previously been defined (Robertson et al., 2015).

3.2. Yoghurt composition

Table 3 shows yoghurt composition on day 7 following incorporation of the *P. lutheri* extract at 0.25% or 0.5%. Moisture, carbohydrate, protein fat and ash contents were not significantly altered by the addition of the extract at 0.25% or 0.5%. In addition, composition of the supplemented yoghurts was similar to those previously reported following addition of an algal oil to yoghurt (Chee et al., 2005). Despite the incorporation of lipid extracts into the fortified yoghurts, fat content did not significantly differ between any of the yoghurts. However, a trend was observed for increasing fat content from the control (1.5% fat) to the 0.25% supplemented (1.56% fat) to the 0.5% supplemented (1.64% fat) yoghurts. Due to the crude method of extraction from the algal biomass, it is possible that a substantial proportion of the algal extract was non-lipid, suggesting that addition of 0.25% extract into the yoghurt may not be reflected by a 0.25% increase in total fat content. In addition, slight variations in lipid content between batches of commercial milk may have contributed to the lack of significance in total lipid content between yoghurts. However, low-fat milk was used for the yoghurt production suggesting that, despite the addition of the lipid extracts, each yoghurt fell within the 'low-fat' (<3g fat/100 g solids) and 'low sugar' (<5g sugars/100 g solids) categories of the European Food Safety Authorities (EFSA) health claims (EFSA, 2006). Overall, addition of the algal extract did not negatively impact the macronutrient composition of yoghurt.

3.3. pH and starter culture viability

Fig. 1a displays pH content of the yoghurts following production over 28 days of storage. Addition of the extract at either concentration did not significantly alter the pH of the yoghurt at any of the time points. At day 1, pH of the control, 0.25% and 0.5% yoghurts were 4.07, 4.05 and 4.1 respectively, which declined to 3.99, 3.97 and 3.99 by

day 28. The low pH of yoghurt is dependent on the growth of starter culture lactic acid bacteria (LAB). LAB produce an acidic environment which contributes to yoghurt flavour and can inhibit the growth of spoilage microorganisms and hence contribute to the long shelf-life of yoghurt (Lindgren and Dobrogosz, 1990). A commercial starter culture mix (Hansen YC-380) was used for yoghurt production, containing strains of LAB species *L. bulgaricus* and *S. thermophilus*. Mean viable counts of *L. bulgaricus* were between 10^6 and 10^7 cfu·mL^{-1} in each of the yoghurts on day one, as outlined in Fig. 1b. Viable counts were not significantly different between the yoghurts at any of the time points, staying at ~ 10^6 cfu·mL^{-1} until day 28, at which point *L. bulgaricus* in the 0.5% supplemented yoghurt dropped to ~ 10^4 cfu·mL^{-1}. Viable counts of *S. thermophilus* were ~10^{10} cfu·mL^{-1} in each of the yoghurts at day 1, which stayed constant in the control yoghurt between ~10^9 and 10^{10} cfu·mL^{-1} over 28 days, as seen in Fig. 1c. A non-significant decline in viable *S. thermophilus* counts was observed in both supplemented yoghurts from day 7 onward in comparison to the control yoghurt. Viable counts were between 10^7 and 10^8 cfu.ml^{-1} in the supplemented yoghurts between day 7 and day 21, until day 28, at which point *S. thermophilus* declined significantly in both supplemented yoghurts compared with the control yoghurt.

The incorporation of microalgae into fermented milk products has been examined previously (Beheshtipour et al., 2013). Beheshtipour et al. examined the addition of *Chlorella vulgaris* and *Arthrospira platensis* into yoghurt and reported that addition of the microalgae significantly increased growth of starter culture LAB compared to control during fermentation, which the authors suggest may be attributed to the array of amino acids, proteins and vitamins within the algal biomass (Beheshtipour et al., 2012). This is similar to the data reported in the present study, in which *L. bulgaricus* displayed a non-significant increase in viable counts in the 0.5% yoghurt

compared to control on day 1. The results reported here, however, demonstrate a trend towards reduced *S. thermophilus* growth over 28 days storage in contrast to previous data for microalgal fortification. The nature and species of the extract used in this study may explain these differences. Algal extracts have previously demonstrated antimicrobial activity (Lordan et al., 2011). In addition, pure DHA and alpha linolenic acid (ALA) have been shown to inhibit Lactobacillus growth and adhesion (Kankaanpää et al., 2001). The addition of fish oil to yoghurt has also been reported to decrease LAB counts over a 28 day period (Estrada et al., 2011). Hence the crude lipid composition of these extracts may have contributed to the non-significant decrease in starter culture viability over 21 days as previously shown for other marine oils (Estrada et al., 2011). The observed decline in *S. thermophilus* growth in the supplemented yoghurt was statistically significant at day 28 which has been observed previously in both marine oil supplemented and control yoghurts (Estrada et al., 2011). Hence fortification of yoghurt with the extract at either 0.25% or 0.5% for 21 days did not negatively affect starter culture viability in comparison with control.

3.4. Syneresis

There were no observed differences in rate of syneresis between control and supplemented yoghurts over 28 days of storage, as shown in Fig. 2. Syneresis values were 62.8%, 65.3%, and 64.1% for control, 0.25% and 0.5% for yoghurts respectively on day 1 and trended towards a non-significant increase to 68.2%, 66.3% and 66.1% respectively on day 28. Mean increase in syneresis value from day 1 to day 28 was 5.4%, 1% and 2% for the control, 0.25% and 0.5% supplemented yoghurts respectively. Syneresis involves spontaneous rearrangement of the yoghurt gel network resulting in separation of whey. Reduced syneresis is associated with more desirable sensory traits in yoghurt (Kearney et al., 2011). The results presented here, exhibiting no differences

between control and fortified yoghurts, are comparable to previous reports for marine oil-supplemented yoghurts (Estrada et al., 2011). As was observed with the composition and starter culture viability, fortification of the yoghurt with 0.25% or 0.5% *P. lutheri* lipid extract did not negatively affect the separation of whey in the yoghurt. Moreover, a non- significant trend was observed between day 1 and day 28 in which the increase in syneresis was less apparent in the 0.25% supplemented (1% increase) and 0.5% supplemented (2% increase) yoghurts in comparison with the control yoghurt (5.4% increase in syneresis), suggesting that incorporation of the extract may reduce the rate of syneresis after 28 days. These data are comparable to previous reports in which n−3 rich oil extracts improved (Dal Bello et al., 2015, Tamjidi et al., 2012) or did not negatively affect yoghurt syneresis (Estrada et al., 2011).

3.5. Rheology

Fig. 3 demonstrated there were no significant differences in viscosity between yoghurts at any of the time points over 28 days as measured by resistance to a stress of 200 s^{-1} shear rate. The control and 0.25% yoghurt had viscosity values of 57 (±11) and 65 (±10) mPa·s respectively on day 1 which declined to 49 (±12) and 47 (±8) mPa·s by day 28. The 0.5% supplemented yoghurt exhibited viscosity of 43 (±5) on day 1, which increased slightly to 49 (±4) by day 28. Rheological properties of yoghurt are dependent on a number of factors including species of starter inoculation, concentration of starter inoculation, handling and storage. Yoghurt exhibits pseudoplastic fluid properties and hence increased viscosity may prevent textural and structural damage to the yoghurt gel matrix. In addition, increased viscosity has been associated with improved sensory properties, and previous investigations have examined the use of exopolysaccharide and B-glucan producing bacteria as techniques to increase yoghurt viscosity (London et al., 2015). Xanthan gum is also used as an additive in industrial yoghurt production to

increase viscosity. Previous reports in fish oil-enriched yoghurt have suggested marine oil enrichment reduces viscosity over 21 days of storage (Tamjidi et al., 2012). Hence, these results are promising, suggesting that this algal extract does not interfere with textural properties of the yoghurt and hence does not impact negatively on viscosity as previously reported for other n−3 PUFA sources.

3.6. Colour

Fig. 4 outlines colorimetric measurements for each of the yoghurts under the L*a*b* colour space during the 28 days storage. Colorimetric measurements remained relatively stable over 28 days storage in all three yoghurts, suggesting storage did not alter colour in each of the individual yoghurts. There were significant differences at all time points between the control yoghurt and supplemented yoghurts. The control yoghurt demonstrated similar values for 'L*' (Lightness) (Fig. 4a) and '-a*' (Greenness) (Fig. 4b) and 'b*' (Yellowness) (Fig. 4c) as reported previously for yoghurt and milk (Hashim et al., 2009, O'Sullivan et al., 2014) with values remaining at ~ 80, ~ − 3, and ~ 5 respectively for the three colour measurements over the 28 day storage period. Both 0.25% and 0.5% supplemented yoghurts, however, displayed significantly lower 'L*' values ($p < 0.0001$) and significantly higher '–a*' ($p < 0.0001$) and 'b*' ($p < 0.0001$) values at each of the time points compared with control. Differences between the control and 0.5% supplemented yoghurt were more pronounced for both 'L*' and '-a*' values compared with the 0.25% yoghurt, reflecting the contribution of the higher concentration of the extract on the extent of surface colour intensity. In addition, there was a significant difference in 'b*' value ($p < 0.05$) between 0.25% and 0.5% supplemented yoghurts at day 14.

The reduced 'lightness' and increased 'Greenness' and 'Yellowness' measurements in the supplemented yoghurts are likely attributed to the carotenoids and

other pigments in the extract including chlorophyll a, β-carotene, fucoxanthin, diadinoxanthin and diatoxanthin (Robertson et al., 2015). O′Sullivan et al. previously demonstrated similar changes in Greenness and Yellowness values following enrichment of milk with seaweed extracts (O'Sullivan et al., 2014). Results from sensory analysis (Fig. 5) suggest that the significant changes in colour are associated negatively with sensory properties. Removal of pigments in the extract through refined extraction procedures may reduce such dramatic changes in colour and hence improve sensory properties, however it is likely that such pigments contribute to the previously observed bioactivity of the extract (Robertson et al., 2015). The influence of the extract on the appearance of the yoghurts is shown in Fig. 4d.

3.7. Fatty acid analysis

GC analysis revealed a number of significant differences between the yoghurts with respect to fatty acid profiles as reported in Table 4. As outlined in Table 1, the *P. lutheri* extract was primarily composed of n−3 PUFA, hence it was hypothesised that incorporation of the extract into the yoghurt during fermentation would enhance n−3 PUFA concentration of the yoghurt. In the control yoghurt, SFA made up 62.7% of the total observed fatty acids whereas these values were lower in the 0.25% and 0.5% supplemented yoghurts at 59.4% and 57.2% respectively. In particular, stearic acid (18:0) was present in significantly lower concentrations in both the 0.25% supplemented ($p < 0.01$) and 0.5% supplemented ($p < 0.01$) yoghurts. Palmitic acid (16:0) ($p < 0.01$) and arachidic acid (20:0) ($p < 0.05$) were also significantly lower in the 0.5% supplemented yoghurt compared with control.

Addition of fish oil to yoghurt has previously been shown to reduce concentrations of SFA (Estrada et al., 2011), similar to the findings presented here for an algal oil. Higher n-3 PUFA intake in place of SFA has been associated with beneficial

cardio-metabolic outcomes (Micha and Mozaffarian, 2010) and hence foods with reduced SFA may be beneficial for consumer preference. There was a trend towards a reduction in total monounsaturated fatty acids (MUFA) in the supplemented yoghurts, which was primarily attributed to significantly reduced proportion of oleic acid (18:1c n−9) in both the 0.25% ($p < 0.05$) and 0.5% ($p < 0.01$) yoghurts. However a small but significant increase was seen in palmitoleic acid (16:1 n−7) in the supplemented yoghurts, which was likely attributed to the relatively high concentration of this fatty acid within the extract (Table 1).

There were few small changes in concentrations of n-6 PUFA within the yoghurts. An increase in total PUFA in the supplemented yoghurts however, was primarily driven by significantly increased levels of a number of n−3 fatty acids. Despite being present in relatively low concentrations in the *P. lutheri* extract, ALA was significantly increased in both the 0.25% ($p < 0.05$) and 0.5% ($p < 0.01$) supplemented yoghurts. ALA has demonstrated anti-inflammatory activity in addition to providing physiological health benefits through its conversion to EPA and DHA (Simopoulos, 1999). SDA, EPA, DHA and total n−3 PUFA were all dose dependently increased in the yoghurt following supplementation of the extract ($p < 0.0001$). ALA was the only n−3 PUFA detected in the control yoghurt, whereas SDA, EPA and DHA were not present in detectable levels. EPA, the most abundant fatty acid within the extract, increased to 3.1% of total fatty acids in the 0.25% supplemented yoghurt ($p < 0.0001$) and to 4.8% in the 0.5% supplemented yoghurt ($p < 0.0001$). In addition to the increases in SDA and DHA, this led to a respective seven-fold ($p < 0.0001$) and ten-fold ($p < 0.0001$) increase in concentration of total n−3 PUFA in the 0.25% and 0.5% supplemented yoghurts compared to the control. Moreover, the increase in n−3 PUFA led the n6/n3 ratio of the yoghurt to decline from 2.2 in the control to 0.3 and 0.2 in the 0.25% and 0.5% yoghurts, respectively.

Yoghurt has previously been assessed as a carrier food for n-3 PUFA-rich oils (Dal Bello et al., 2015, Chee et al., 2005). The global decline in n−3 PUFA intake has led to research examining foods to act as vehicles for n-3 PUFA for incorporation into habitual diet. n-3 PUFA-supplemented yoghurt has been demonstrated as a suitable method to increase plasma DHA and reduce plasma AA in human subjects (McCowen et al., 2010). In this study, a crude algal lipid extract significantly increased concentrations of DHA and EPA in yoghurt in addition to increases in other fatty acids such as SDA, which has previously demonstrated cardio-protective and anti-tumorigenic activity (Whelan, 2009). These data are the first to report details of a yoghurt enriched with an n-3 PUFA-rich lipid extract from *P. lutheri*. The results thus suggest that this algal extract is a suitable method to enhance n-3 PUFA content of yoghurt and hence deliver a wide range of essential n−3 PUFA and bioactive pigments to the consumer. It is necessary to note indeed that yoghurt contains a number of other shorter chain fatty acids other than those reported here, particularly caproic (6:0), caprylic (8:0) and capric (10:0) acids. However, it was not within the scope of this study to examine these fatty acids, in addition no changes were observed to these fatty acids previously, following addition of an n-3 PUFA-rich oil to yoghurt (Estrada et al., 2011).

3.8. Sensory analysis

Hedonic and descriptive sensory analysis from naïve untrained assessors on days 7 and 28 revealed a number of significant differences between the yoghurts as outlined in Fig. 5 and Table S1. Significance of regression correlations are outlined in Table S1 as positive or negative values. In general, positive values indicate preferable, and negative scores indicate non-preferable scores for each of the sensory attributes. Analysis was carried out at two time points to assess whether storage altered sensory attributes of each of the yoghurts.

Hedonic analysis revealed that supplementation of the yoghurt at both concentrations was negatively correlated with all metrics ('colour', 'liking of flavour', 'liking of texture' and 'overall acceptability') on day 7 ($p < 0.001$) and day 28. Negative correlations with scores for 'colour' correlate with the colorimetric surface colour measurements of the yoghurt outlined in Fig. 5c. Higher '-a*' (Greenness) and 'b*' (Yellowness) values likely contributed to the negative scores in the hedonic sensory analysis. Tamjidi et al. found similar results in which fish oil supplemented yoghurts scored lower for flavour and overall acceptance (Tamjidi et al., 2012) and O′Sullivan et al. also reported negative correlations for sensory, colour and overall acceptability scores in milk enriched with algal extracts (O'Sullivan et al., 2014).

Descriptive sensory analysis found no significant differences between yoghurts for 'odour' or 'sour taste' on day 7 or day 28. 'Thickness' was significantly negatively correlated with both supplemented yoghurts on day 7 ($p < 0.05$) however not on day 28. Results from viscosity measurements (Fig. 3) showed non-significant differences at day 7 but not at day 28 and hence may reflect the data seen here in the sensory analysis. 'Yoghurt flavour' was also negatively correlated for both supplemented yoghurts at both time points. 'Fishy taste' has previously been negatively correlated with both supplemented yoghurts and contributed greatly to the separation of correlation loadings as seen in Fig. 5c. 'Fishy taste' has previously shown negative correlation with sensory attributes in yoghurt enriched with algal oil (Chee et al., 2005) and in milk enriched with seaweed extracts (O'Sullivan et al., 2014), and hence poses sensory issues for enrichment of carrier foods with marine nutraceuticals. Indeed, methods such as microencapsulation or the addition of flavourings may help improve sensory attributes of marine oil enriched foods (Estrada et al., 2011).

4. CONCLUSION

The novel data reported in this study reveal that yoghurt is a suitable food to act as a carrier for an n-3 PUFA-rich lipid extract from *P*. *lutheri*. In general, addition of the extract did not affect overall quality measures of the yoghurt. Moreover, addition of the extract to the yoghurt significantly increased concentrations of n-3 PUFA in the yoghurt. Incorporation of n−3 PUFA into vehicle foods may be an effective strategy to enhance tissue n-3 PUFA status and hence improve inflammatory and metabolic clinical outcomes. The *P*. *lutheri* lipid extract used in the present study has previously exhibited high bioactive pigment content, high n-3 PUFA content and potent anti-inflammatory activity in vitro. Therefore, this extract, as an active ingredient in yoghurt, may exhibit potential as an anti-inflammatory functional food and confer a health benefit following consumption, however further research in warranted to address this in vivo. Indeed, for commercial production of such a food, more extensive sanitation and purification of the extract may be required. Further research is warranted to assess how such purification procedures may influence the composition and bioactivity of the extract. The limitations to this, from a consumer perspective however, can be attributed to negative correlations with sensory scores. Colorimetry and sensory analysis indicated that supplementation of the extract into the yoghurt contributed to poor acceptability. However, due to the minimal impact that the extract had on other functional properties of the yoghurt, further research into methods to improve sensory quality may pose potential for such algal extracts to become commercial nutraceuticals in yoghurt or other carrier foods and act as novel sources of n−3 PUFA.

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TABLES

Table 1. Fatty acid composition of a food-grade lipid extract of *P. lutheri*

Fatty acids	***P. lutheri* extract**
Saturated fatty acids (SFA)	
14:0	3.21 ± 0.19
16:0	2.99 ± 0.35
18:0	0.10 ± 0.01
Total SFA	6.30 ± 0.53
Monounsaturated fatty acids (MUFA)	
14:1	0.26 ± 0.04
15:1	0.20 ± 0.02
16:1c n-7	4.07 ± 0.49
18:1t n-9	0.57 ± 0.08
18:1t n-7	0.17 ± 0.01
Total MUFA	5.27 ± 0.63
Polyunsaturated fatty acids (PUFA)	
n-6 PUFA	
16:2 n-6	0.34 ± 0.04
18:2 n-6	0.32 ± 0.04
18:3 n-6	0.46 ± 0.06
20:4 n-6	0.40 ± 0.32
Total n-6 PUFA	1.52 ± 0.39
n-3 PUFA	
16:4 n-3	0.36 ± 0.05
18:3 n-3	0.56 ± 0.08
18:4 n-3	2.21 ± 0.34
20:5 n-3	8.90 ± 1.40
22:5 n-3	0.33 ± 0.09
22:6 n-3	3.48 ± 0.66
Total n-3 PUFA	15.85 ± 2.58
Other PUFA	1.77 ± 0.20
Total PUFA	19.15 ± 3.13
n6/n3	0.10 ± 0.01

Data are expressed as g/100g of crude extract and are presented as mean values ± standard deviation (SD)

Table 2. Pigment composition of a food-grade lipid extract of *P. lutheri*

Pigments (% Molar)	***P. lutheri* extract**
diadinoxanthin + diatoxanthin + deg.	2.82 ± 0.01
chlorophyll a + deg.	4.00 ± 0.42
fucoxanthin + *cys*-fucoxanthin + deg.	2.75 ± 0.08
β-carotene	1.10 ± 0.01
chlorophyll c + deg.	0.30 ± 0.01
Total Pigments	10.96 ± 0.35

Data are expressed as g/100g of crude extract and are presented as mean values ± standard deviation (SD); deg., degradation products.

Table 3. Mean composition of supplemented and control yoghurts

	Composition %				
	Moisture	**Carbohydrate**	**Protein**	**Fat**	**Ash**
Control	89.7±0.1	4.7±0.2	3.4±0.1	1.5±0.1	0.7±0.1
0.25%	89.6±0.1	4.8±0.1	3.3±0.1	1.56±0.1	0.7±0.1
0.5%	89.5±0.1	5.0±0.2	3.2±0.1	1.64±0.1	0.7±0.1

Data are expressed as mean values of triplicate trials ± standard error of the mean (SEM).

Table 4. Fatty acid profiles of supplemented and control yoghurts

	Yoghurt FA profile		
g/100g total fatty acids	**Control**	**0.25%**	**0.5%**
Saturated fatty acids (SFA)			
14:0	12.6 ± 0.3	12.6 ± 0.3	12 ± 0.3
16:0	34 ± 0.7	32.3 ± 0.5	30.8 ± 0.7 *
18:0	15.8 ± 0.2	14.4 ± 0.3 *	14.3 ± 0.2 *
20:0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1 *
Total SFA	62.7	59.4	57.2
Monounsaturated fatty acids (MUFA)			
16:1c n-7	1.6 ± 0.1	2.7 ± 0.1 *	3.3 ± 0.1 *#
18:1t n-9	5.9 ± 0.4	5.3 ± 0.4	4.9 ± 0.2
18:1c n-9	26.3 ± 0.4	24.2 ± 0.4 *	23.5 ± 0.4 *
18:1 t n-7	0.6 ± 0.1	0.7 ± 0.1 *	0.8 ± 0.1 *#
Total MUFA	34.9	33.4	32.7
Polyunsaturated fatty acids (PUFA)			
n-6 PUFA			
18:2 n-6	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
18:3 n-6	0.5 ± 0.1	0.2 ± 0.1 *	0.3 ± 0.1 *
20:4 n-6	*n.d.*	0.2 ± 0.1 *	0.2 ± 0.1 *
Total n-6 PUFA	1.7 ± 0.1	1.5 ± 0.1	1.7 ± 0.1
n-3 PUFA			
18:3 n-3	0.8 ± 0.1	0.9 ± 0.1 *	0.9 ± 0.1 *
C18:4 n-3	*n.d.*	0.5 ± 0.1 *	0.8 ± 0.1 *#
20:5 n-3	*n.d.*	3.1 ± 0.3 *	4.8 ± 0.1 *#
22:6 n-3	*n.d.*	1.2 ± 0.1 *	2 ± 0.1 *#
Total n-3 PUFA	0.8 ± 0.1	5.7 ± 0.4 *	8.4 ± 0.1 *#
Total PUFA	2.5	6.7	10.1
n6:n3	2.2	0.3	0.2

Data expressed as g/100g of total fatty acid methyl esters (FAME) and are presented as mean values of triplicate trials ± standard error of the mean (SEM).
* significantly different vs. control yoghurt ($p<0.05$)
# significantly different vs. 0.25% yoghurt ($p<0.05$)

FIGURES

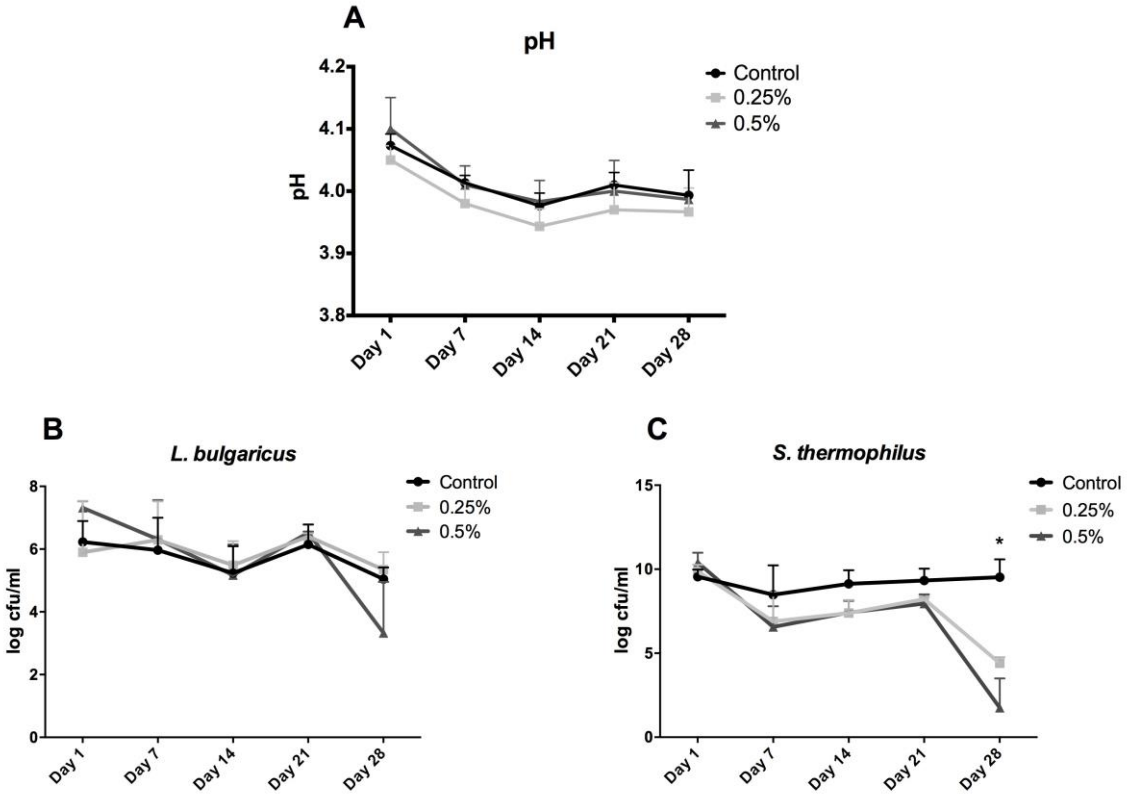


Figure 1. pH values and starter culture counts. (A) pH values of yoghurts over the storage period. (B) *L*. *bulgaricus* survival over the storage period. (C) *S*. *thermophilus* survival over the storage period. Data are expressed as means of triplicate trials. Control yoghurt, black line and circles; 0.25% supplemented yoghurt, light grey line and squares; 0.5% supplemented yoghurt, dark grey line and triangles. *$p<0.05$ control vs 0.25% and 0.5% yoghurts.

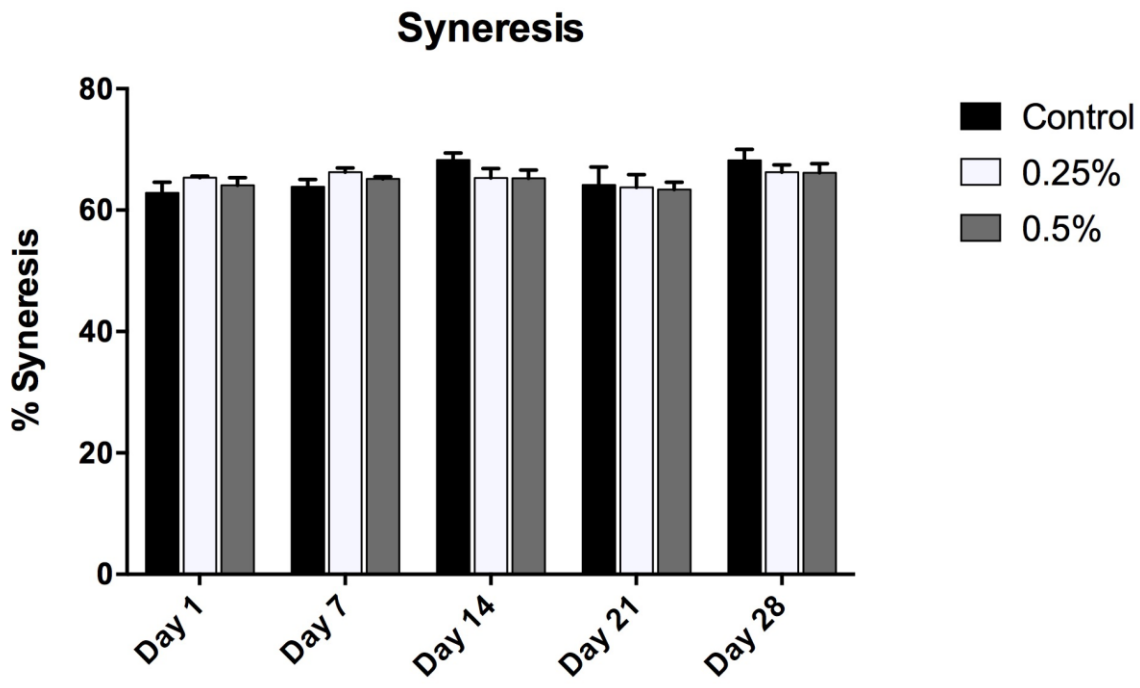


Figure 2. Syneresis values of the yoghurts. Data are expressed as means of whey separation values ± standard error of the mean (SEM) of triplicate trials. Control, black bars; 0.25% supplemented yoghurt, white bars; 0.5% supplemented yoghurt, grey bars.

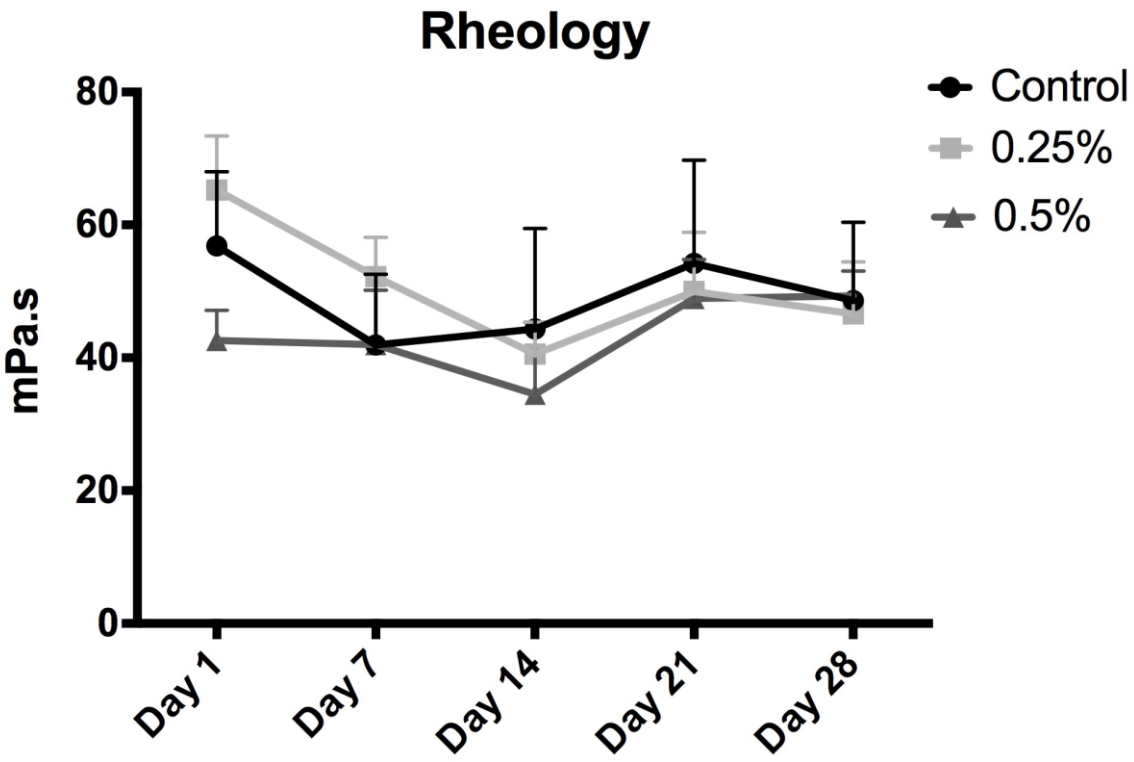


Figure 3. Rheology of stirred yoghurts over the storage period. Data are expressed as mean mPa.s of triplicate trials. Control yoghurt, black line and circles; 0.25% supplemented yoghurt, light grey line and squares; 0.5% supplemented yoghurt, dark grey line and triangles.

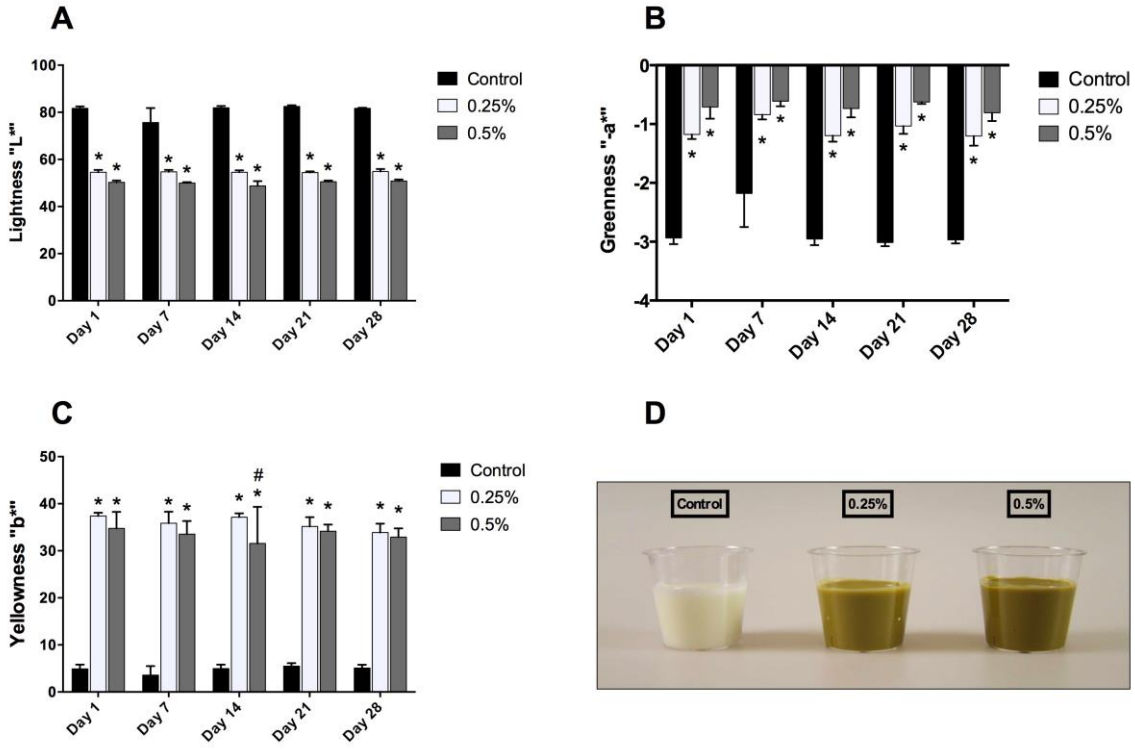


Figure 4. Colorimetry of the yoghurts over the storage period. (A) 'L*' Lightness values of the yoghurts over the storage period. (B) '-a*' Greenness values of the yoghurts over the storage period. (A) 'b*' Yellowness values of the yoghurts over the storage period. Control, black bars; 0.25% supplemented yoghurt, white bars; 0.5% supplemented yoghurt, grey bars. Values are means ± standard error of the mean (SEM) of triplicate trials. * significantly different vs. control yoghurt ($p<0.05$). # significantly different vs. 0.25% yoghurt ($p<0.05$). (D) Image of the control, 0.25% and 0.5% supplemented yoghurts.

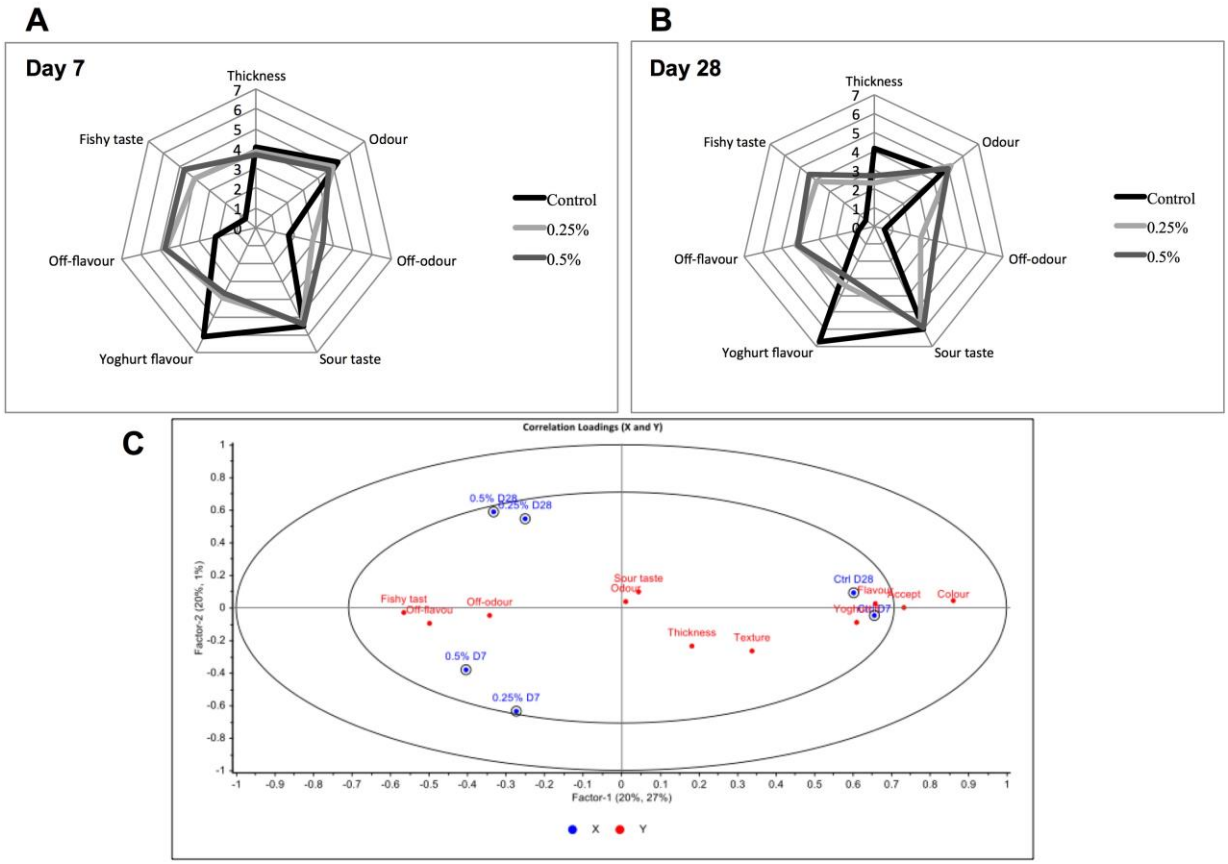


Figure 5. Sensory analysis of the yoghurts at day 7 and day 28 of storage. (A) Intensity sensory values of the yoghurts at day 7. (B) Intensity sensory values of the yoghurts at day 28. Control yoghurt, black line; 0.25% supplemented yoghurt, light grey line; 0.5% supplemented yoghurt, dark grey line. (C) APLSR plot for the yoghurts. Circles represent sensory descriptors; Circles with outer rings represent yoghurt samples.

SUPPLEMENTARY DATA

Table S1. Significance of regression coefficients (ANOVA values) for hedonic and intensity sensory analysis of yoghurt as derived by jack-knife uncertainty testing

Descriptor	**Storage Time, days / Treatment**					
	Control		**0.25%**		**0.5%**	
Hedonic	**7**	**28**	**7**	**28**	**7**	**28**
Colour	0.000***	0.000***	-0.000***	-0.001**	-0.000***	-0.000***
Liking of flavour	0.000***	0.000***	-0.000***	-0.002**	-0.000***	-0.000***
Liking of texture	0.000***	0.000***	-0.000***	-0.013*	-0.000***	-0.009**
Overall	0.000***	0.000***	-0.000***	-0.002**	-0.000***	-0.000***
Intensity						
Thickness	0.021*	0.024*	-0.02*	-0.088	-0.015*	-0.054
Odour	0.865	0.863	0.863	-0.864	-0.865	-0.864
Off-odour	-0.000***	-0.000***	-0.002**	0.002**	0.000***	0.001**
Sour taste	0.541	0.558	0.561	-0.554	-0.554	-0.541
Yoghurt flavour	0.000***	0.000***	-0.000***	-0.003**	-0.000***	-0.000***
Off-flavour	-0.000***	-0.000***	0.001***	0.002**	0.000***	0.000***
Fishy taste	-0.000***	-0.000***	0.000***	0.001**	0.000***	0.000***

Data expressed as significance of regression coefficients (p values); *P < 0.05, **P < 0.01, ***P < 0.001.

Chapter 7

General Discussion

GENERAL DISCUSSION

There is a plethora of evidence for the health benefits of omega-3 polyunsaturated fatty acids (n-3 PUFA), which have been scrutinized in the scientific literature as much, if not more than any other nutrient. The monumental shift in the dietary ratio of n-6/n-3 PUFA over the last number of decades has likely contributed to this research (Simopoulos, 2002). n-3 PUFA play critical roles in cardiovascular health through processes that regulate cholesterol metabolism, atherogenesis and hypertension (Wang et al., 2006). As discussed in Chapters 1.1 and 2, brain health is critically dependent on n-3 PUFA by regulating neurogenesis, synaptic plasticity and neurotransmitter activity (Bazinet and Layé, 2014). Metabolic health is uniquely modified by n-3 PUFA through interactions with glucose homeostasis, adipogenesis and inflammation (Calder, 2006, Kim et al., 2015). Bone health (Weiss et al., 2005), tumorigenesis (Larsson et al., 2004), liver function (Parker et al., 2012) and a number of other physiological functions appear to be strongly influenced by n-3 PUFA. Indeed, as fatty acids form an integral component of the phospholipid bilayer in all mammalian cell membranes, it is evident that n-3 PUFA and other fatty acids play integral structural and functional roles in human tissues. Interestingly, however, many clinical trials involving n-3 PUFA supplementation have failed to replicate the promising results observed in animal studies (Du et al., 2015), leading many to question the true significance of dietary n-3 PUFA. Many of these conflicting results may be attributed to ineffective supplementation doses, confounding dietary factors and most importantly, differences in baseline tissue fatty acid composition, which widen inter-individual differences.

The recent discovery of the significance of the gut microbiota however, primarily as a result of advanced sequencing technologies, unveiled a novel mechanism and target through which n-3 PUFA may exert their beneficial health effects. Indeed, recent research has begun to uncover the opposing effects that n-3 PUFA exert on the gut

microbiota composition in comparison to n-6 PUFA or saturated fatty acids (SFA) (Kaliannan et al., 2015, Caesar et al., 2015). A number of dietary n-3 PUFA supplementation studies suggest that n-3 PUFA have a direct interaction with the commensal gut microbiota (Patterson et al., 2014, Robertson et al., 2016). However, transgenic studies using the *fat-1* model suggest that there is also an indirect relationship between n-3 PUFA and the microbiota through altered tissue fatty acid status and a modified intestinal environment mediated by inflammation and antimicrobial peptide production (Kaliannan et al., 2016). Therefore, the observed variation in n-3 PUFA supplementation efficacy in clinical trials may also be somewhat attributed to underlying differences in the gut microbiota.

Research investigating the interaction between n-3 PUFA and the gut microbiota, using deep sequencing technologies, while still a novel area of research appears to be expanding (Patterson et al., 2014, Caesar et al., 2015). These studies have shed light on the compositional effects that dietary lipids exert on the gut microbiota, however none have yet examined these interactions in the context of different ages or disease contexts. This thesis aimed to achieve this, to examine the interaction of n-3 PUFA with the gut microbiota in the context of different ages and disease contexts, notably obesity and behavioural deficits. Secondly, from a translational viewpoint, this thesis aimed to examine algae as novel source of bioactive n-3 PUFA and its potential as a functional food to increase n-3 PUFA intake.

n-3 PUFA account for a significant proportion of lipids in the brain and play vital roles in the fetal and neonatal developing brain. Moreover, n-3 PUFA have been shown to prevent some of the processes that contribute to neurodegeneration (Bazinet and Layé, 2014). Adjacently, the gut microbiota plays a crucial role in central nervous system (CNS) development and functioning, which has implications for mammalian behaviour (Borre et al., 2014). In Chapter 2, the role of n-3 PUFA, or absence of n-3

PUFA, was assessed within the gut-brain-microbiota axis. The data presented highlighted the crucial role of n-3 PUFA in early-life and adult behaviours. n-3 PUFA deficiency significantly impaired communicative behaviour in very early-life while being significantly enhanced by n-3 PUFA supplementation. These neurobehavioural differences induced by varying n-3 PUFA availability were augmented at later stages of life whereby n-3 PUFA supplementation enhanced cognition at both adolescence and adulthood, and n-3 PUFA deficiency induced depressive-like behaviour and impaired social behaviour in adulthood. Some of these n-3 PUFA-induced behavioural alterations have been reported previously, however the mechanisms behind them remain unclear. n-3 PUFA supplementation reduced the corticosterone response to a forced stress suggesting that their role in negative feedback within the hypothalamic-pituitary-adrenal (HPA) axis may contribute to the alterations in stress and depressive-like behaviours. Furthermore, n-3 PUFA deficiency induced changes to the immune response resulting in muted systemic lipopolysaccharide (LPS)-responsiveness, which was previously observed in offspring of high fat diet-fed mothers (Myles et al., 2013). Disruptions within the immune system also play strong roles in behavioural disorders (Dantzer et al., 2008).

We also closely tracked the development of the gut microbiota at both timepoints, i.e. adolescence and adulthood, to examine whether the behavioural alterations were associated with modifications to the gut microbiome. Indeed, a number of beneficial species known to positively enhance CNS functioning and behaviour, including *Bifidobacterium*, *Lactobacillus* and the *Bifidobacterium*/*Enterobacteriaceae* ratio, trended towards higher abundance in n-3 PUFA supplemented mice in adolescence and became significantly greater in adulthood. Conversely, n-3 PUFA deficiency induced a greater ratio of Firmicutes:Bacteroidetes, a marker of obesity, and *Peptostreptococcaceae* which have previously been associated with non-alcoholic fatty

liver disease (NAFLD) (Jiang et al., 2015). These novel findings not only characterized the composition of the adolescent and adulthood gut microbiota following n-3 PUFA supplementation or deficiency but also revealed that this altered microbiota is closely associated with comprehensive changes to CNS functioning and behaviour. Hence, the previously observed benefits of n-3 PUFA for brain health may be mediated through the gut microbiota and hence gut microbiota-targeted interventions may provide novel therapeutic strategies for neuropsychiatric disorders.

Although much knowledge has been gained from microbiota research regarding its composition under different disease states and its use as a therapeutic target for intervention, there is still a lack of knowledge regarding its functionality. It has been estimated that in the human body, microbial cells outnumber human cells by 10:1, however the microbial gene count compared with humans may be as high as 100:1 (Hamady and Knight, 2009). Therefore, there is still a lot of knowledge to be gained surrounding the functional activity and output of the microbiota rather than its composition alone. Advances in metabolomics technologies will help to achieve this. In Chapter 3, using a similar experimental design as in Chapter 2, an investigation was performed to examine the cecal microbiota composition of n-3 deficient and n-3 supplemented mothers and their offspring, in addition to the metabolic output of the microbiota in the cecal content. A unique cecal metabolite signature has previously been identified in a high fat diet (Daniel et al., 2014), and a unique liver metabolite signature following n-3 PUFA supplementation (Depner et al., 2013). We first showed that low n-3 PUFA intake induced a state of 'dysbiosis' or microbial imbalance in the cecal microbiota of both mothers and their offspring by elevating relative abundance of *Tenericutes*, *Anaeroplasma* and *Coriobacteriaceae*. Conversely, n-3 PUFA supplementation reduced *Lachnospiraceae* abundance and the Firmicutes:Bacteroidetes ratio in mothers. The composition of the cecal microbiota appeared to differ somewhat

to that of the fecal microbiota as was reported previously in Chapter 2 (Robertson et al., 2016). Microbiota composition differs between the intestinal segments likely reflecting different roles in digestion and absorption for each region (Marteau et al., 2001).

The subsequent metabolomics analysis revealed that n-3 PUFA supplementation stimulated an increase in the production of cecal metabolites involved in energy metabolism, namely fumaric acid, malic acid and α-ketoglucaric acid. It has previously been reported that these energy metabolites and others involved in the tricarboxylic acid (TCA) cycle were increased in the serum of conventionally raised animals compared with germ free animals, suggesting the microbiota plays a crucial role in energy metabolism (Velagapudi et al., 2010). However, there is conflicting evidence about the upregulation or downregulation of these metabolites in different animal models of obesity, therefore more research is warranted to identify their true role in metabolic disease. Using targeted analysis, we also observed a significant decrease of cecal short chain fatty acids (SCFA) in n-3 deficiency. SCFA play a number of immunomodulatory roles in the gut as well as maintaining intestinal epithelial integrity (den Besten et al., 2013). Therefore, these results suggest that a lack of n-3 PUFA could contribute to the impaired intestinal homeostasis observed in chronic metabolic disease. Finally the alterations to microbiota composition and function in n-3 deficiency were associated with a reduced ratio of IL-10/TNFα in stimulated spenocytes, a marker of increased inflammation. This in-depth analysis provides novel data about how n-3 PUFA and indeed, lack of n-3 PUFA, interact with the intestinal environment, notably microbiota composition but also function. This study and others could provide useful information for future dietary interventions in human subjects that target the microbiota and the plethora of metabolites that it produces.

As previously discussed, a transgenic model of endogenous n-3 PUFA production, the *fat-1* model, has revealed that n-3 PUFA not only have a direct

interaction with the gut microbiota through the diet but also an indirect relationship by modifying the inflammatory activity of the intestinal environment (Kaliannan et al., 2015). The *fat-1* model is important for nutrition research, especially related to the microbiota, as it eliminates the confounding effects of feeding different diets and in addition allows any observed effect to be attributed solely to n-3 PUFA, rather than other nutrients that may be present in dietary oils. This model however, has not been used to examine maternal n-3 PUFA status on the offspring microbiota and subsequent metabolic outcomes. Maternal n-3 PUFA status during pregnancy significantly regulate offspring growth and weight through a number of mechanisms, however it is not known whether an altered microbiota is one of these. In Chapter 4, the *fat-1* model was utilized to examine whether maternal n-3 PUFA status could influence offspring weight gain in the context of obesity. Interestingly, it was observed that higher maternal n-3 PUFA status either during gestation or lactation significantly reduced weight gain in high fat diet (HFD)-fed offspring compared with high maternal n-6 PUFA (wild-type, WT). However this effect was only observed in male offspring, not females. The significant changes in weight were associated with differences in the intestinal environment. Maternal n-3 PUFA significantly reduced intestinal permeability in the offspring. In addition, comprehensive alterations were observed to the microbiota in mothers, which appeared to be transferred to the offspring both before introduction of the HFD and after. *Firmicutes*, *Clostridia* and *Lachnospiraceae* were all elevated in offspring of mice whom had a WT mother during lactation, which correlated with weight gain, n-6/n-3 ratio and intestinal permeability. Interestingly the mothers n-3 PUFA status and microbiota during lactation appeared to have a greater effect on offspring phenotypes and microbiota than the biological mother. These results suggested that maternal n-3 PUFA, especially during lactation, may be an effective measure to reduce weight gain in

the context of offspring obesity and that it is associated with comprehensive alterations to the gut microbiota.

Despite the comprehensive literature reporting the health benefits of n-3 PUFA, there are a number of barriers to achieving global increased consumption. The main metabolically active n-3 PUFA, eicospentaenoic acid (EPA) and docosahexaenoic acid (DHA), are formed through the elongation of their precursor a-linoleic acid (ALA). ALA is typically found in plant sources such as flaxseed oil, and itself has been reported to exert a number of health promoting bioactivites. EPA and DHA are only obtained from marine sources such as oily fish and have been reported to exert more potent anti-inflammatory, cardioprotective and neuroprotective properties than ALA (Anderson and Ma, 2009). The enzymatic conversion of ALA to EPA and DHA is an inefficient process in mammalian tissues therefore it is recommended to directly increase consumption of EPA and DHA from marine sources such as oily fish. There are doubts however, over the sustainability of fish, as global stocks appear to be rapidly declining (Branch et al., 2011). Indeed, pollution also poses threats to fish production and consumption. Algae, particularly microalgae, pose potential as a novel sustainable, industrial and economical alternative for n-3 PUFA production than fish (Robertson et al., 2013). A number of microalgae species have been closely studied for their ability to produce n-3 PUFA under varying environmental conditions, however little is known about the bioactivity of algal n-3 PUFA and their potential as functional ingredients. In Chapter 5, a number of macroalgal and microalgal lipid extracts were examined for their n-3 PUFA and bioactive pigment composition in addition to their anti-inflammatory activity *in vitro*. Previous studies had reported the fatty acid profiles of the algae species involved, while in this study, an in-depth analysis of the pigment and lipid partitioning profiles of each extract was performed. The lipid partitioning profiles are of particular interest as it has been shown that n-3 PUFA bioavailability and bioactivity differs within different lipid

classes such as triglycerides and phosphoplipids (Rossmeisl et al., 2012). The algae lipid extracts were rich in n-3 PUFA with EPA being the most abundant fatty acid in each extract. Human THP-1 macrophages were subsequently stimulated with LPS to mimic *in vivo* inflammation. Exposure of the algae extracts to the stimulated macrophages significantly dampened the production of inflammatory cytokines including IL-6 and IL-8. In addition, an array of inflammatory gene expression assays were performed which identified a number of genes involved in toll-like receptor activity and chemokine activity that were down-regulated by the extracts. There is growing evidence of the bioactivity of n-3 PUFA algal oils. However, due to the differing fatty acid profiles and lipid partitioning profiles in particular, algal oils may significantly differ to fish oils in their bioactivity. Furthermore, little research into algal oil bioactivity has provided mechanistic evidence for their observed effects. The market for vegetarian sources of n-3 PUFA is increasing and hence more research is warranted to identify suitable species for production and their potential health benefits. The results presented here not only characterized the lipid and pigment profiles of novel extracts but also provided mechanistic evidence as to how such extracts dampen inflammation. This may have further implications for intestinal health. The immune system is uniquely regulated by the intestinal environment through interaction with the gut microbiota. Indeed, as n-3 PUFA modulate microbiota and inflammation as described in Chapters 2 to 4, it is possible that these n-3 PUFA-rich algae extracts have similar effects. Hence, there is potential for these extracts to act as anti-inflammatory functional ingredients, as was investigated in Chapter 6.

Another barrier to n-3 PUFA consumption is undesirable sensory attributes of fish and fish oil. Therefore, some research has begun to examine various food matrices that could act as carriers for n-3 PUFA-rich oils. Dairy products in particular appear to be suitable carrier for n-3 PUFA (Chee et al., 2005) and may act as suitable foods in

which to incorporate functional ingredients into habitual diets. Due to the previously reported high n-3 PUFA composition and potent anti-inflammatory activity of a lipid extract from the microalgae *P. lutheri* in Chapter 5 (Robertson et al., 2015), the study outlined in Chapter 6 aimed to investigate whether yoghurt could act as a suitable carrier for the extract. The data reported that the primary techno-functional properties of the yoghurt (pH, whey separation, rheology, starter culture viability and macronutrient composition) were all unaffected by addition of the extract during processing at two different doses (0.25% and 0.5%). Another study reported similar results using a different algal extract (Chee et al., 2005). Furthermore, addition of the extract to the yoghurt dose-dependently increased n-3 PUFA content of the yoghurt. However, extensive sensory analysis reported that the supplemented yoghurt was not well accepted under a number of sensory parameters. The novel data reported in this study reveal that yoghurt is a suitable food to act as a carrier for an n-3 PUFA-rich lipid extract from *P. lutheri*. Incorporation of n-3 PUFA into vehicle foods may be an effective strategy to enhance tissue n-3 PUFA status and hence improve inflammatory and metabolic clinical outcomes as has been suggested previously for enriched yoghurt (McCowen et al., 2010). Although there were perceived negative sensory attributes, the lack of effect of the extract on the techno-functional properties of the yoghurt as well as the increasing n-3 PUFA content warrants further research into how sensory properties could be improved in order to commercially manufacture such a product.

The data presented in this thesis reveals a novel mechanism by which n-3 PUFA mediate health and disease, through interaction with the microbiota. Furthermore, this has been reported in the context of different ages (mothers, adolescence, adulthood) and disease states (obesity, inflammatory disorders and behavioural deficits). Increasing evidence is showing that a perturbed microbiota can be a causal factor in a number of chronic diseases and that microbiota-targeted nutritional interventions can be

therapeutically effective. However, microbiota research is only in its infancy therefore further insights into gastrointestinal ecology and its interaction with the diet will aid in future disease interventions and preventative measures. Uniquely, this thesis has also reported evidence for potential novel methods of increasing n-3 PUFA production and consumption. The use of algae as novel sustainable sources of n-3 PUFA may help to increase n-3 PUFA intake. With further research, this may benefit gut microbiota and inflammation-linked chronic disease through novel sustainable foods. From a public health perspective, such research may aid public health recommendations in order to define recommended intake levels for n-3 PUFA, which currently are lacking. Indeed increasing awareness about the health benefits of n-3 PUFA and the risks of high dietary n-6 PUFA may improve public nutrition and health outcomes.

Despite much research already reporting the beneficial health effects of n-3 PUFA, their interaction with gut microbiota is relatively unexplored in addition to their bioactivity from algae. The data presented here represents the beginning of such research however further work is necessary. Ongoing research should address and consider this n-3 PUFA-microbiota interaction in different disease states. Clinical trials of n-3 PUFA supplementation in certain inflammatory diseases such as inflammatory bowel disease (IBD) have generated conflicting and inconclusive results. Future research should examine whether a lack of effect in n-3 PUFA clinical trials involving disorders such as IBD are due to differences in gut microbiota composition and function. Regarding metabolic health and the data generated from chapter 4, further research is warranted to examine whether the observed effects on weight are truly mediated by changes to the gut microbiota. A co-housing study or germ free study in which the microbiota of the *fat-1* mother's offspring is transplanted into that of the WT mother's offspring would answer such a question. Indeed, as low-dose antibiotics induce a similar state of dysbiosis and weight gain, it would be beneficial to examine whether maternal

n-3 PUFA can protect against such antibiotic-induced disturbances in the offspring. Importantly, the observed gender difference should be further addressed. A similar co-housing or germ-free design could be employed to further investigate the behavioural and neurological outcomes observed in chapter 2, to assess whether a true gut-microbiota-brain interaction exists in this design. Indeed, the longer term effects on ageing brain health and the associated interaction between n-3 PUFA and the microbiota would answer important questions related to dementia. Finally, to further develop the knowledge and research regarding algae-derived n-3 PUFA, research that compares the anti-inflammatory effect or anti-obesigenic effect of algae oils in comparison to fish oil is warranted in addition to their interaction with the gut microbiota. By continuing the research described in chapters 5 and 6 using *in vivo* or clinical models of obesity or inflammatory disease, important knowledge would be obtained regarding the true potential for algae to replace fish as a source of n-3 PUFA and act as viable functional foods.

Such research could help promote greater global n-3 PUFA intake in place of other dietary lipids and help stem and prevent the growing epidemic of obesity, neurobehavioural and inflammation-linked chronic diseases.

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