IMPACT OF DIFFERENT DIETARY LONG-CHAIN POLYUNSATURATED FATTY ACIDS ON SURVIVAL OF PROBIOTIC BACTERIA IN COLONIC CELL LINES

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I certify that the work contained in this thesis is entirely the result of my own work, except where reference is made to other authors. It has not been submitted in any form to any other University.

Authors declaration

Erika de-guzman completed mRNA and protein analysis of cell lines treated with pathogenic bacteria and Kathryn Cross completed scanning electron microscopy pictures.

Impact of Dietary Long-Chain Polyunsaturated Fatty Acids on the Survival of Probiotic Bacteria in Colonic Cell Lines

Abstract

Probiotic bacteria have been shown to be potentially useful in maintaining colonic health and improving mucosal immune homeostasis. In addition, polyunsaturated fatty acids (PUFAs) are known to be potentially beneficial in this respect and so manufacturers may try to combine both ingredients in 'functional foods' but we know nothing about how these two factors interact.

Two *in vitro* approaches were used in these studies. Firstly the impact of physiologically relevant doses of dietary PUFAs on growth rates of different strains of lactobacilli were measured using turbidity measurements (Bioscreen). Secondly, the same strains of lactobacilli were co-cultured with human colon cell lines (HT29, HT29-MTX-mucin secreting & Caco-2). Confluent cell cultures were pre-treated with a range of different PUFAs to modify cell lipid profiles prior to exposure to lactobacilli and measurement of bacterial cell adhesion and mammalian cell cytokine release will be measured. Data from gas chromatographic analysis of PUFA incorporation into human and bacterial cells will be used to gain further insight into the how they might influence the end-points being assessed.

Results showed that human isolates survive in concentrations of PUFAs that are biologically relevant in the colon suggesting a mechanism to avoid the anionic detergent agents of PUFAs.

Secondly that docosahexaenoic acid up-regulates lactobacilli adhesion to human epithelial cells. However, arachidonic acid up regulates adhesion of some lactobacilli

to mucus secreting HT29-MTX cells, possibility involving modification of mucin production or composition to favour binding. Therefore these results suggest that DHA and AA act through different mechanisms to support the beneficial effects of lactobacilli in the colon. In regulation to immune modulation, lactobacilli can up-regulate TGF-β that maybe involved in survival by down-regulation of inflammatory signalling, thus avoiding recognition and removal from the host cell surface. While EPA can enhance the production of TGF-β and therefore may aid in lactobacilli survival.

Studies have indicated that all PUFAs tested had a positive impact on adhesion of certain strains of lactobacilli to at least one cell line tested. Although it is likely that each predominately acts through a different mechanism.

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CHAPTER 1: General introduction

SECTION 1.1 The Colon

The colon is the most colonised region of the gastrointestinal tract, containing approximately 10^{12} bacteria per gram of gut contents and therefore is an obvious choice for human microbiological research. Many species of bacteria provide a mutual relationship with the colon and can support its function, for example the fermentation of complex carbohydrates into short chain fatty acids used as an energy source for colonic cells. Although the colon and its bacteria have been thoroughly studied, there is still much to learn and even to this day there are many undiscovered species and strains.

1.1.1 Structure and function

The wall of the whole gastrointestinal tract follows a general structure (figure 1). Along the tract is a layer of epithelial cells and amongst these, exocrine cells secrete mucus into the lumen and endocrine cells secrete hormones into the bloodstream. The epithelial layer consists of some invaginations into the underlying tissue forming exocrine glands that secrete acid (stomach), enzymes, water and ions. Typical of such epithelial cell layers these cells have an apical brush border membrane to increase surface area. Below the epithelial cells layer there is the lamina propria consisting of blood vessels, nerve fibres and lymphatic ducts. The muscularis mucosa is a thin layer of smooth muscle that sits beneath the lamina propria and all three of these layers form the mucosa.

A connective tissue layer lies below the mucosa termed the submucosa, this contains submucous plexus (nerve cells) and blood lymphatic vessels that penetrate

both mucosa and muscularis externa (smooth muscle below). Muscularis externa consists of two types of muscle, circular (narrows lumen) and longitudinal (shortens tract), between which lies the myenteric plexus nerve cells. Outside these layers connective tissue exists named the serosa which is attached to the abdominal wall thus supporting the gastrointestinal tract in the abdominal cavity[1].

Regulation of the reflexes is by luminal stimuli such as:

- Distension of wall by volumes of luminal contents.
- Chyme osmolarity.
- Chyme acidity.
- Chyme concentrations of specific digestive products, i.e. fatty acids and amino acids.

The stimuli act on mechanoreceptors, osmoreceptors, and chemoreceptors in the tract wall.

The enteric nervous system is the digestive systems specific local nervous system consisting of the myenteric plexus and the submucosal plexus. The nerves end at epithelial cells or smooth muscle cells and glands (effectors). It should be noted that the central nervous system can also impact upon the motility and secretory activities of the tract. In addition to signals in the tract stimulating neural reflexes, sight, smell and emotional state have significant impact via autonomic neurons mediated by the central nervous system. Hormones also regulate the gastrointestinal tract, this having influence predominately in the stomach region and small intestine.

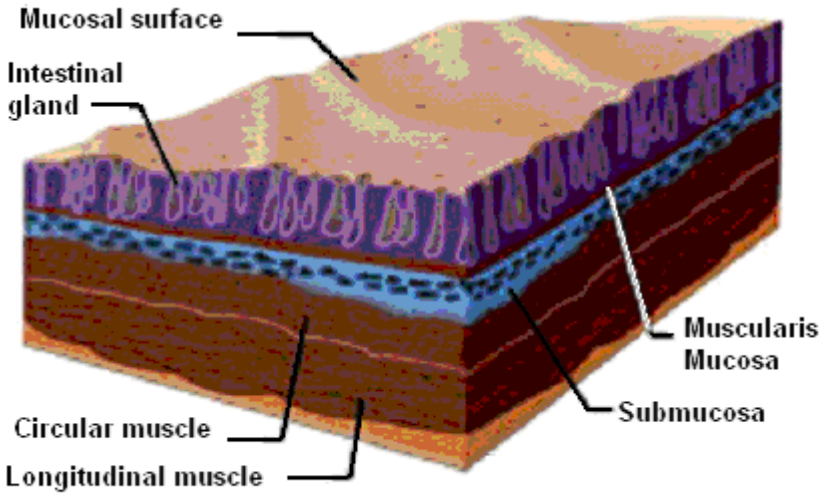

Figure 1 *The structure of the gastrointestinal wall in general is made up of many layers. The mucosal surface is pitted with invaginations into the underlying tissue forming exocrine glands. Below the surface epithelial layer are the lamina propria consisting of blood vessels, nerve fibres and lymphatic ducts. Beneath are subsequent layers of muscularis mucosa, submucosa, circular muscle and longitudinal muscle (image by C. Hogan [2]).*

The colon, otherwise known as the large intestine, extends from the *Cecocolic ostrium* (caecum) to the rectum. It is approximately 70 to 75 cm long and is dark green in colour when full. In the colon the highly convoluted surface has a layer of epithelial cells positioned along it linked together with tight junctions (figure 2). At the base of the crypts mucus is secreted hence preventing bacterial adhesion in the crypts. The colon is divided into three parts

- Ascending colon - decreases in diameter, and runs close to the caecum. It loops to form a spiral, with two centripetal coils, and two centrifugal coils;
- Transverse colon - running left, and dorsally to the duodenum;
- Descending colon - on the left side, and is quite straight. The surface is finely folded.

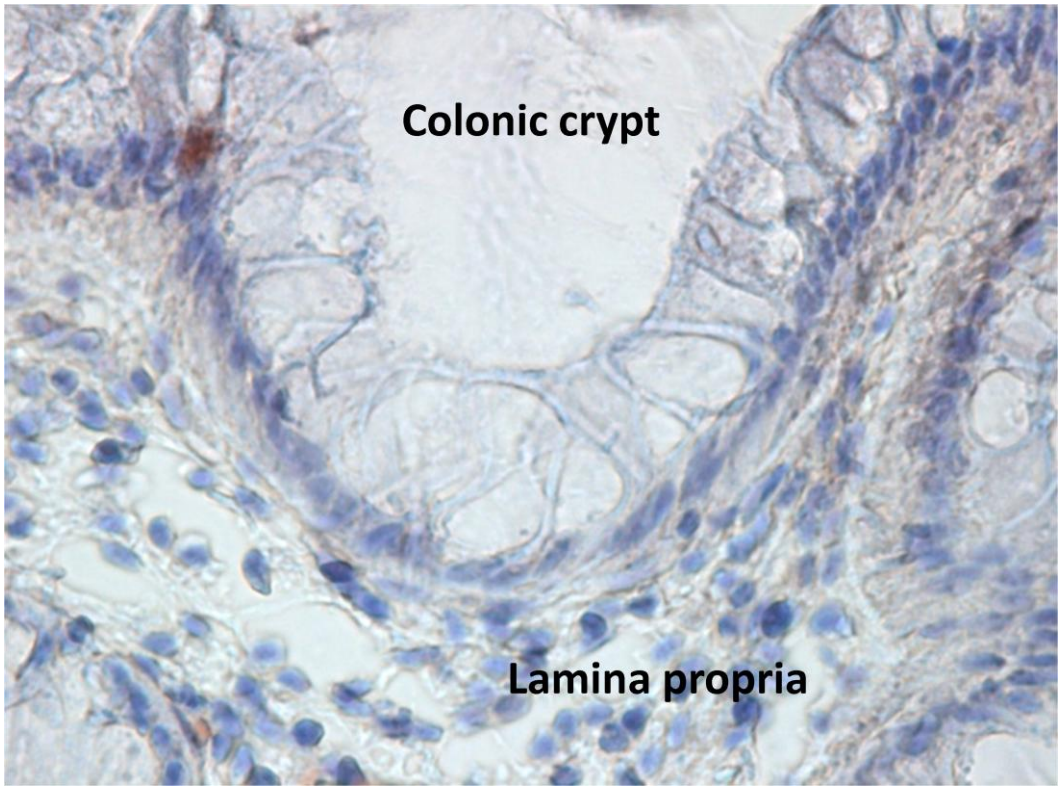

Figure 2 *Colonic crypts along the surface of the colon wall. The highly convoluted surface consists of epithelial cells linked together with tight junctions. At the base of the crypts mucus is secreted out onto the surface as a mechanism of protection from pathogen (Image by Claudia Lötsch, Institute of Food Research, Norwich).*

The large intestine is a highly specialized organ that is responsible for processing waste so that emptying the bowels is easy and convenient. The stool passes through the colon by peristalsis; it consists of mostly food debris and commensal non-pathogenic microflora. These types of bacteria perform several useful functions, such as synthesizing various vitamins, processing waste products and food particles, and protecting against harmful bacteria. A vast number of the gut bacteria are vital for the development of the intestine. The array of ingested antigens and the gut flora form the major constituents of the gut lumen. This is particularly true for the distal ileum and the colon where the resident anaerobic bacteria exist in very high (10^8 to 10^{12} bacteria/gram of luminal contents) concentrations. To deal with this

continuous and diverse antigenic exposure, a unique immune system exists at the surface of the gastrointestinal tract, which is distinct from the systemic immune system [1].

Secretions from the large intestine consist of mucus and fluid containing bicarbonate and potassium ions transferred from the blood during the processing of faecal material. The main absorptive process is active transport of sodium from lumen to the blood system coupled with the osmotic absorption of water. Other materials absorbed include products of the microflora, such as fibre which is metabolized to short chain fatty acids (neutralised by bicarbonate) and absorbed by passive diffusion. Vitamin K is also produced from microflora and is absorbed into the blood, thus being an integral part of vitamin K intake if dietary intake is low. Gases are produced by intestinal bacteria via fermentation of undigested polysaccharides; carbon dioxide and nitrogen being most predominant.

Overall the structure of the colon is highly specific to its function and due to this, its contact with the outside world results in it being very susceptible to mutation possibly leading to cancer. Much research is being undertaken on the colon to find prevention and cures for colonic diseases, many of which could be related to optimising the diet [3-4].

SECTION 1.2 Polyunsaturated fatty acids

1.2.1 Structure and function

The characteristic feature of fatty acids is the carboxy group attached to the aliphatic body. Long chain fatty acids are those with 14-26 carbons and the majority of these sit in cis formation, some contain carbon-carbon double bonds and their number, type and position all affect their metabolic properties. In particular, polyunsaturated fatty acids (PUFAs) will contain more than one double bond between carbon atoms. Two families of PUFAs are the n-3 fatty acids (double bond between 3rd and 4th carbon from acyl end) and the n-6 fatty acids (double bond between 6th and 7th carbon from acyl end). These essential fatty acids are of particular importance in normal human functions and must be sourced from the diet. A major source of n-3 fatty acids is fish oil, although linolenic acid is found in several seed oils; while n-6 fatty acids are present in vegetable oils, such as sunflower oil. One affect these PUFAs can have on the human system is via immunomodulatory mechanisms and these can be split into six main avenues[5].

- **Modulation by eicosanoids**- Eicosanoids from arachidonic acid (AA), derived from omega-6, is more pro-inflammatory than those derived from fish oils, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Increased levels of EPA derived eicosanoids in the blood, result in significant effects on platelet aggregation, vasoconstriction, neutrophil function, inflammation and immunity[6].

- **n-3 PUFAs, membrane fluidity and lipid rafts**-An increase in membrane fluidity is found in response to increased n-3 consumption[7]. Evidence suggests that in vitro omega-3 PUFAs suppress T cell activation by

displacing acylated signalling proteins from lipid rafts (place in membrane where signalling in immune cells occur) by altering the rafts composition[8].

- **n-3 PUFAs and signal transduction**-Lipids are involved in intracellular signalling pathways[5]. For example, phospholipids such as phosphatidylserine, containing fatty acid chains, can generate secondary messengers that are involved in activating enzymes, i.e. activation of protein kinase C (PKC). However, omega-3 fatty acids can inhibit PKC as they decrease the generation of the relevant secondary messengers[9].

- **n-3 PUFAs and gene expression**- Fatty acids effect the expression of genes for proteins involved in hepatic fatty acid and lipoprotein metabolism, by covalent modification, redox state, proteolytic modification or through binding of peroxisome-proliferator activated receptors (see section 1.2.5).

- **n-3 PUFAs- modulation of antigen presenting capacity**- Dietary fish oil supplementation for 21 days decreases the intensity of expression of MHCII antigen presenting molecules on peripheral blood monocytes [10].

- **n-3 PUFA- modulation of the gastrointestinal flora**- May affect the mucosal adhesion sites for gastrointestinal bacteria by modifying the composition of the intestinal wall [5].

1.2.2 Digestion, absorption and transport

The digestion, absorption and transport of lipids has been well summarised by Kohlmeier [11]. On consumption of fats the triglyceride components are hydrolysed by lipases in the upper digestive tract to monoglyceride and free fatty acids. The

dietary triglycerides, monoglycerides, bile phospholipids and bile acid mix to form micelles and are further hydrolysed by bile-salt activated lipase in the small intestine. Free acids and monoglycerides from the micelles transfer to enterocytes of the small intestine by diffusion and facilitated transport. During transfer through epithelial cells they are re-synthesised into triacylglycerol, this lowers free acid concentration that can maintain diffusion. Inside the cell fat aggregates are formed and amphipathic proteins emulsify. To exit the cell vesicles containing a fat droplet pinch of the endoplasmic reticulum and are processed through golgi apparatus, they then bind the plasma membrane and the fat is released into the interstitial fluid. The extracellular fat droplets are called chylomicrons and are transported via lacteals lymphatic capillaries in the intestinal villi. The free fatty acids are released by endothelial lipoprotein lipase, enter adipocytes and combine with α-glycerol phosphate to form triacylglycerols. Much of these are oxidised to produce energy for organs and some is stored.

Long chain fatty acids (LFCAs) are used in cell processes, including membrane synthesis, intracellular signalling, transcriptional regulation, post-translational protein modification and metabolism. The latter is used by most vertebrate tissues in situations of metabolic stress. LFCAs are insoluble in aqueous environments and are bound with high affinity to proteins in plasma, predominantly albumin. Inside cells LFCAs are bound to cytosolic fatty acid binding proteins (FABPs), that assists solubility and play a role in intracellular movements of these molecules to various organelles. Intestinal FABP binding affinities are similar to aqueous partition for many fatty acids. However, DHA appears to have a lower affinity than predicted [12]. Another protein involved in fatty acid uptake in mammalian cells was identified by Abrumrad et al. [13], named fatty acid translocase (FAT). This binds long chain fatty acids and has no preference for particular fatty acids. More recently another fatty acid transport protein (FATP) has been identified [14], suggested to act with fatty

acyl CoA synthease (FACS),that prevents efflux of fatty acids. FATP is regulated by peroxisome proliferator-activated receptor (PPAR) ligands, which include many LFCAs, therefore can be up regulated by the substrate it binds.

Inside the cell LFCAs are esterified by long chain FACS and the long chain fatty acyl CoAs subsequently transported to the mitochondria to be degraded by β oxidation or very long chain fatty acids are transported into peroxisomes. In β oxidation the resulting acyl coA diffuses across the outer mitochondrial membrane and is transformed to carnitine by palmitoyl-coA: L-cartinine O-palmitoyltransferase I to cross the inner membrane. Once inside the carnitine is transferred back to coA. During each round of β oxidation two carbons are removed and one acetyl-coA is released. The process is repeated until two acetyl-coAs are formed or one acetyl coA and one propionyl-coA. In addition to the process in the case of PUFAs the double bonds are oxidised by microsomal cytochrome P450-enzymes.

As mentioned previously fatty acid can cross the plasma membrane by simple diffusion, followed by lateral movement within the membrane and flip-flop to their inner leaflet. For accelerated dissociation from albumin that is bound by high affinity, intracellular proteins or enzymes that bind or metabolise LCFA may shift equilibrium towards uptake.

1.2.3 Arachidonic acid metabolism

Fatty acids are not always subject to oxidation and can play a role in many immune responses. Important PUFAs such as arachidonic acid are incorporated into membrane phospholipids prior to being used as a precursor for prostaglandins, prostacyclin and thromoxanes. The primary step of the process involves Cox-1, Cox-2 and arachidonate 5-lipoxygenase catalysing the first step in leukotriene

synthesis. Both enzymes can accept other long-chain fatty acids, such as EPA, resulting in a variety of eicosanoids with differing characteristic activity profiles.

1.2.4 Eicosapentaenoic acid and Docosahexaenoic acid metabolism

α linolenic acid, EPA and other n-3 fatty acids can be converted to DHA via elongation and desaturation, predominately by the liver. Tetracosahexaenoic acid is produced by the endoplasmic reticulum and then the 24 carbons are shortened via peroxisomal β-oxidation to DHA. The synthesis requires supply of many substrates including B vitamins and iron and conversion can be limited. DHA can undergo β oxidation in mitochondria and peroxisomes (described in section 1.1.2). Approximately one tenth of consumed DHA is shortened to EPA. EPA is a precursor of 3-series prostaglandins and 5-series leukotrienes and inhibits the formation of omega-6 prostaglandins by direct competition for key enzymes[15].

1.2.5 Peroxisome-proliferator activated receptors (PPARs)

PPARs have been shown to bind PUFAs and give a potential mechanism through which PUFAs act [16].

PPAR cDNA was isolated relatively recently [17], since then several isoforms of PPAR protein have been discovered, i.e. PPAR α, β and γ. They are members of the nuclear receptor family and form heterodimers with RXR, resulting in an active conformation in the presence of an agonist [18]. Following activation co-activator proteins recruit to create a complex that binds peroxisome proliferator response elements (PPRE) in target genes, stimulating their expression (PPAR can also function without RXR [19]).

The mammalian isoforms have varied tissue distribution: PPAR α, found in muscle, heart, kidney and small intestine is important for fatty acid uptake and oxidation; PPAR γ, found in fat, large intestine and macrophages is important for adipocytes differentiation and PPAR δ, which is found in most cell types, is important in cell differentiation in the nervous system, colon and other organs. For my studies I have been interested in the latter two isoforms, due to the characterised localisation in colonic and immune cells. The ligand binding structure is varied between isoforms and results in varied binding affinities of PUFAs. All isoforms fold into a single domain containing 13 helices and a 4-stranded β sheet. Between helices H2 and 3 is a very flexible loop situated at the binding site entrance, allowing adaption to ligand size [20].

Dietary ligands of PPARs include conjugated linoleic acid (CLA), which has been shown to induce PPARs γ and δ, and result in repression of inflammatory cytokine TNF-α and induce immunomodulatory cytokine TGF-β [21]. The latter being a molecular target for PPAR δ only [22]. Studies have detailed fatty acid properties i.e. chain length in relation to PPAR binding and have distinguished variance in isoforms for which fatty acid binds with the greatest affinity [23]. For example PPAR α will efficiently bind saturated fatty acids of 14 to 16 carbons. However, PPAR γ failed to have any interaction with unsaturated fatty acids of fewer than 20 carbons. Saturated fatty acids bound neither PPAR γ nor δ isoforms. In contrast, long chain unsaturated fatty acids (C>20) bound to all isoforms, in particular AA and EPA interacted efficiently with PPAR γ and δ, whilst PPAR α did not bind them anymore than the saturated analogue. Detailed co-crystal structure of PPAR δ revealed that EPA occupied the ligand-binding pocket in two conformations, completely burying it within the domain, further stability resulted through hydrogen bonds and hydrophobic interaction, which would not be present if the fatty acid was 14 carbons or fewer. In addition longer chain fatty acids, greater than 20 carbons (i.e. DHA

C22:6) would not fit the binding pocket and thus be destabilized by solvent. However DHA is shown to bind PPAR γ with greater affinity than AA and EPA [24], giving complex examples on how fatty acids may modulate immune responses via PPARs using very different mechanisms, hence resulting in varied end products, with potential to affect immune responses. Thus, binding affinity is not a good predictor of receptor activation.

SECTION 1.3 Probiotic bacteria

1.3.1 Commensal bacteria

The human body contains more commensal bacteria than its own cells. In the colon alone an estimated 2 x 10^{13} bacteria are present, most of which are uncultured and unknown. Bacteria are the majority of microorganisms present in the colon and are dominated by phyla; *Firmicutes*, *Bacterioidetes*, *Proteobacteria* and *Actinobacteria*. They survive on polysaccharides and other left-overs from the diet, that reach the colon and in return food may confer benefits to the host, including competitive exclusion, whereby occupying space and taking nutrients that would otherwise be available to pathogenic bacteria.

Probiotics are defined as " live microbes that when administered in adequate amounts confer a health benefit on the host" [25]. In infants *Bifidobacterium* is mostly dominant, particularly in breastfed babies, until about the age of four when they have become a minority group found in faeces. The colonisation of the gut in early life has been associated with immune development, since children that had numerous antibiotic treatments tend to have a Th2 cells skew, leading to a proneness of allergies [26-27]

1.3.2 Bacterial adherence to epithelial cells

Commensal bacteria living in the colon provide a symbiotic relationship with the host through: metabolism of nutrients and organic substrates, development of intestinal epithelium, resistance of pathogens and a range of other mechanisms [28]. However, problems can occur i.e. in Crohn's disease where an inappropriate immune response to the bacteria results in inflammation of the mucosa [29].

Up to now very little work has been published on commensal adhesion to the colonic mucosa. However we can deduce likely mechanisms or interaction between bacteria and the epithelial layer from work published on pathogens in the small intestine. The epithelial layer provides a barrier linking the outside and internal environment of the human body. If antigens do in fact manage to cross the enterocytes of the epithelium while being presented signals are sent producing T cell activation, cytokine production and release, thus aiding protection from antigen damage. The barriers to antigen adsorption in the intestine are either non immunological or immunological mechanisms, such as mucus and IgA respectively (figure 3). Commensals compete for substrates and receptor binding along the colonic epithelium and the binding differs widely between species of bacteria. However, many pathogenic bacteria share similar binding factors with certain commensals. An example of commensal bacteria inhibiting a pathogen is *Bifidobacterium profringins* that compete for mannose sugar with pathogenic *E.coli* in attachment to the epithelium [30].

Bacterial-epithelial cross talk is defined by the interaction between epithelial cells, (particularly enterocytes) and micro-organisms. To colonise bacteria must adhere to epithelial cells, for example to glycoconjugates on their cell membrane (figure 4). The glycoconjugate is often a receptor for a physiological ligand, such as a growth factor that's domain is linked to a signal transduction pathway and can activate genes through transcription factors. Therefore by co-opting the receptor the bacteria can facilitate its translocation into the host's intravascular space, resulting in inflammation and tissue destruction.

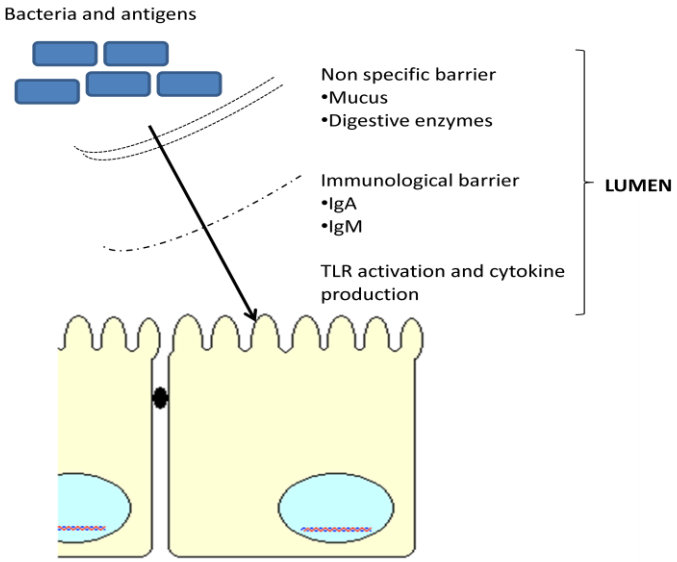

Figure 3 *Barriers of the gastrointestinal tract used to prevent bacterial and antigen invasion, such as mucus containing IgA and M antibody. Subsequently methods of defence are illustrated following bacteria contact, giving protection if bacteria breakthrough the external barriers set in place. These will predominately lead to T cell activation and phagocytosis of invading bacteria.*

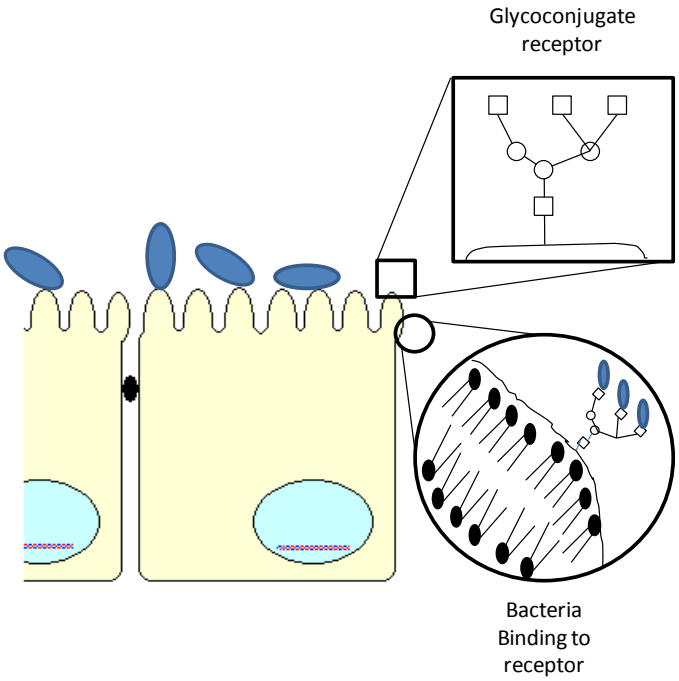

Figure 4 *Glycoconjugate receptors of human cells can act as adhesion molecules for bacteria. Following the attachment bacteria could facilitate its translocation into the host resulting in tissue destruction.*

Bacterial components also interact with enterocytes to produce pathogenic responses e.g. lipopolysaccharide components (endotoxin) of gram negative cell walls. Endotoxin can interact with enterocytes to activate transcription and translocation of inflammatory cytokines i.e. TNF and IL-8, which help recruit neutrophils, thus leading to the inflammatory response. The endotoxin signals induce a pathway leading to transcription factor NF-κB that leads to an increase in mRNA transcription for these cytokines.

Buck et al. [31] studied *Lactobacillus acidophilus* for its adhesion factors in the gastrointestinal tract by inactivating surface layer genes and measuring the rate of attachment. A fibronectin binding protein was found to reduce attachment significantly but not completely, suggesting another binding protein is involved. This was found to be a mucin binding protein, which alone would most probably be easily washed away with the flow of mucus but along with the fibronectin binding protein, produces good binding affinity, hence taking space that pathogens can not access. It is also thought that the mucin binding may be essential to the host's realisation of certain properties that distinguishes many commensals as beneficial bacteria.

Different strains of bacteria possess different binding abilities. This new area of research is currently limited to investigating a few bacterial types and a limited number of binding mechanisms. The diversity of the binding mechanisms of bacteria makes their study complex. However it is this diversity in commensals that leads to an advantage in their inhibition of a wide range of pathogens aiming to adhere to host cells.

1.3.3 Lactobacilli

Lactobacilli belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and family *Lactobacillaceae*. They are gram positive, rod shaped bacteria. Their optimal

growth conditions are between 30 and 40°C in temperature and a pH between 5.5 - 6.2. These anaerobes can tolerate an aerobic environment. Most lactobacilli have one or more plasmids ranging from 1.2 - 150 kb. They contain genes for lactase metabolism, drug resistance and bacteriocin synthesis. Lactobacilli prefer to grow in habitats with high levels of soluble carbohydrates, protein breakdown products and vitamins, in addition to low oxygen. The production of lactic acid reduces the pH of the surroundings which can suppress the growth of other bacteria.

Lactobacilli are commonly used as probiotics, because of their safety, functional and technological aspects. Many lactobacilli strains are considered commensal microorganisms with no pathogenic potential. For the majority of their commensal functions they must adhere and colonise well, under a particular physiological condition present in the designated area. These aspects have been closely related to their immune effects due to the prolonged contact with the lymphoid tissues. Their antimicrobial protection including production of bacteriocins, as well as lactic acid and hydrogen peroxide is thought to be important in preventing pathogen colonisation. The ability to adhere to epithelial cells has been shown to be an important factor in colonisation of mucous membranes, through involvement of cell surface glycoproteins, carbohydrates and lipoteichoic acid [32]. Increasing evidence has indicated that lactobacilli can modulate immunity by enhancing the local and systemic immune function [33-36].

1.3.4 Bacterial recognition by host

Toll-like receptors (TLRs) are important in immune activation by bacteria in the colon. They are transmembrane receptors that recognise repetitive patterns termed pathogen-associated molecular patterns (PAMPs) present on a diverse range of microbes, including gram positive and gram negative bacteria (figure 5). There are

10 TLRs identified to date that have been discovered in mammals, each with differing recognition products. Most commonly studied are TLR 2,3,4,5 and 9 whose recognition products are shown in table 1[37-46]. TLR4 signalling involves CD14 and MD-2 (lipopolysaccharide binding protein) and mice deficient in either protein are resistant to the effects of injected lipopolysaccharide (LPS)[47]. Combinations of TLRs are used to recognise certain PAMPs, for example TLR2 and TLR6 to recognise peptidoglycan and TLR1 and TLR2 to recognise triacylated bacterial lipopeptide, although in the case of over-expression systems TLR1 can inhibit TLR2 and TLR4[38, 48]. TLRs 2 and 4 can be inhibited by Tollip, an inhibitory adaptor protein that interferes with IL-1R-activated kinase (IRAK) [49]. Thus could be a possible mechanism used by commensal bacteria in allowing tolerance towards them in the human colon. TLRs are mainly expressed on innate immune system cells such as macrophages and dendritic cells; their activation results in the TLR's attached cell becoming mature, thus enhancing adaptive immunity via an increase in antigen uptake, cell surface presentation and cytokine production[50].

Table 1 *Toll-like receptors and the foreign particles they detect.*

TLR2	Gram positive products (LTA, PGN, STF[a]) and mycobacterial pathogens
TLR3	Double stranded viral RNA
TLR4	Lipopolysaccharide (Gram negative bacteria)
TLR5	Flagella
TLR9	Bacterial DNA

[a]LTA-Lipotechoic acid, PGN-Peptidoglycan (Requires TLR6 also) and STF-Soluble tuberculosis factor

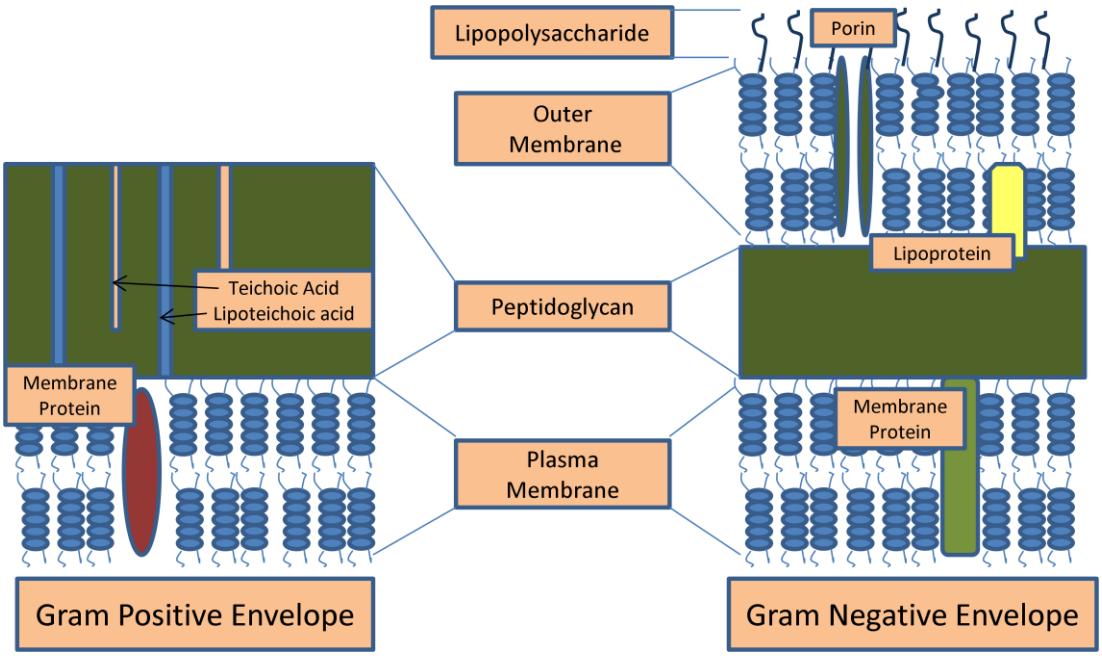

Figure 5 *The differences between gram positive and gram negative bacterial envelopes, to show that different receptors are often required to label and possibly destroy the varying types. The most distinguishing feature of gram negative bacteria are the lipopolysaccharides that can initiate immune response on attachment to many immune cells, although this layer can give protection against lysozymes and penicillin. Gram positive bacteria differs from gram negative bacteria predominately through its contents of teichoic acid and lipoteichoic acid, which can be used as chelating agents or for adherence to immune cells.*

Signalling is mostly documented to be through the Toll/IL-1R domain leading to the TIR domain recruiting TIR-containing adaptor molecules, including MyD88. This recruits IRAK and then TRAF6 making up the IKK complex. The subunits of the complex phosphorylate I-κB-α that inhibits NF-κB subsequently activating the molecule that translocates from the cytoplasm to the nucleus and drives expression of pro-inflammatory genes, such as cytokines, for example neutrophil chemoattractant IL-8 [51] (figure 6).

Figure 7 demonstrates a variety of pathways in which the TLRs can signal, showing that TLR4 also can lead to a MyD88 independent pathway. Eisenbarth et al. [52] found low LPS can cause a T helper 2 cell (Th2) response of IgE and an increase of IL-4, IL-5 and IL-13 but high LPS gives a T helper 1 cell (Th1) response with

neutophils and IgG2a. This research was supported by Kaisho et al. [53] that discovered the MyD88 independent pathway that may be the instruction for the Th2 development for response to LPS, whereas the MyD88 dependant pathway results in the Th1 response. Mast cells are an example of cells that respond to Th2 cytokines when TLR4 is exposed to its ligands[54-55]. It must be noted that TLR activation predominately favours a Th1 response as will be described later. Kalliomaki et al. [56] and Dabbagh et al. [57] proposed that this contradictory effect of responses can still be beneficial to prevent atopic disease (produced by an enhanced Th2 response,) since Th2 cytokine IL-10 does down regulate Th2 and Th1 responses that will not only halt allergic disease, but also could be valuable for inhibition of autoimmune disease (produced by a predominant Th1 response).

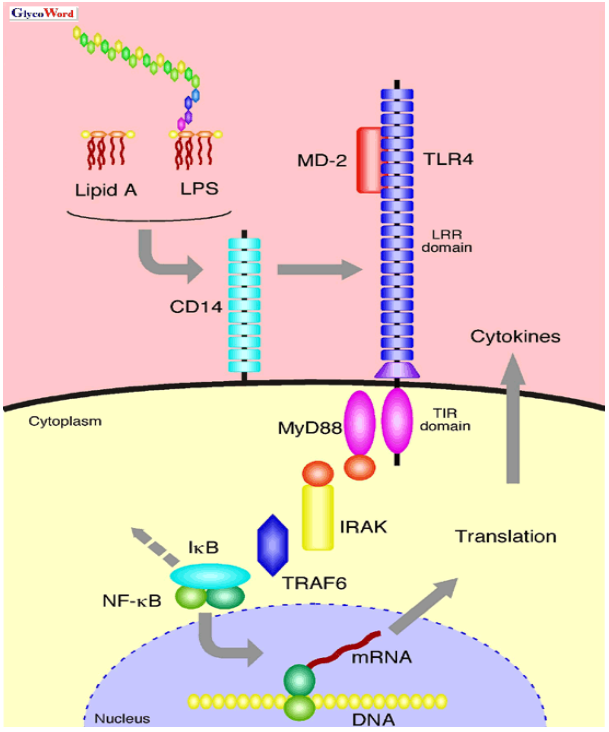

Figure 6 *TLR4 signalling, leading to the recruitment of MyD88 through the TIR domain. MyD88 aids IRAK and TRAF6 to form IKK complex resulting in the phosphorylation of I-κB-α that subsequently inhibits NF-κB. The result is expression of pro-inflammatory genes via NF-κB translocation to the nucleus. (Takada et al. [58])*

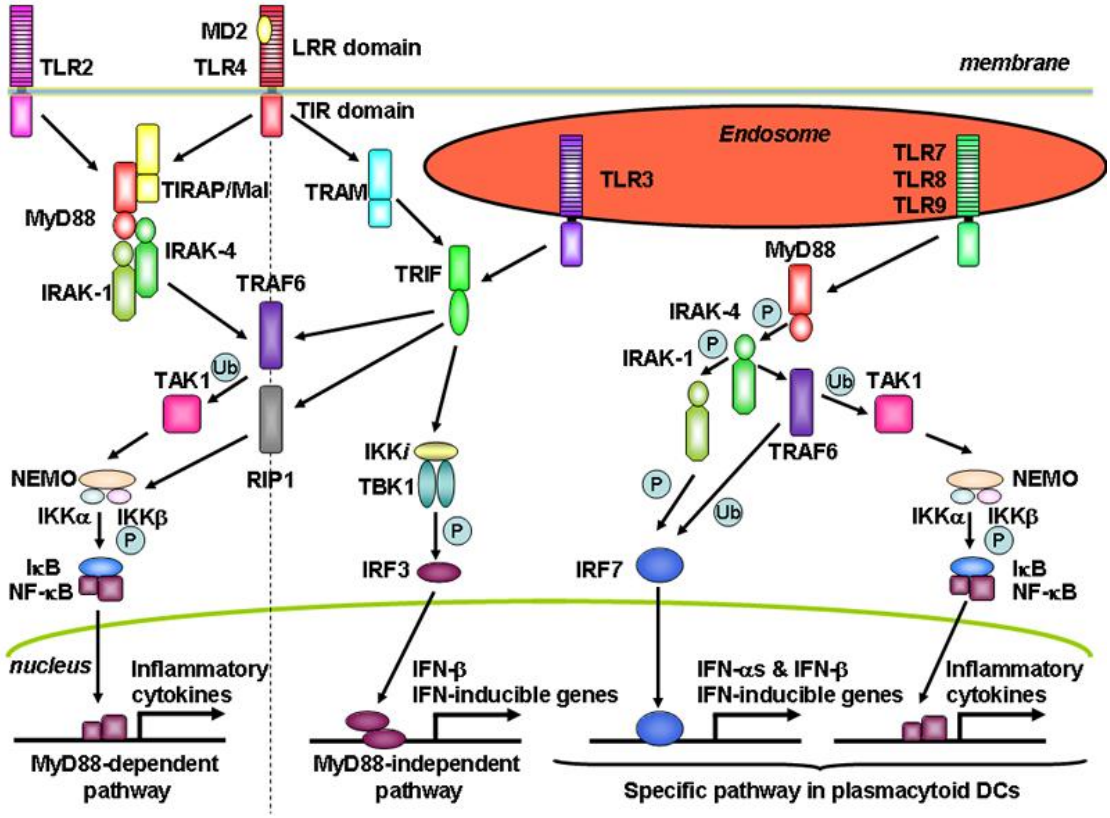

Figure 7 *Alternative pathways of TLR signalling that can bypass MyD88. The TLR4 signalling through the TIR domain leads to recruitment of TRAM followed by TRIF. As shown TLR3 also signals through TRIF, and both TLR 3 and 4 result in induction of type I interferons (IFNs) and IFN-inducible genes via IFN regulatory factor–3 activation. A recently identified adaptor molecule, Toll-IL receptor domain–containing adaptor protein/MyD88 adaptor–like, may participate in the MyD88-independent pathway[59]. (Takada et al. [60])*

TLRs trigger the secretion of antimicrobial peptides [61] and provide interactions between the innate and adaptive immune response by attracting adjacent lamina propria immune cells for the adaptive responses [62]. It has been more recently noted that commensally-derived TLR signalling can maintain intestinal homeostasis via enhanced barrier function, promotion of intestinal epithelial cells (IECs) proliferating and differentiating and a possible role in repair mechanisms following colonic cell injury [63]. The colonic IECs produce a low constitutive expression of key TLRs 2 and 4, but also TLR4s cofactors CD14 or MD-2, therefore limiting excessive TLR signalling [64] . Another protection from the stimulation from the

normal microfloral environment is that TLR5 is expressed only on the basolateral part of surface epithelial cells in the gut, thus requiring flagellin translocation or bacterial penetration of the epithelial barrier for stimulation [65].

Following TLR activation CD4+ cells may differentiate into either Th1 or Th2 cells as depicted in figure 8. Subsequently Th1 cells produce TNF-α, TNF-β (both lead to colitis), ref A, IL-2, IL-12 and IFN-γ. IFN-γ promotes TLR4 and MD-2 signalling, leading to IL-8 secretion in the presence of gram negative LPS, and the subsequent inflammation that can also inhibit Th2 proliferation. Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-13 and IL-10, that inhibits Th1 function which can be beneficial in halting colitis [66]. Th1 cells give immunity against intracellular pathogens and are important for the activation of macrophages [67], whereas Th2 cells result in increased antihelminthic immunity, elevated allergic inflammation and production of IgE and IgG1, although IL-10 acts by suppressing atopy by anti-inflammatory actions [68].

Mueller et al. [69] observed cytokine affects in TLR mRNA expression. Th2 cytokines IL-4 and IL-13 gave a marked decrease in TLR3, TLR4 and MD-2 in T84 and HT29 cells. Also a 23 % and 29 % decrease in TLR2 by IL-4 and IL-13 was observed respectively, therefore giving a potential role in desensitising cells to viral RNA, gram negative bacteria and gram positive. This could be a positive effect in probiotic binding but can make the cell susceptible to bacterial and viral infection. However, the experiment showed that IFN-γ (Th1) increased mRNA expression of TLR2, TLR3, TLR4, TLR5 and MD-2 in T84 and HT29 cells, hence the potential of increasing sensitivity to bacteria and viruses. Highlighting that the ratio of Th1 to Th2 is important to obtain the correct efficiency of TLRs, so as to benefit both, tolerance to probiotics yet sufficient immunity against pathogens. In fact once competition between IL-4 and IFN-gamma was studied, IL-4 could almost completely attenuate

IFN-γ up regulation of TLRs and MD-2 mRNA. Comparison of TLR4 protein in IL-4, IL-13 but not IL-5 primed T84 and HT29 cells reflects TLR4 mRNA expression; the reasoning behind the lack of response by IL-5 is possibly due to absence of IL-5 receptor on the cell lines [69].

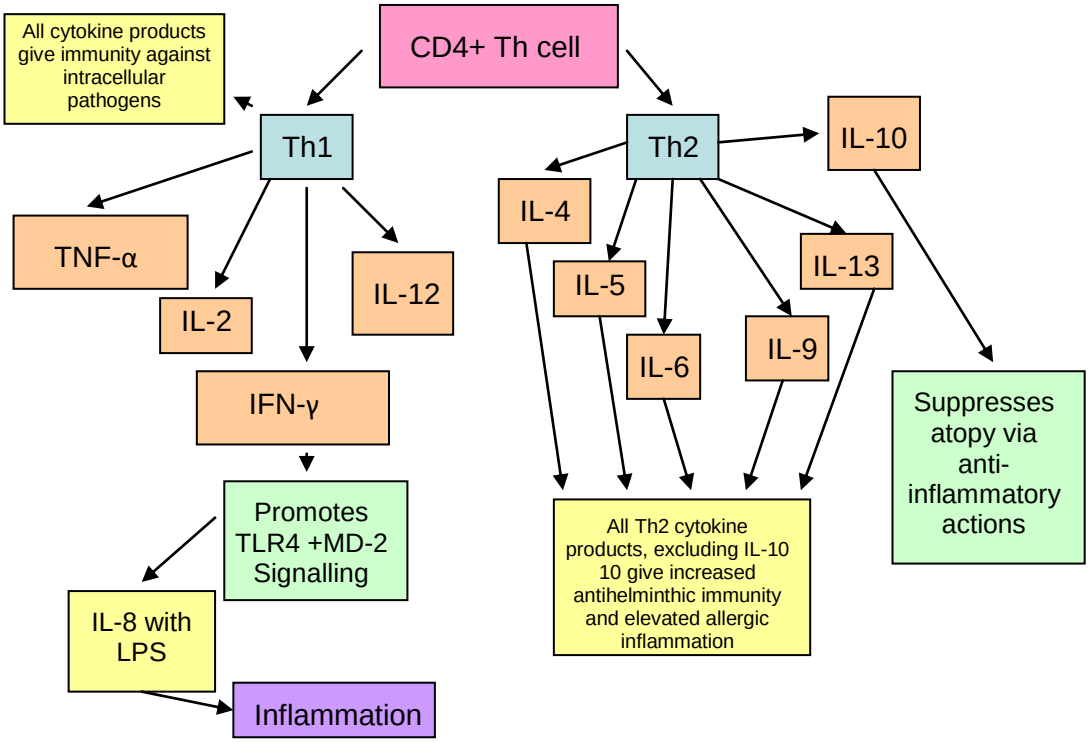

Figure 8 *T Helper Cell Differentiation into either T helper cell 1 or 2, each producing a different set of cytokines. Each cytokine has its own specific roles but many roles overlap between cytokines so they can act together, such as the T helper 1 cytokines all give immunity against intracellular pathogens. IFN-γ has a highly important role in detection of gram negative bacteria via increasing TLR4 signalling giving rise to inflammation and removal of the bacteria from intracellular compartments.*

Signal transducers Stat 4 and 6 are essential in differentiating Th1 or Th2 cells as shown in figure 9. IL-12 stimulates stat 4 to produce IFN-γ producing cells that lead to Th1 cell differentiation subsequently inducing more IFN-γ which can suppress Th2 differentiation. IL-4 leads to transcription of stat 6 leading to Th2 cell differentiation and blocking IFN-γ [68]. TLR activation increases Th1 response due to the

production of IL-12, IL-23 and IL-27 that also inhibits Th2 responses [70]. It has been found that MyD88 and Rip2 are important components in pathways leading to the Th1 response. Bacterial DNA and DNA containing unmethylated CpG motifs act as adjuvants to stimulate Th1 responses and this is through TLR9 activation that leads to CD4+ T cell stimulation to increase IFN-γ in response to the antigenic stimuli and therefore elevate Th1 cell differentiation [71].

TLR activation leads to antigen presenting cell (APC) maturation and expression of CD80/CD86, which are members of the B7 family that interact with CD28 on T cells. These are critical for the co-stimulation signal for activation and differentiation of naïve CD4+ T cells plus accelerated APC maturation and activation in an indirect manner where CD154 (a CD40 ligand on CD4+ T cells), binds to APC's CD40 resulting in APCs production of cytokines that increases it's own expression. APCs particularly dendritic cells can favour CD4+ differentiation into Th1 or Th2 cells dependant on cytokine milieu. Il-12, IL-23 and IL-27 give rise to Th1 cells producing IFN-γ and IgG2a, while IL-4 and other poorly understood factors produce Th2 cells. TLR's predominately induce IL-12 cytokine production and thus Th1 cell types are favoured [57].

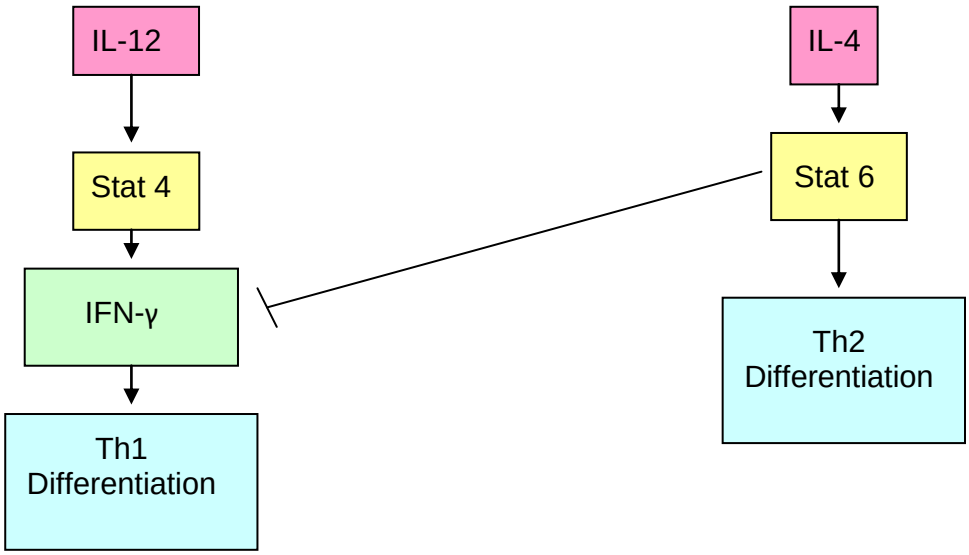

Figure 9 *Signal transducers involved in T helper cell differentiation. Certain cytokines produced by the specific T helper cell type play a role in their differentiation, for example T helper cell 1 (Th1) cytokine products IL-12 and IFN-γ. The pathways are linked by the inhibitory action of Stat 6 on IFN-γ resulting in more T helper 2 (Th2) and less T helper 1.*

More recently other molecules have been shown to be involved in the innate immune response such as nucleotide-binding olligomerisation domain (Nod) molecules. To date, the cytoplasmic surveillance molecules Nod1 and Nod2 have been discovered, both recognising peptidoglycan (PGN) that exists on the outer layer of gram positive bacteria but also under the outer membrane of gram negative bacteria. Nod1, present in intestinal epithelial cells[72] detects muropeptide PGN that presents diaminopilemicacid (DAP) which is derived from gram negative bacteria. Nod2, present in macrophages, dendritic cells and induced on enterocytes[72], detects muramyl dipeptide (MDP) fragment of PGN that derives from both gram positive and negative bacteria [73]. The stimulatory activity of MDP is poor and a vehicle such as mineral oil or lipid modification is required to enhance its activity and these factors possibly aid the internalisation of the molecule into cells for interaction with Nod2[73]. Patients with Crohn's disease have been found to lack

Nod2 and possibly Nod1, a loss of this surveillance activity may result in the inability of local responses, therefore intestinal mucosa cannot control bacterial infection resulting in systemic response and aberrant inflammation [74]. For infection, a pathogen must gain access to the internal environment of the host by invading tissue or closely interacting with the cells. In the colon it has been previously discussed that there is a low level of bacterial detection due to the requirement of commensal bacteria colonisation. These alone provide a variety of mechanisms to eliminate pathogenic bacteria, but in the event of pathogen invasion the Nods have an important role in the detection of them. Macrophages and dendritic cells (DCs) within the submucosa are surveillance cells ready to engulf bacteria that cross the epithelial barrier and destroy the microbes. If this mechanism fails the systemic immune response is activated [73].

SECTION 1.4 Interactions between Polyunsaturated fatty acids and lactic acid bacteria.

1.4.1 Current evidence

Previous studies have shown that PUFAs can modify adhesion of lactobacilli in the intestinal mucosa. In particular Bomba et al. showed that diets fed to gnotobiotic piglets, containing high n-3 PUFAs, increased adhesion of inoculated *Lactobacillus paracasei* to jejunal mucosa [75]. In addition Ringo et al. showed n-3 linolenic acid fed arctic charr have a higher frequency of intestinal lactic acid bacteria [76]. Both studies suggest that PUFAs may affect adhesion sites of lactobacilli by modifying the lipid composition of the intestinal epithelial cell membrane. Studies by Kankaanpaa et al. showed an inhibitory effect of PUFA on the adhesion of many *Lactobacillus sp.* However, one strain, *Lactobacillus casei* Shirota, adhered better to human intestinal epithelial cells grown with n-3 α linolenic acid [77]. Kankaanpaa et al. suggest that within the genus of lactobacilli there are many strain specificities and there is a possibility that the beneficial effects of PUFAs on the adhesion of lactobacilli are not effective in human epithelial cells. Broiler chickens are another model used for studying the effects of PUFAs on lactobacilli and studies have shown that diets high in PUFAs can change *Lactobacillus sp*. profile in the intestinal contents[78]. This evidence supports the hypothesis that adherence of lactobacilli is strain specific and some adhere better to epithelial surfaces following PUFA exposure.

In humans it is estimated that 2 % of ingested PUFAs reach the colonic contents[79], thus they will have direct contact with the resident bacteria. Kankaanpaa et al. showed that direct interaction of n-3 PUFAs with lactobacilli inhibited their growth, although n-6 arachidonic acid in low concentrations (5 µM)

promoted growth[77]. Additional studies have also shown that: bacterial strain, fatty acid concentration and fatty acid type, all affect the result of interaction of PUFAs and lactic acid bacteria. For example n-6 linoleic acid inhibited *L. reuteri* ATCC 55739 growth, while there was no effect on *L. reuteri* ATCC 23272 [80].

In conclusion, there are only a few studies to support the varied effects of PUFAs on lactic acid bacteria, either in relation to survival or adhesion. More strains need to be studied to give a clearer picture of strain specificities and the mechanisms of PUFAs actions need to be explored. For example, looking at the uptake of fatty acid in human epithelial cell membranes and also experimenting on other avenues that could influence an effect of PUFAs on bacteria, such as immune modulation.

1.4.2 Modification of immune responses

Probiotics have been shown to reduce dietary antigens and allergens by degradation and modification, thus leading to the Th1/Th2 shift to move towards Th1 (figure 10) [81]. This alteration towards Th1 domination is aided by probiotics ability to increase TGF-β that can suppress Th2 cells in the colon and induce oral tolerance (beneficial to probiotic survival when in large numbers)[68], but can also suppress TNF-α (Th1 cytokine) aiding in the prevention of increased intestinal permeability, thus inhibiting pathogen invasion. This is modulated further by probiotic enhancement of tight junctions that can increase protection from pathogens gaining entry to cells beneath the epithelial layer of the colon [82]. An elevation of IgA is a response to probiotic leading to protection against pathogens on the mucosal surface [83]. Probiotics produce an anti-bacterial substance, and compete for enterocyte adherence with pathogens, explaining protective mechanisms in which probiotics enhance the immune function [82]. In newborns,

Th2 responses are dominant; therefore probiotics are important to move the shift towards Th1 responses to give elevated pathogenic immunity.

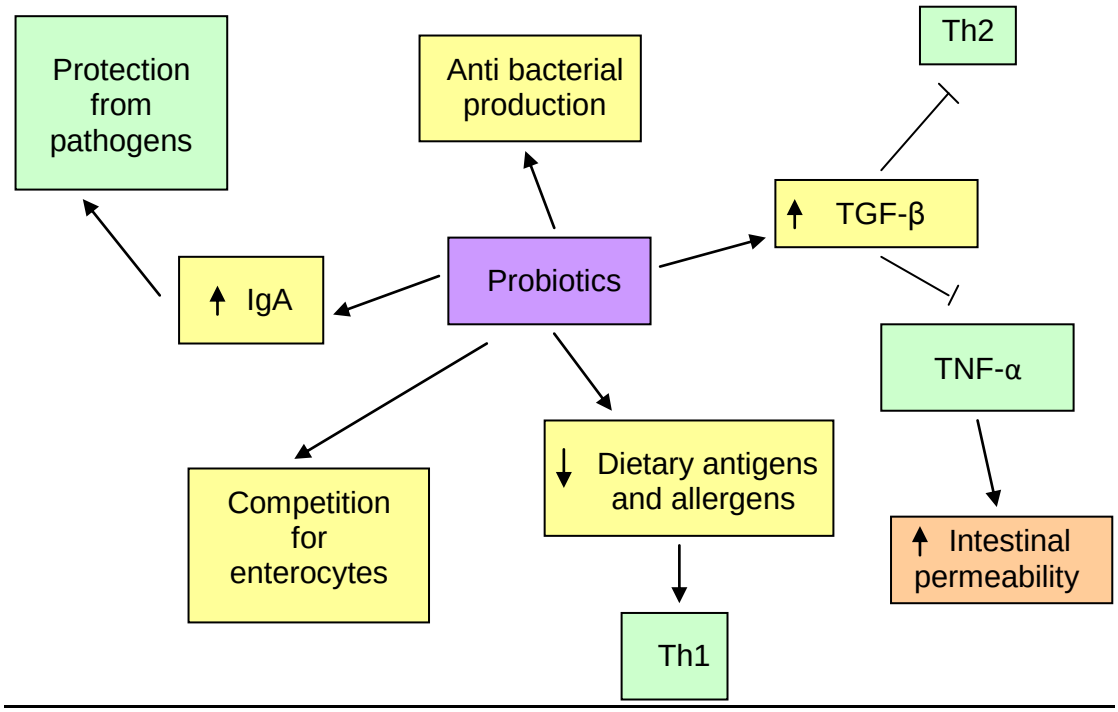

Figure 10 *Probiotics shift T helper cells towards Th1 via an increase in TGF-β subsequently inhibiting Th2 cell differentiation, leading to a decline in inducer Stat 6 that can inhibit IFN-γ and subsequently stop Th1 cell differentiation. Alternatively, a decrease in dietary antigen and allergens through probiotic innate surveillance will lead to the production of more Th1 cells as Th2 cell production will not be required to remove them.*

In contrast, Das et als. reviewed [68] PUFAs effects on the Th1/Th2 shift and found that γ-linolenic and AA, both n-6 fatty acids, and EPA and DHA, both n-3 fatty acids, suppressed immune response by reducing cytokines IL-1, IL-2 and TNF-α, predominately in blood cells[84-85]. However, this effect is enhanced in the EPA and DHA treated cells. Thus n-3s are shown to induce immune suppression more than n-6s. This effect is verified when considering individual PUFAs. AA significantly increased pro-inflammatory leukotriene B4 and prostaglandin E2 but had no effect on Th1 cytokines whereas n-3 fatty acids decrease TNF-α by peripheral blood monocytes and reduction of inflammatory mediators thromboxanes

B2 and B3, but n-6 gave no change [86]. EPA and DHA augmented TGF-β that, as mentioned previously can suppress the Th2 cytokines, but also suppresses TNF-α (Th1 cytokine) biosynthesis and release[87], hence giving comparable effects between long chain polyunsaturated fatty acids (LCPUFAs) and probiotics, through an ill-defined characterisation of the Th1/Th2 shift. Even though the many other factors involved mostly favour the fact that probiotic will give a Th1 shift while n-3 PUFAs give a Th2 shift in theory. This may be problematic when considering immunity against pathogens via Th cell differentiation, but there is protection through PUFAs antibiotic-like actions [88]. Interesting, evidence summarised in Calder et al. [89], showed fish oils decrease inflammatory eicosanoids and adhesion molecules i.e. VCAM1, possibly revealing a reason for n-3 fatty acid elevation of probiotic adhesion via a decrease in cytokines. Although, if VCAM1 were to be shown to be a factor in certain probiotic adhesion it may have the opposite effect. It is important to note that this work was in endothelial cells and that the effects maybe very different in the colonic mucosa as Lima-Storegjerde et al. [90] previously described.

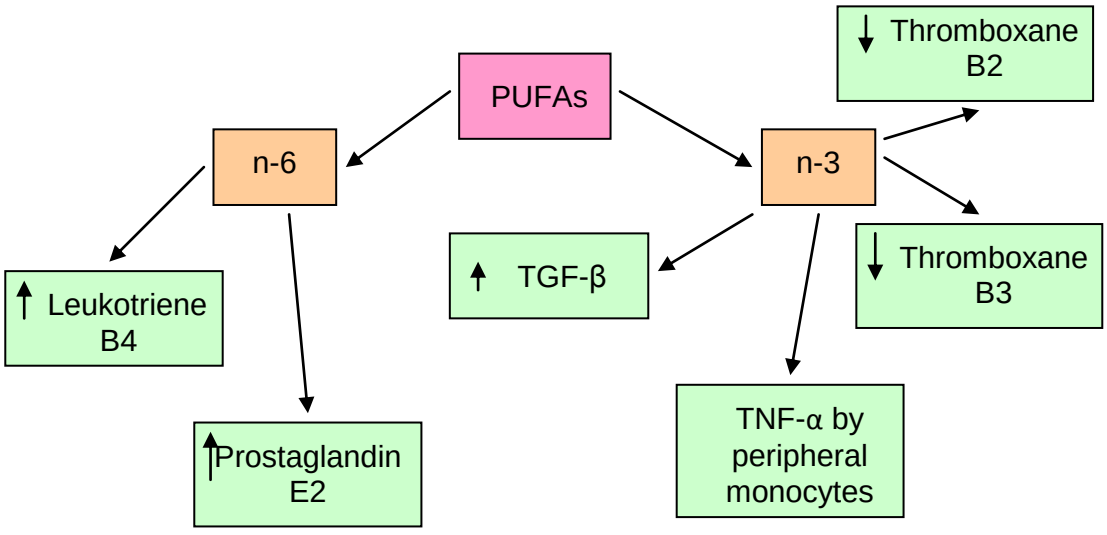

Figure 11 *N-3 PUFAs shift T helper cells to Th2. EPA and DHA fatty acids suppress Th1 cytokines IL-1, IL-2 and TNF-α, also inflammatory mediators; thromboxane B2 and B3. Although n-6 PUFA's increase pro-inflammatory leukotriene B4 and prostaglandin E2. The elevation of TGF-β can inhibit Th2 cell differentiation, although there is evidence that it can also inhibit TNF-α biosynthesis and release.*

Although, probiotics and LCPUFAs have been shown to act together by promoting the adhesion of probiotics to IECs, this area is a new field of research. However, Kankaanpaa et al. [77] demonstrated elevated adhesion of *Lactobacilli casei* by α-linolenic (n-3) fatty acid. The increased adhesion to intestinal mucosal cells may enhance development of gut-associated lymphoid tissue by direct interaction and by probiotics and LCPUFAs ability to augment growth factors, such as TGF-β and various cytokines. Overall both are necessary for gut development and physiological balance between Th1 and Th2 responses[68].

Prebiotics are becoming increasingly studied as the non-digestible oligosaccharides fermented by probiotics. These augment the levels of short chain fatty acids that again elevate IFN-γ (Th1 cytokine) [91], up regulate TLR expression and are an energy source for colonocytes, thus aiding in maintenance of the intestinal epithelium [92]. Another important role is their ability to decrease the pH of the environment, which favours the survival of probiotics in the colon, therefore on administering probiotics it is equally as important to include prebiotics to raise the potential beneficial effects.

There is currently a focus on research investigating the recognition of probiotic bacteria and their protection from our immune systems, as yet poorly understood tolerogenic mechanisms. At birth the immune system is compromised, one reason being delayed maturation of CD4+ cells and inhibition of self-reactive T cells by α-fetoprotein present in the foetus. In neonatal life Th2 cells are dominant, thus humans lack immunity against pathogens at this time of life [93]. It is therefore possible that when Th1 cells develop the microflora has already become resident and due to adhesions with self cells is also recognised as self. The innate immune response will ignore self cells unless they change their surface structure and the

adaptive immune response allows immature clones of lymphocytes with antigen receptors resulting in an unresponsive state on interaction with an antigen. This gives reasoning to the fact that it is hard to modulate microflora content during later life supported by long-lived T cell memory [94], although there are ways of gaining acquired tolerance. One path is via active suppression by Th2 cells due to the production of inhibitory cytokines TGF-β and IL-10. Since probiotics lead to TGF-β production, this is an interesting potential mechanism by which probiotics can survive host immune response. Another route of acquired tolerance is by administering high doses of antigen leading to T cell tolerance [95], this explains the binding of probiotics by a high dose live yogurt for example [96]. This is still thought to be short lived giving protection for only up to one week, thus finding ways to increase their adhesion would be useful in enhanced probiotic protection.

As discussed previously (section 1.3.4), TLRs are used for recognition of bacterial components and thus will not distinguish between probiotics and pathogenic bacteria. Fortunately TLR2 and TLR4 are inhibited in the colon by Tollip [49] allowing avoidance of immune response towards probiotics. Although Tollip is not present in the lamina propria with macrophages expressing these receptors, therefore if a pathogen invades the epithelial layer recognition can still occur by TLRs.

SECTION 1.5 Cell lines

1.5.1 Isolation and characterisation

Development of normal colon cell lines has been difficult, due to lack of viability and loss of differentiation markers [97]. This has resulted in the wide use of colorectal tumour cell lines that most importantly have immortality. The first human colon carcinoma cell line to be developed was HT29 [98] and since then many lines have been developed from human carcinomas, giving a highly diverse range of cell type and differentiation. By varying culture conditions some of the cell lines were able to express differentiation characteristics of mature intestinal epithelial cells, e.g. mucus secretion (HT29-MTX) [99]. These cell lines are important models for intestinal biological research and provide a basis for many studies since they are relatively inexpensive and produce replicable results. Currently they are used extensively in experiments involving adhesion and penetration of bacteria and viruses. Although, in many cases studies will need to be followed up by primary cell line, animal models or human testing before results can be verified, the tumour models can give a good idea of what is worth focusing future research on and what may benefit humans or animals.

1.5.2 Caco-2 cells

Caco-2 cells were isolated from a well differentiated tumour; however in early culture the cells remain undifferentiated. Once confluent, the monolayer become polarised and are joined by tight junctions that result in resistance much higher than that of the normal colon [100]. As cells differentiate apical microvilli become well developed and cells produce disaccharides and peptidases commonly associated with small intestinal cells. The cell line is of particular interest to those studying transport

properties, since Caco-2 cells transport ions and water to the basolateral membrane [101].

1.5.3 HT29 and HT29-MTX cells

HT29 cells were isolated from a human adenocarcinoma. With glucose and serum supplementation as standard culture conditions they remain undifferentiated and grow as a multilayer of un-polarised cells, often described as exhibiting human fetal colon analogies [102-103]. However, changes in culture condition can lead to differentiation, for example treatment with the anticancer drug methotrexate (MTX) with increasing concentration up to 10^{-7} mol/L results in a mixed population of adsorptive and goblet cells. Higher concentrations (10^{-6} mol/L) result in the goblet cells secreting gastric mucins, whilst 10^{-4} mol/L results in differentiation from mucus to adsorptive phenotype. The changes have been linked to amplification of a gene encoding the target enzyme for MTX, named dihydrofolate reductase [104].

1.5.4 Innate immune responses

In the normal healthy colon mRNA levels of TLR3, TLR4, TLR5 and TLR7 are expressed [105] and a low level of mRNA for TLR2 is reported expression of the protein does not occur in IECs due to the presence of Tollip. However, macrophages in the lamina propria do express TLR2; thus allowing adherence of gram positive bacteria to IEC while maintaining protection if the pathogen enters the cells to reach lamina propria. Inflammation has been shown to increase levels of TLR2 and TLR4 by decrease of TLR1 rather than change in mRNA expression [106]. It is important to note that mRNA levels do not always match the level of the protein expressed and are therefore not a realistic view of TLR activity.

Experimental lines HT29 and Caco-2 revealed differing levels of TLR mRNA. The undifferentiated HT29 cell line increased TLR2 mRNA in response to gram positive bacteria, TLR3 with *Bifidobacterium bifidum* and TLR 4 with *Escherichia coli faecalis* only, indicating possible interference with probiotic bacteria adherence. Since the HT29 cells have been compared to newly formed crypt cells [103] it is hypothesised that probiotics only have an effect in newly formed cells and no later effects as shown via Caco-2's more mature cells. Caco-2 showed no significant difference in TLR 1, TLR2, TLR3 and TLR4 mRNA levels and even a slight decrease in TLR 2 and TLR4 on addition of a selection of bacterium. Also it is important to note that in this experiment only live bacteria affected the HT29s [103]. Melmed et al. [106] showed no presence of TLR2 protein expression in the HT29 cell line. Therefore it is likely there are inhibitors of TLR2 in HT29 as in human IECs, again showing they are potentially more similar and a better line to study for TLR work in the colon. However, Caco-2 showed that TLR2 protein was present with a lower TLR1 level, thus this is likely to be the important receptor for bacterial immune recognition. Melmed et al. [106] also compared Caco-2 cells to the cells of the small intestine while HT29s are similar to colonic cells, thus experimental results demonstrate a varied response to bacteria along the intestine.

SECTION 1.6 Objectives

The project aims to assess the impact of dietary PUFAs such as those found in plant and fish oils on probiotic bacterial survival in the colon. Probiotic bacteria have been shown to be potentially useful in maintaining colonic health and improving mucosal immune homeostasis, an effect which may be important in relation to healthy ageing as well as ameliorating the symptoms of inflammatory bowel disease (Ulcerative Colitis & Crohn's disease). PUFAs are also known to be potentially beneficial in this respect and so manufacturers may try to combine both ingredients in 'functional foods' but we know nothing about how these two factors interact.

Two *in vitro* approaches will be used in these studies. Firstly, the impact of physiologically relevant doses of dietary PUFAs on growth rates of different strains of lactobacilli will be measured using turbidity measurements (Bioscreen). Secondly, the same strains of lactobacilli will be co-cultured with human colon cell lines (HT29, MTX-mucin secreting & Caco-2). Confluent cell cultures will be pre-treated with a range of different PUFAs to modify cell lipid profiles prior to exposure to lactobacilli and measurement of bacterial cell adhesion and mammalian cell cytokine release will be measured. Data from gas chromatographic analysis of PUFA incorporation into human and bacterial cells will be used to gain further insight into how they might influence the end-points being assessed. Thus this study is particularly novel in that it investigates the three way interaction of: host-bacteria, diet-host and diet-bacteria in considering the colon as a complex system integrating knowledge from three different biological specialities (figure 12).

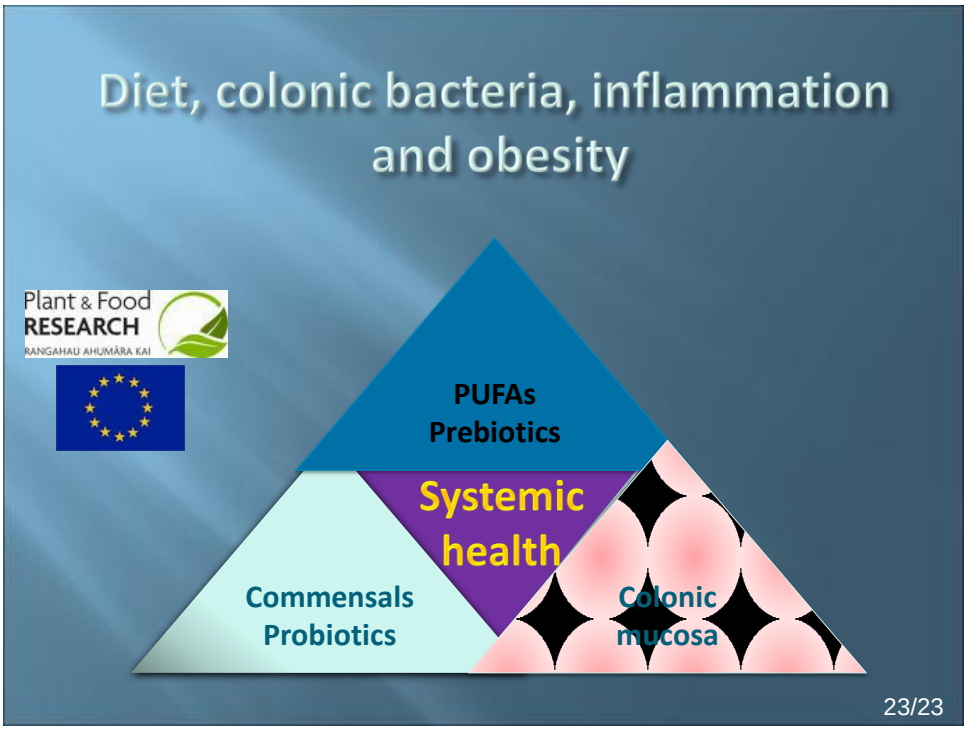

Figure 12 *The interactions between dietary PUFAs, prebiotics, commensals and colonic mucosa with regards to systemic health.*

CHAPTER 2: Methods

2.1.0 Cell culture

2.1.1 Maintenance of cell stocks

Stock cultures of HT29◊, HT29-MTX† and Caco-2◊ cells (European collection of cultured cells◊ and Dr. T. Lesuffleur, INSERM, France†) were grown in 75 cm^2 flasks at 37°C with 5%CO_2/95% atmospheric air at constant humidity in RPMI +Glutamax (Invitrogen) supplemented with 10%v/v foetal calf serum (FCS) (Sigma-Aldrich) and 2%v/v streptomycin penicillin (Invitrogen). The volume of medium in each flask was 20 ml and was changed every two days. Cells were used between passages 1-10 from purchased stocks.

2.1.2 Cell passaging and seeding

Cell lines were passaged at 5-7 days at 80-90% confluence by removing medium from cells and adding 6 ml of 1xTryspin EDTA (Invitrogen). Cells were then incubated at 37°C for 5 minutes and 1xTryspin EDTA was deactivated by 6 ml RPMI +Glutamax. In the case of HT29-MTX, cells were scraped using a cell scraper after tryspin treatment. Cell suspensions were centrifuged at 1000 rpm for 5 min, after which the medium was removed. 10 ml fresh medium was added to the resultant cell pellet and mixed thoroughly by titration to break-up cell clumps and give a homogenous cell suspension. A 10 µl sample of the cell suspension was then counted using a bright line haemocytometer with 4 fields counted and averaged.

The resulting cell count was corrected by multiplying by 10,000 to obtain the amount of cells in 1 ml of cell suspension. The figure was used to calculate the volume of

cell suspension needed for a specific cell seeding density in a flask, well plate or Transwell. The µl volume of cell suspension required for a seeding density follows a formula of Z= (1000/n) x; where Z =µl volume of cell suspension; n=cell count/ml and x= total number of cells required.

2.1.3 Freezing and thawing cells

Cells were harvested with tryspin (see section 2.1.2) at 70-100% confluency. 900 µl cell suspension were aliquoted to a 1 ml cryopreservation vial (Nunc) and 10 % (v/v) DMSO added. Tubes were place in "Mr Frosty" (Fisher Scientific, Leicestershire, UK), and placed in -80°C for 24 h after which tubes were transferred to liquid nitrogen.

Frozen vials of cells were taken from liquid nitrogen and warmed at 37 °C until partially defrosted. Cells were then added to pre-warmed medium and centrifuged at 1000 rpm for 5 minutes. Finally the cells were resuspended in 20 ml fresh medium and seeded in a 75 cm^2 culture flasks.

2.2.0 Bacterial culture

2.2.1 Strains

The following strains were used in this study

- *Lactobacillus johnsonii FI9785* (*L. FI9785*) -Chicken faecal isolate from in house culture collection, Institute of Food Research.

- *Lactobacillus gasseri* ATCC 33323 (*L. gasseri*) -Human isolate from the German Resource Centre for Biological Material.

- *Lactobacillus casei R1 (L. R1)* -Human faecal isolate from Carmen Nueno-palop, Institute of Food Research.

- *Lactobacillus casei 44* (*L. 44*) -Human faecal isolate from in house culture collection, Institute of Food Research.

- *Lactobacillus L2* (*L. 2*) -Human faecal isolate from in house culture collection, Institute of Food Research.

- *Lactobacillus L10* (*L.10*) -Human faecal isolate from in house culture collection, Institute of Food Research.

2.2.2 Maintenance of cell stocks

Stock cultures were grown in De Mann, Rogosa and Sharpe media (MRS) inoculated at 1%v/v for 9 or 16h at 37°C.

2.3.0 Fatty acid uptake

2.3.1 Cell preparation

HT29, HT29-MTX and Caco-2 cells were grown in 75 cm2 flasks and seeded at 50 x 10^4 cell/ml until confluent (approximately 2 days). HT29 cells were treated with 50, 100, 150, 200 µM EPA, DHA, AA or 10 µl ethanol (control) in RPMI + Glutamax (5%v/v FBS and 2%v/v streptomycin penicillin) for 48 h, whilst HT29-MTX and Caco-2 cells were treated with 50 µM fatty acid treatments only. Cells were removed with 6 ml tryspin-EDTA, washed in RPMI + Glutamax and pelleted to remove RPMI. Cells were frozen at -20°C until further analysis.

2.3.2 Bacterial preparation

L. FI9785, *L. 44* and *L. R1* were inoculated at 1 % in MRS and grown for 24 h with supplementation 20 µM EPA, DHA, AA or oleic acid (OA) 0.06 % ethanol (control) in 20 ml bijous. Cells were washed in phosphate buffered saline (PBS) 3 times and pelleted.

2.3.3 Total fatty acid extraction

Lipids were extracted using the Bligh and Dyer method (1959)[107]. Cells were homogenised with 0.8 ml water in a 2 ml dram vial (bacterial samples were boiled for 10 mins at 100 °C), 1 ml of chloroform/BHT (49:1) and 1 ml of methanol was added to each sample and vortexed. 1 ml of water and 1 ml of chloroform/BHT (49:1) was added to the dram vial and vortexed. Samples were centrifuged at 1800 rpm at 4^{o}C for 5 mins. The bottom layer was extracted via pipetting and added to a pre-weighed 2 ml dram vial. To non-lipid residue 1 ml of chloroform/BHT (49:1) was added and centrifuged at 1800 rpm at 4^{o}C for 5 mins and the bottom layer was extracted once again into the pre-weighed dram vial. The extract was dried under oxygen-free nitrogen and stored at -20 ^{o}C.

2.3.4 Phospholipid extraction

1 ml of chloroform/BHT was added to the dried extracts (section 2.3.2) and 0.5 ml of the mixture was set aside for phospholipid extraction, prior to re-drying the remainder for total lipid analysis of samples. The phospholipid fraction was extracted using Sep-Pak Light silica cartridge columns (Waters Corporation, Milford, Massachusetts, USA). A vacuum tank was assembled with 2 dram vials in place for each sample; a syringe was placed above the cartridge column of dram vial 1. The syringe was rinsed with chloroform without allowing it to run dry. 1 ml chloroform

was added to the syringe followed by 0.5 ml total lipid extraction sub-sample. 1 ml chloroform was added, then 2 ml chloroform/methanol (49:1). The syringe was moved to a 2nd position on the vacuum and phospholipid fraction collected with 2.5 ml methanol x 2 added to the syringe until dry. Samples are then dried under oxygen free nitrogen and stored at -20 °C.

2.3.5 Fatty acid methylation

The extracted phospholipid and total lipid samples were converted to fatty acid methyl esters (FAMES) by acid methylation prior to gas chromatography (GC) analysis[108]. 0.5 ml dry toluene was added to dried lipid extractions and vortexed to dissolve lipid. 1 ml of 2 % (v/v) sulphuric acid in methanol was added and the solution was heated at 50 °C for 2h. After cooling, 1 ml neutralising solution (0.025M $KHCO_3$ AND 0.5M K_2CO_3) and 1 ml dry hexane was added and the solution was vortexed. The sample was centrifuged at 1000 rpm for 2 mins at room temperate with low brake and the upper phase collected in a dram vial. The methylated sample was dried under oxygen-free nitrogen at 40 °C and resuspended in 150 µl hexane. The sample was then transferred to a gas chromatography vial and stored at -20 °C[109].

2.3.6 Gas Chromatography analysis

Lipids were quantified by GC using Trace MS plus with GC ultra and triplus autosampler (Thermo Electron Corporation). GC column specifications were 30m x 0.22 mm x 0.25mm (SGE BPX70) and samples were injected split flow 22 ml/min. Program temperature was 140 °C to 200 °C at 5 °C/min and held for 11 mins, then to 220 °C at 10 °C/min and held for 5 mins with a helium flow rate at constant pressure (1 ml/min). Samples were calibrated using a standard FAME mix

(Supelco PA-USA, F.A.M.E Mix GLC-10) and each peak using mass spectrometer data to predict the compound using NIST mass spectral library with search program (version 2.0). Areas beneath each peak were converted to percentages of the total area of all fatty acid methyl ester peaks, those found not to be a fatty acid methyl ester were omitted from total area.

2.4.0 Method development

2.4.1 Scanning electron microscopy

HT29, HT29-MTX and Caco-2 cell were seeded at 50 x 10^4 cell/ml in 24 well transwell plates until confluent, washed 3 times with RPMI + Glutamax, followed by 48 hours in RPMI + Glutamax (5 % fetal calf serum only), followed by 4 washes with RPMI + Glutamax leaving the final solution on the cells prior to fixing with 3 % glutaraldehyde (Agar Scientific, Stansted, UK) in 0.1 M cacodylate buffer (pH 7.2) for 2 hours. The fixative was then replaced with 3 changes of 0.1M cacodylate buffer. The cells were then dehydrated in a series of ethanol solutions (10, 20, 30, 40, 50, 60, 70, 80, 90, 3x 100 %) for at least 30 mins in each. Samples were dried in a Polaron E3000 critical point drier using liquid carbon dioxide as the transition fluid. The filters were then removed from the wells and attached, using sticky tabs, to aluminium SEM stubs (Agar Scientific, Stansted, UK) with the cell layer facing upwards. The samples were coated with gold in an Agar high resolution sputter-coater apparatus. Scanning electron microscopy was carried out using a Zeiss Supra 55 VP FEG SEM, operating at 3kV.

2.4.2 Bacterial identification using 16S rDNA gene sequencing

To determine the species of faecal isolates *L. 44* and *L. R1* 16S sequencing was preformed to compare with the 16S genome database (NCBI, Blast search). 16S sequence of the DNA was amplified using 1 µl of each primer 08F 5'-AGAGTTTGATCATGGCTCAG-3' (forward) 1391R 5'-GACGGGCGGTGTGTRCA-3' (reverse) mixed with 1 colony of *L. 44* or *L. R1* suspended in 39 µl sterile water, 5 µl 10 x buffer, 4 µl dNTP (2.5mM), 0.2 µl Taq polymerase (Qiagen). A PCR program heated sample at 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 11 min. PCR product was checked by running on an agarose gel and purity was assessed by nanodrop. PCR product was cleaned with Qiagen RNA/DNA maxi kit following manufacturer's instructions prior to sending to John Innes Centre for sequence analysis. Forward and reverse sequences were aligned using DNA star (SeqMan NGen v2.1) to generate a consensus, prior to input of the sequence into a NCBI Blast search.

2.4.3. Quantification of optical density

To allow estimation of bacterial colony forming units (CFUs), calibration curves were constructed to produce equations that would predict bacterial numbers from the optical density of a solution in PBS. *L. FI9785*, *L. gasseri*, *L. 44* and *L. R1* grown for 16 h were washed 3 times in PBS. Dilutions of 1/2, 1/4, 1/16 and 1/32 were made and optical densities were read. 1/10 dilutions of each previous dilution were made until 10^7 and 3 times 20 µl 10^4-10^7 dilutions were added to MRS plates to CFUs. These were converted to actual bacterial numbers/ml by the equation Z=50yx; where Z= actual bacterial number; y= dilution and x= average of three CFUs. Number of bacteria was plotted against optical density read for each bacterial strain and the equation for the curve was calculated in Microsoft Excel (2003).

2.4.4 Growth in PBS

To check whether bacteria number would not increase during a 3 h incubation period in PBS, which was the planned time that subsequent adhesion experiments would use for exposure to bacteria, approximately 10^9 CFUs of *L. FI9785*, *L. 44* and *L. R1* where added to 1 ml PBS. Growth was assessed by counting CFUs after plating a series of dilutions on MRS plates following 0, 1, 2 and 3 h.

2.4.5 Numbers of washes required to remove un-adhered bacteria

To assess the number of washes required for sufficient removal of un-adhered bacteria following 3 h exposure of human colonic cells to bacteria, thus to obtain an accurate adhesion number, 10^9 CFU s *L. FI9785*, *L. 44* and *L. R1* in 1 ml PBS were added to confluent 24 well plates of HT29 cells for 3 h. Bacteria were removed with 2- 5 washes of 1 ml PBS followed by addition of 1 ml of 1% triton (Sigma-Aldrich). HT29 cells with un-adhered bacteria were removed with magnetic fleas on a magnetic stirrer for 10 mins. Dilutions were plated on MRS plates to determine cell numbers, the wash that gave no significant difference in bacteria number from the previous number of washes was taken as the washes required.

2.5.0 Adhesion experiments

2.5.1 Cell preparation

HT29, HT29-MTX and Caco-2 cells were grown in 24 well plates seeded at 50 x 10^4 cells/ml until confluent. Cells were washed in RPMI + Glutamax 3 times to remove antibiotic medium and treated with 50 µM EPA, DHA, AA or 0.3 µl ethanol (control)

in RPMI + Glutamax (5 % FCS) for 48 h. Cells were washed in RPMI + Glutamax 3 times prior to bacterial adhesion.

2.5.2 Bacteria viable count method

L. FI9785, *L. 44* and *L. R1* were inoculated at 1 % in MRS and grown for 16 h. The bacteria were washed 3 times in PBS and diluted to 1 x 10^9 CFU/ml using optical density conversion graphs (method section 2.2.3). 1 ml of bacterial solution in RPMI +Glutamax were added to each fatty acid pre-treatment in triplicate and allowed to adhere for 3 h of incubation at 37°C. Bacteria were washed 4 times in PBS followed by 1 ml of 1% triton (Sigma-Aldrich) and a magnetic flea. Plates were placed on a magnetic stirrer for 10 mins to remove bacteria and cells. The bacteria were diluted to 10^7 and plated 3 times 20 µl on MRS plates. Average CFUs were used to quantify bacteria adhered.

2.5.3 Radiation method

L. 44, *L. R1* and *L. FI9785* were inoculated at 1 % in MRS and grown for 9 h at 37 °C. 2 ml of all bacteria were then inoculated separately into 8 ml MRS media followed by addition of 25 ul tritiated methyl thymidine, concentration 37 MBq/ml (GE healthcare) and incubated for 3 h at 37 ° C. Bacteria were washed 3 times in PBS by centrifugation at 4000g for 10 mins, followed by suspension in 1 ml PBS. Bacteria were diluted to approximately 1 x 10^9 CFU/ ml and 1 ml of radioactively labelled bacteria were added to 10 ml scintilent and 1 ml Tryspin-EDTA for obtaining a total radioactivity of bacteria added to human cells.

HT29, HT29-MTX and Caco-2 cells were grown in 24 well plates seeded at 50 x 10^4 cells/ml until confluent at 37 °C, 5 % CO_2 (approximately 48 h), washed in RPMI +

Glutamax 3 times to remove antibiotic and treated with 50 µM EPA, DHA, AA or 0.3 ul ethanol (control) in RPMI + Glutamax (5 % fetal calf serum) for 48 h at 37 °C, 5 % CO_2. Cells were washed in RPMI + Glutamax 3 times and 1 x 10^9 CFUs of tritiated labelled *L. 44*, *L. R1* or *L. FI9875* in RPMI were added for 3 h and incubated at 37 °C. Un-attached bacteria were removed by washing in RPMI (4 times) and cells with attached bacteria were removed using 1 ml Trypsin-EDTA. Cell/bacterial solutions were added to 10 ml scintilent and numbers of bacteria were quantified after measurement of radioactivity using a Liquid scintillation analyzer Tri-Carb 27000TR (Packard).

2.6.0 Immunomodulatory gene expression in intestinal cell lines

2.6.1 Culture of human cell lines

Human intestinal cell lines; HT29 and HT29-MTX were grown in RPMI + Glutamax (10 % fetal calf serum, 2 % Penicillin-Streptomycin) used unless stated otherwise. After at least 2 passages cells were grown in 6 well transwell plates seeded at 50 x 10^4 cells/ml until confluent. Then washed in RPMI + Glutamax 3 times and treated with 50 uM EPA, DHA, AA or 0.6 ul ethanol (control) in RPMI + Glutamax (5 % FCS) to apical side and 1.9 x 10^6 cells/ml peripheral blood mononuclear cells (PBMC) in RPMI + Glutamax (5 % FCS) to basolateral side of cells for 12 h. PBMC were isolated from 500 ml blood (New Zealand Blood Service, Wellington) pooled for 4 adult individuals, using 1.077 g/mL Histopaque (Sigma-Aldrich) following manufacturer instructions. The apical side of cells were then washed 3 times in RPMI +Glutamax without FCS or antibiotics prior to bacterial adhesion.

2.6.2 Bacterial culture

Human isolates; *L. gasseri, Escherichia coli LF82* (*E. coli)* and *Staphylococcus aureus 8325-4 (S. aureus)* were grown in MRS, Trypticase soy broth (TSB) or Brain heart infusion broth (BHI) respectively. *L. gasseri* were inoculated at 1 % in MRS media and grown overnight and *E. coli* and *S. aureus* at 0.1 % into refresh broth. Bacteria were then washed 3 times is PBS and re-suspended in 1ml RPMI + Glutamax.

2.6.3 Co-incubation of bacteria with human cell lines

The cell culture assay included three bacterial treatments (*L. gasseri, E. coli, S. aureus*) as well as a no-bacteria control group for each cell line (HT29 and HT29-MTX), each incubated with four PUFA treatments (AA, DHA, EPA, and ethanol negative control). Each PUFA treatment was represented by three biological replicates (wells) in a tissue culture plate.

After at least 2 passages, cells were grown in 6 well transwell plates seeded at 50 x 10^4 cells/ml until confluent. Then washed in RPMI + Glutamax 3 times and treated with 50 uM EPA, DHA, AA or 0.06 % ethanol (control) in RPMI + Glutamax (5 % Fetal calf serum) to apical side and 1.9 x 10^6 cells/ml PBMC in RPMI + Glutamax (5 % FCS) to basolateral side of cells for 12 h. PBMC were isolated from 500 ml blood (New Zealand Blood Service) using 1.077 g/mL Histopaque (Sigma-Aldrich) following manufacturer instructions.

After pre-treatment with PUFAs for 12 hours, *L. gasseri* (approximately 1 x 10^9 CFUs), *E. coli LF82* (approximately 1 x 10^6 CFUs) and *S. aureus* (approximately 1 x 10^6 CFUs) in RPMI + Glutamax were added to thrice washed cell monolayers (apical) and incubated for 3 hours at 37˚C and 5% CO_2. Unattached bacteria were

then removed by washing in RPMI 3 times. Cells were re-suspended in RPMI + Glutamax for a further 6 h to allow for mRNA expression of cytokines after which supernatants were collected for protein ELISA s for cytokines that have significant variation in their mRNA expression. Cells were removed by applying 300uL RNA extraction lysis buffer (QIAGEN –Rneasy Mini Kit) directly onto the cell insert membrane. Samples were stored at -80˚C.

2.6.4 Gene expression analysis

RNA was extracted using the RNeasy Mini Kit (QIAGEN) in combination with the RNase-free DNase kit (QIAGEN) carried out according to the manufacturer's instructions. RNA yield and purity ($1.8< OD_{260}/OD_{280} <2.0$) was measured using a NanoDrop® ND-1000 (NanoDrop Technologies Inc., Wilmington, DE USA) while RNA integrity was determined by gel electrophoresis. All reverse transcription reactions were done using 1 µg of total RNA with the Transcriptor First Strand cDNA Synthesis kit (Roche) according to manufacturer's instructions for oligo-dT primed reactions. cDNA were stored at -20˚C.

A sample of cDNA from each sample in a cell line set was pooled and all target genes; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta actin (ACTB) heat shock protein 25 (HSP 25),heat shock protein 72 (HSP 72), transforming growth factor beta (TGF-β1), interleukin-8 (IL-8), tumour necrosis factor alpha (TNF-α), interleukin-10 (IL-10),interleukin-2 (IL-2), nucleotide-binding oligomerization domain 2 (NOD-2), toll-like receptor 4 (TLR-4), interferon (IFN-γ) and GATA binding protein 3 (GATA-3) were analysed. Genes of interest were selected from those showing fluorescent signals prior to 30 cycles (see below information on Real-time PCR techniques used). Target genes analysed include IL-8, TNFα, TGFβ1, HSP25, HSP75 with reference gene GAPDH. Primers and primer-probe combinations (table 1) were designed online using the Universal probe library

system assay design centre (Roche Applied Science). Dual-hybridization probes from the Universal Probe Library (Roche Diagnostics) were paired with unmodified, desalted primers (Invitrogen New Zealand Ltd). A manually set-up, 96-well format, reverse-transcription qPCR (RT-qPCR) assay was performed using the Lightcycler® 480 system (Roche) with three reactions (technical replicates) for each sample. Each reaction contained 5µL of cDNA template, primers (200mM), probes (100mM), and Lightcycler® 480 Probes Master (FastStart Taq DNA Polymerase, 6.4 mM $MgCl_2$; Roche). Real-time PCR parameters are as follows: 10 minutes (0:10:00) pre-incubation at 95˚C, 40 cycles of amplification from 95˚C (0:00:10), to 58˚C (0:00:20), to 72˚C (0:00:01), followed by cooling at 40˚C (0:00:10). No-template-controls included in reverse-transcription reactions and RT-qPCR runs were negative for amplification products. Standard curves for each gene and cell line were generated on separate runs using up to 7 serial dilutions (1/10-1/1000) of pooled cDNA samples.

Table 2 *qPCR oligonucleotides and RT-qPCR efficiencies for (i) HT29, (ii) HT29-MTX, and (iii) Caco-2 assays*

Gene name	Genebank access n°	Primer and probe sequences 5'-3'	Amplicon size	PCR efficiency, slope (y-intercept)
GAPDH Glyceraldehyde-3-phosphate dehydrogenase	NM_002046.3	F: AGCCACATCGCTCAGACAC R: GCCAATACGACCAAATCC Probe #60: TGGGAAG	66	1.942[i], -3.470 (20.83) 1.933[iii], -3.495 (23.24) 1.937[iii], -3.482 (16.61)
HSPA1A Heat shock 70kDa protein 1a, (HSP72)	NM_005345.4	F: GGAGTCCTACGCCTTCAACA R: CCAGCACCTTCTTCTTGTCG Probe #88: GGAGGATG	89	1.841[i], -3.772 (24.68) 1.849[ii], -3.745 (21.46) 1.836[iii], -3.788 (19.89)
IL8 Interleukin 8	NM_000584.2	F: AGACAGCAGAGCACACAAGC R: ATGGTTCCTTCCGGTGGT Probe #72: GCCAGGAA	62	1.773[i], -4.021 (26.16) 1.801[ii], -3.913 (17.89) 1.833[iii], -3.800 (22.53)
TGFB1 Transforming growth factor B1,	NM_000660.3	F: GCAGCACGTGGAGCTGTA R: CAGCCGGTTGCTGAGGTA Probe #72: TTCCTGGC	64	2.107[i], -3.090 (29.18) 1.782[ii], -3.987 (23.57) 1.797[ii], -3.927 (23.46)

2.6.5 Protein expression Enzyme-linked Immunosorbent assay (ELISAs)

IL-8 and TGFβ1 protein expression were analysed according to manufacturers' instructions using enzyme-linked immunosorbant assay (ELISA) kits from Invitrogen (Catalog No. KHC0081 and KAC1688 respectively).

2.7.0 Exploring theories behind immunomodulation results

2.7.1 Peroxisome proliferator-activated receptor (PPAR) ELISA

HT29, HT29-MTX and Caco-2 cells were grown in 75 cm^2 cell culture flasks, standard culture media with 5 % fetal calf serum until confluent. Cells were treated for 48 hours with either 50 µM AA, EPA, DHA or 0.06 % ethanol (control) in duplicate and removed with 6 ml 0.05% Trypsin 0.53mM EDTA.4Na (Invitrogen). Nuclear extracts were isolated using a Nuclear Extraction Kit (Cayman Chemical, Catalog No. 10009277) and samples were tested for protein concentration using BCA Protein Assay Kit (Pierce). Nuclear extracts were added to the PPARα, δ, γ Complete Transcription Factor Assay plate (cayman Chemical, Catalog No. 10008878) using each biological replicate testing for each PPAR (δ and γ) following manufacturers instructions. Samples optical densities' (450 nm) were corrected for protein concentration.

2.7.2 Lactate dehydrogenase (LDH) assay

HT29, HT29-MTX and Caco-2 cells were grown in 24 well plates until 90 % confluency in experimental culture conditions (i.e. 5 % FCS), omitting phenol red. Cells were treated for 48 h with 50 µM AA, EPA, DHA or ethanol (control) in triplicate. A background control of culture media and a high control of cells to be lysed were included in the plate design for % cytotoxicity calculations. LDH cytotoxicity Assay Kit (ams Biotechnoglogy, Catolog No. K6330400) was used to obtain optical density 490 nm and values were used to calculate (exp. Value-low control)/ (high control-low control) x 100 = % cytotoxicity.

2.7.3 Transepithelial electrical resistance (TEER) reading

HT29, HT29-MTX and Caco-2 cells were grown in 6 well transwell plates seeded at 50 x 10^4 cells/ml until confluent, then washed in RPMI + Glutamax 3 times and treated 0.06 % ethanol (control) in RPMI + Glutamax (5 % Fetal calf serum) to apical side for 12 h, using RPMI + Glutamax without supplementation in the bottom compartment. An epithelial voltohmmeter was used to complete TEER readings by placing the split probe so that one prong was in each compartment. A control well without cells was used to measure resistance of the transwell membrane and this was deducted from each result.

2.7.4 PUFA transport through cell lines

HT29, HT29-MTX and Caco-2 cells were grown in 6 well transwell plates seeded at 50 x 10^4 cells/ml until confluent, then washed in RPMI + Glutamax 3 times and treated with 50 uM EPA, DHA, AA or 0.06 % ethanol (control) in RPMI + Glutamax (5 % Fetal calf serum) to apical side of cells, using RPMI + Glutamax without supplementation in the bottom compartment. After 12 h media in the bottom compartment was collected for fatty acid extraction (section 2.3.3), fatty acid methylation (section 2.3.5) and gas chromatography (section 2.3.6).

2.8.0 Bioscreen

L. FI9785, *L. 44*, L.2, L.10 and *L.R1* were inoculated at 1 % in MRS and grown aerobically at 37 °C overnight prior to experiments. Each species of bacteria was inoculated at approximately 10^8-10^9 CFUs (10 % inoculum) in MRS and grown for 24 h at 37 °C in the presence of 0, 5, 10, or 20 µM AA, EPA, DHA or OA in 100 honeycomb well plates (Thermo Scientific, USA). Optical density (OD) was

measured at 600 nM over 24 h every 20 min using the Bioscreen –C Microbiology Reader (Transgalactic New Jersey, USA). Conversion from OD values to bacterial numbers was estimated by measuring the number of colony forming units on MRS plates at 0 (lag growth phase), 8 (log growth phase) and 24 h (stationary growth phase), associated with known OD values. PUFAs were made up in 1 ml ethanol prior to addition to growth media to give a final ethanol concentration of 0.03 %.

The concentrations of PUFAs used in this study were estimated from typical dietary intakes of PUFAs [110-112], the assumption that 2 % escape absorption based on the ileostomy study described in the introduction [79] and an approximate volume of the colon of 700 ml. We estimated the physiological concentrations of AA, EPA and AA reaching the colon to be 16 µM AA, 18 µM EPA and 17 µM DHA as a consequence of consuming a typical western diet, where as potentially 61 µM EPA and 57 µM DHA could be present if recommended amounts where consumed. Taking into consideration the potential errors relating to these estimates in concentration four different concentrations were used in the study ranging from 0-20 µM to observe how lactobacilli would react directly to the PUFAs used.

CHAPTER 3: Method development

3.1.0 Introduction

Many cell lines and bacteria cells undergo rapid growth and in a short period of time it is inevitable that some mutations in copied genes will occur. Therefore the same cell lines at different passage number or bacteria that have been through many inoculations may vary significantly and it is important to characterise the cell that will be used in the subsequent experiments.

3.1.1 Cell culture

Human epithelial cell lines used during the course of experiments all vary in their characteristics (described in 1.5.1). However in addition, the cells used in my experiments may differ further to those described in the literature, due to a number of factors including: passage number; culture conditions and time allowed following confluency. My experiments are kept consistent by maintaining these conditions as closely as possible when using all cell lines. This has resulted in Caco-2 cells being used for experiments earlier in confluency than often stated in literature to maintain the time allowed post confluency between all cell types used. For example if HT29 were to be left for longer periods, instead of becoming more columnar like a Caco-2 cell, they start to climb on top of each other forming an uneven monolayer. Due to the differences that may exist between previous images shown in the literature and cells used in my experiments I observed cell growth on transwell membranes for scanning electron microscopy to determine visible characteristics of the cells.

3.1.2 Microbiology

Lactobacilli used during the course of my studies vary slightly in their growth rates and thus a simple way to perform estimates of bacterial number required for experiments is via OD 600 nm readings using known numbers of bacteria. The results can then be plotted on graphs (x= OD, y= bacterial number) and an equation is determined to allow prediction of bacterial number from subsequent OD 600 nm readings.

With respect to strains *L. R1* and *L. 44,* 16S rDNA gene sequencing was employed to identify which specific species of lactobacilli they maybe, via database matching of gene sequences that have previously been identified. In addition, factors that may influence bacterial number during the course of experiments were investigated. In particular growth whilst bacteria are suspended in PBS and number of washes required to remove un-adhered bacteria from human epithelial cells.

3.2.0 Methods

3.2.1 Scanning electron microscopy (SEM)

Cells were grown as stated in section 2.1.4 and prepared for SEM photography as described. In addition cells were grown to confluence (as in 2.1.4) with approximately 10^9 CFUs of *L. FI9785*, *L. R1* or *L. 44* added following the 48 hour incubation with RPMI + Glutamax (5% fetal calf serum). The subsequent steps in 2.1.4 were followed as described.

3.2.2 Species identification

16 S rRNA gene sequencing was preformed as described in section 2.4.2.

3.2.0 Microbiology method development

Methods stated in 2.4.3, 2.4.4 and 2.4.5 were completed to: quantify the number of bacteria from optical density readings, find if bacteria grow in a 3 h incubation period at 37 °C when suspended in PBS and to find the number of washes required to remove un-adhered bacteria.

3.3.0 Results

3.3.1 SEM

Cells show difference in morphological phenotypes between the more differentiated cell lines Caco-2 and HT29-MTX compared to the HT29 line, with regards to the closeness of individual cells (figure 13). For example, although cells were always used when they appeared confluent using the cell culture inverted light microscope, SEM revealed that under my cell culture conditions HT29s have clear gaps between the cells, apparently linked by filaments. In contrast, Caco-2 and HT29-MTX cells have no visible gaps at the same 10000 X magnification. Despite this, all differ from each other when observing the brush border membrane that covers the cell. For example Caco-2 and HT29-MTX cells have many more projections near where the cells join where as HT29 cells are consistently covered across the cell. The least coverage is found in the HT29-MTX line where large patches on the cell surface remain completely flat.

Bacterial adhesion SEM pictures were selected as those showing clearest bacterial adhesion and therefore only show lactobacilli on either HT29 and Caco-2 cells. The chicken strain (*L. FI9785*) looks very different to the human isolates (figure 14).

Firstly they appear to be longer, at approximately 2 μM; however, it may be the result of its straightness compared with *L. R1* and *L. 44*. Secondly it remains singular when adhered to the surface of cells, unlike the human isolates that seem to curl around each other in clumps.

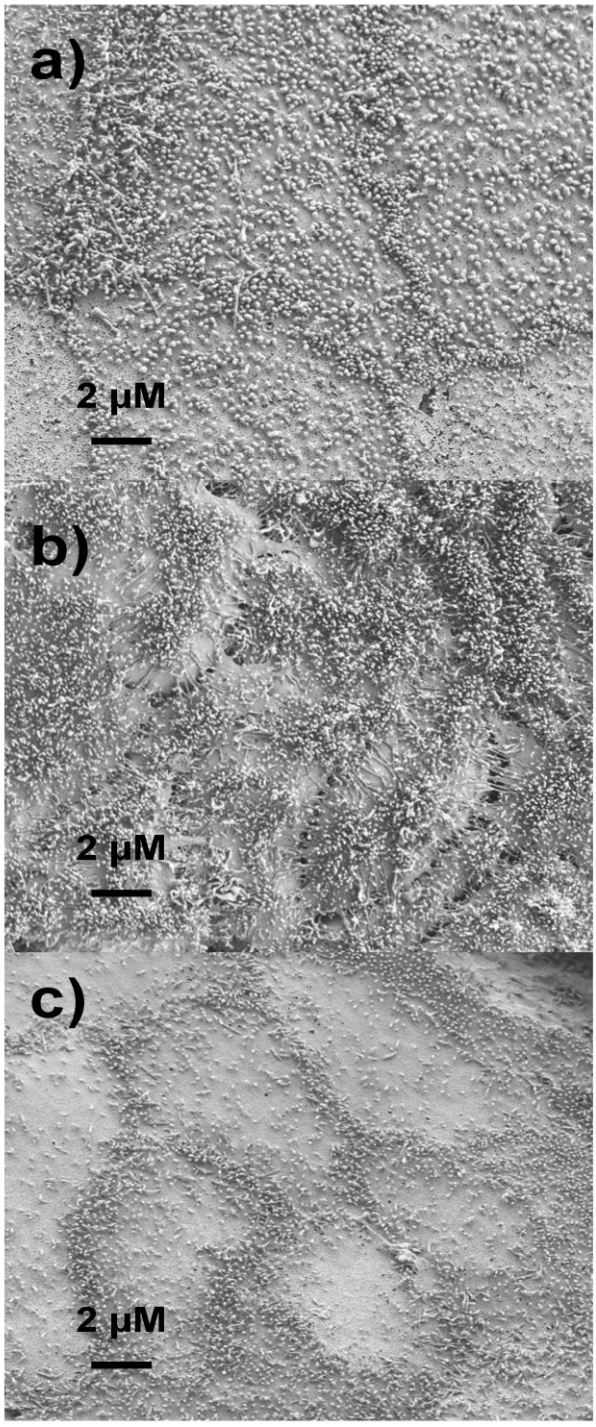

Figure 13 *SEM pictures of cell lines a) Caco-2 b) HT29 c) HT29-MTX, grown 2 day post confluency.*

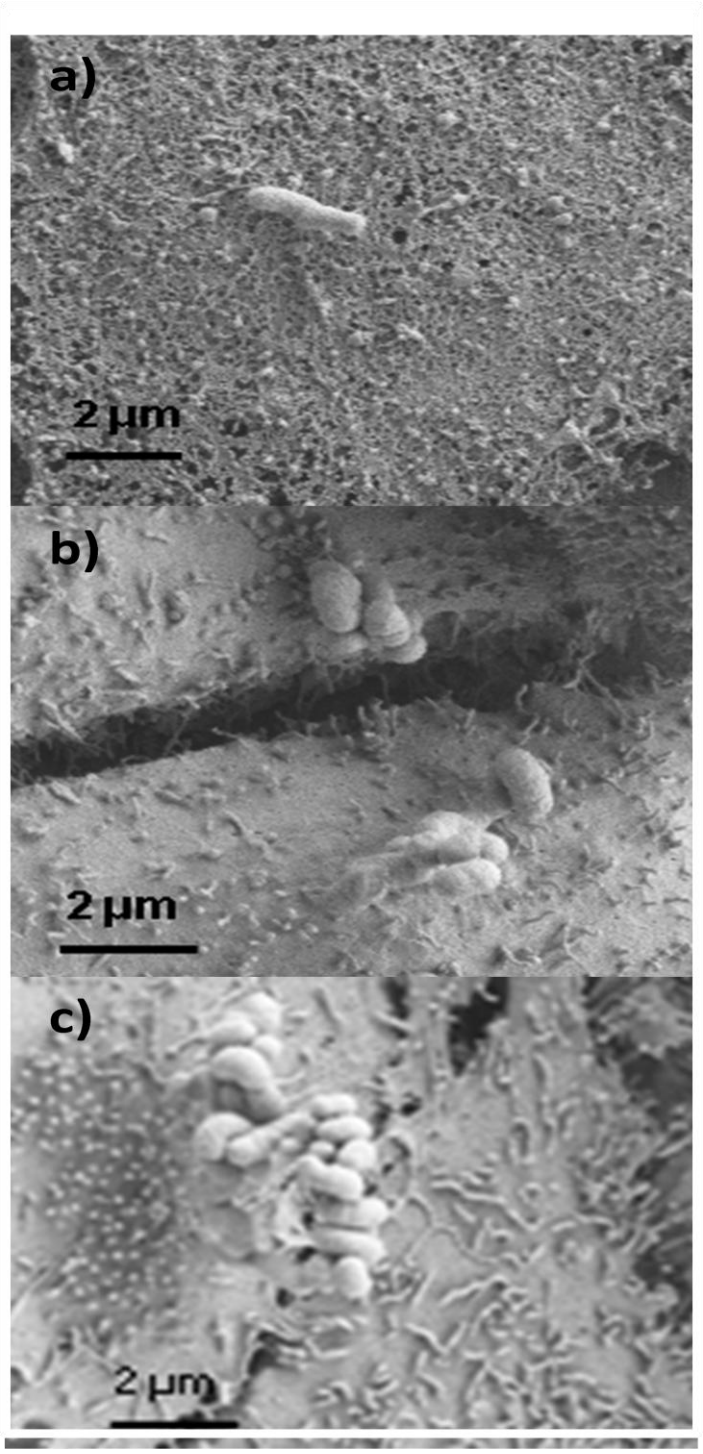

Figure 14 *SEM pictures of cell lines a) Caco-2 with L. FI9785 b) HT29 with L. R1 c) HT29 with L. 44, grown 2 day post confluency.*

3.3.2 16S rRNA gene sequencing

16S Sequences obtained for *L. R1* and *L. 44* were 100 % identical and results from NCBI Blast database revealed that they were identical matches to many *L. casei* species.

3.3.3 Survival of lactobacilli in PBS

During 3 h incubation in PBS there were no significant differences between numbers of bacteria at 0 h and 3 h for all lactobacilli tested (figure 15).

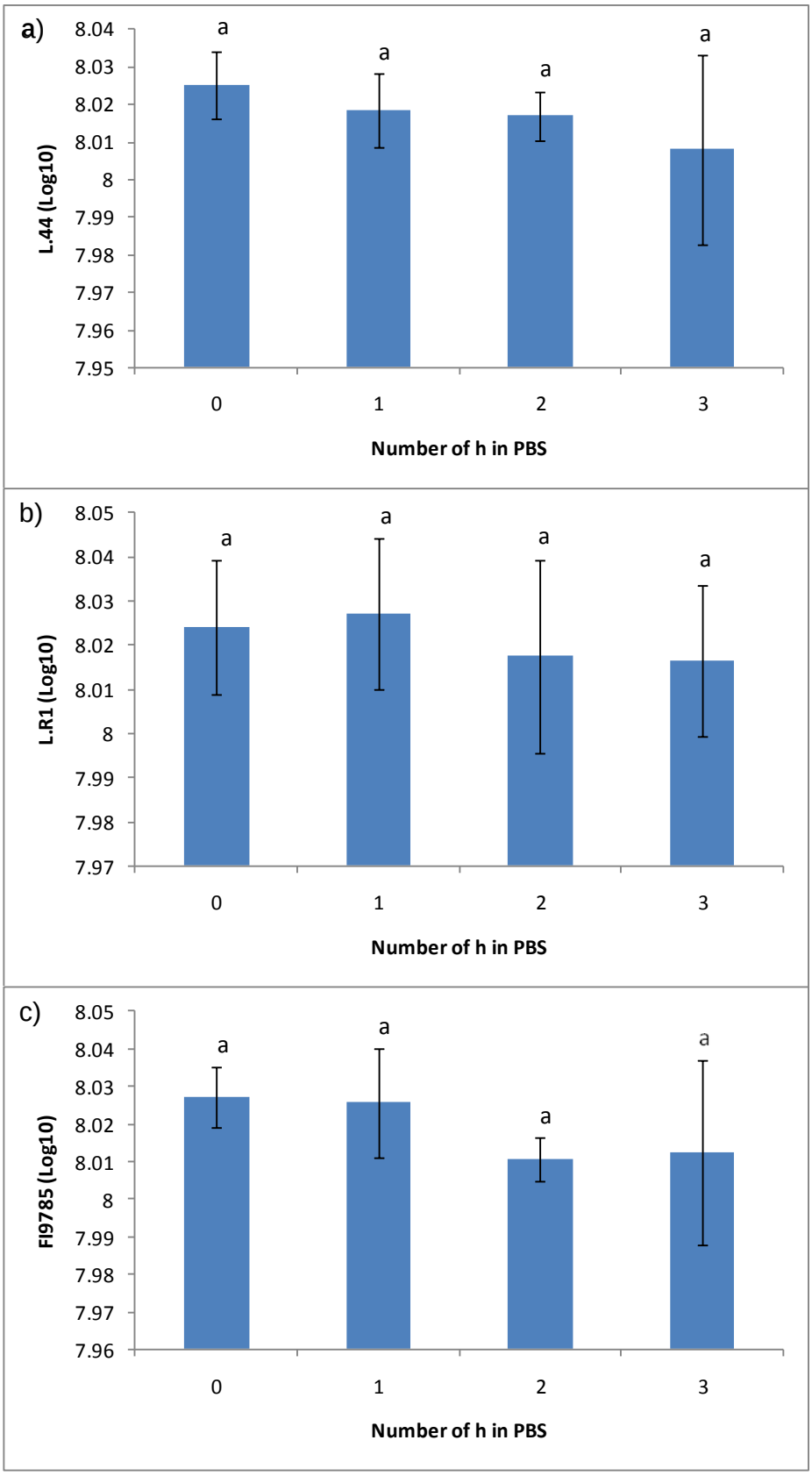

Figure 15 *Numbers of lactobacilli (Log10) CFU/ml added to PBS between 0-3 h of incubation, showing standard deviations of three biological replicates. Each graph represents a strain of lactobacilli a) L. 44 b) L. R1 c) L. FI9785.*

3.3.4 Numbers of washes required to remove un-adhered bacteria

The lactobacilli that were tested required four washes to remove un-adhered bacteria, following 3 h incubations of the bacteria on human colonic cell lines. Wash number 5 showed that there was no significant difference in carrying out an extra wash (figure 16).

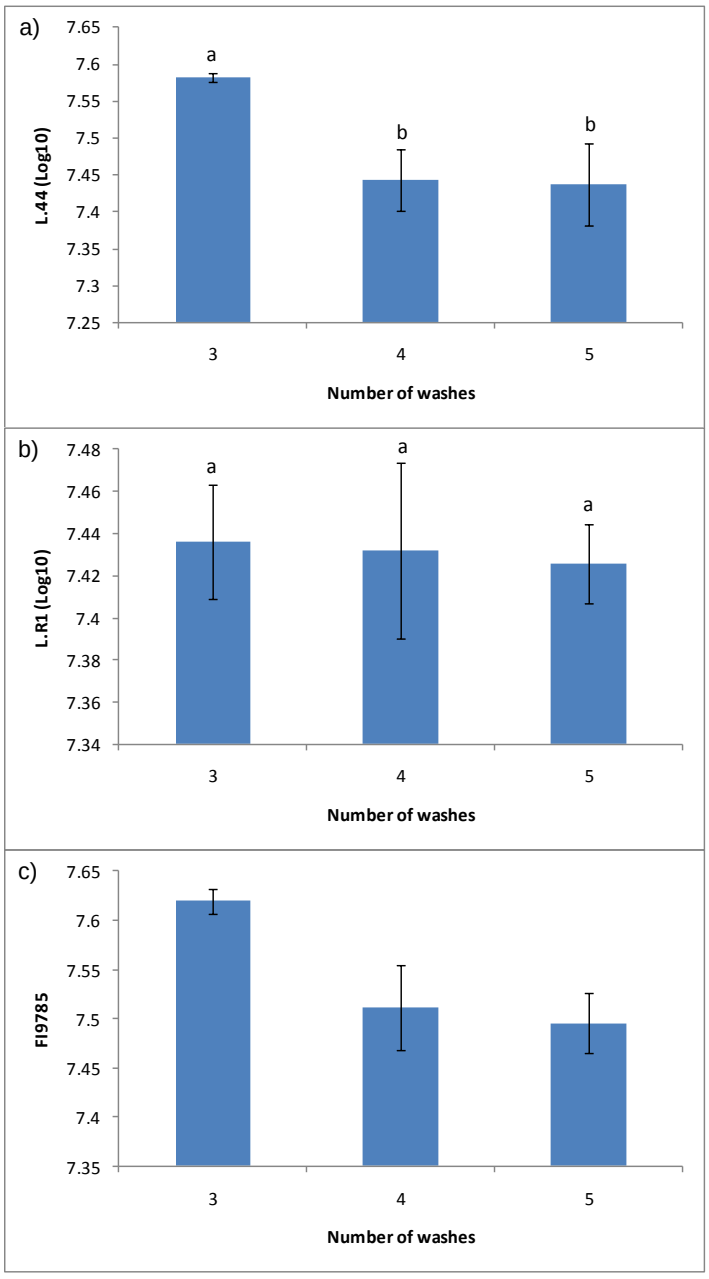

Figure 16 *Numbers of lactobacilli (Log10) CFU/ml per well (1.74 cm^2) adhered to HT29 cells after 3-5 washes in PBS, showing standard deviations of three biological replicates. Each graph represents a strain of lactobacilli a) L. 44 b) L. R1 c) L. FI9785.*

3.3.5 Calculation of formulas to estimate bacterial number from OD 600 nm

Results primarily fitted an exponential curve with best accuracy, however this was modified further by a statistician to $y= Aexp^{k.x} +Bexp^{k2x}$, where A is the value prior to exp (exponential) calculated after adding an Excel exponential equation, k is the value prior to x calculated after adding an Excel exponential equation and B is predicted using solver (Excel 2003) to minimize the sum of the squares between the actual and predicted results. The modifications result in an R^2 range between 0.9987-0.9999. The extended curve shows that OD 600 nm will reach a maximum although bacteria number could still increase in the presence of adequate media. Equations were used for subsequent methods to estimate 1×10^9 from OD 600 nm and are shown in figure 17.

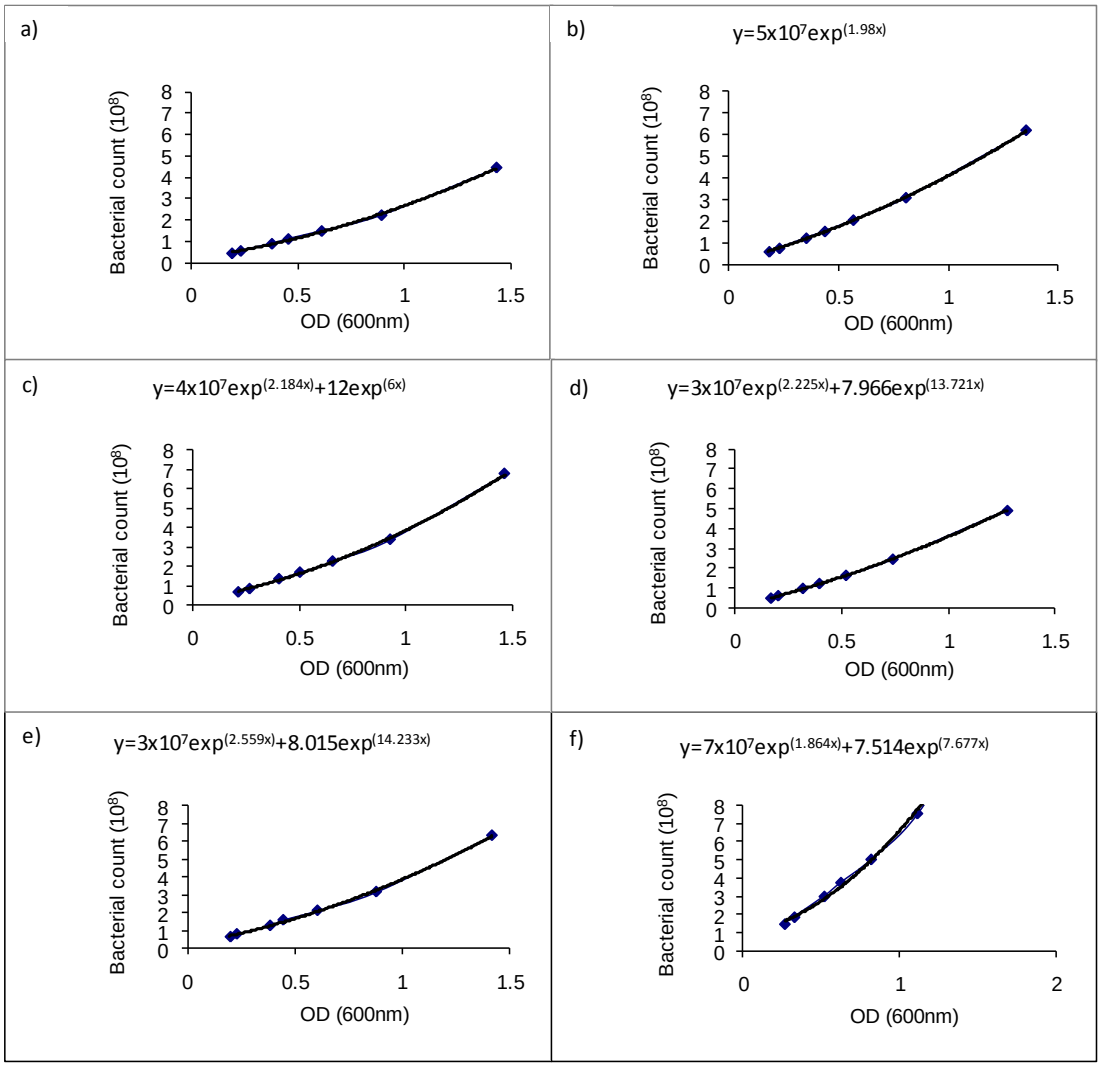

Figure 17 Equations and graphs showing the association between optical density and lactobacilli number (CFUs). *Dilutions of bacterial suspensions in PBS gave a range of OD (0.2-1.5) that were quantified using colony forming units grown on MRS plates. Results for each bacteria a) L. FI9785 b) L. R1 c) L.44 d) L. gasseri e) L. 2 f) L. 10 were plotted OD against bacteria number and the best curve was fitted to give a formula for conversion of OD (600 nm) to bacterial numbers.*

3.4.0 Discussion

Cells lines used have clear differences between them; therefore it is important to analysis further experimental results in view of these variations. SEM images display three distinctly different cell lines, in particular the HT29-MTX cells have many fewer brush border projections. Certain bacterial species have been shown to adhere to the brush border [113] and therefore density of microvilli may directly

affect the number of bacteria that adhere. Also HT29 cells have clear gaps between cells, whilst the same magnification of more differentiated cells: HT29-MTX and Caco-2 cells are more tightly packed. This factor may impact on adhesion due to the additional exposure of structures that may support adhesion of specific bacteria or due to weaker tight junctions they may make the cell more susceptible to invasion of certain bacteria.

Species identification of bacteria has shown that both *L.R1* and *L.44* are likely to be the same species of lactobacilli, therefore any variation in results using these bacteria will demonstrate a strain specific difference. It is clear from SEM pictures (figure 14) that *L. FI9785* differs from the human isolates (*L. R1* and *L. 44*); the curling and clumping formed by the latter species indicate either co-aggregation or dividing of cells. The assumption from these pictures would imply that the human isolate may show higher adhesion due to their ability to form clumps together and thus increase numbers that would adhere.

Quantification of bacteria during microbiology method development revealed a second series of exponential equation fits data points from experiments comparing OD readings to bacterial number and describes the OD saturation when bacteria will not let light pass through, but can still gain higher numbers in solution. Experiments involving observation of survival in PBS showed that bacterial numbers were not significantly different between 0 and 3 hours, therefore PBS was used when adding bacteria to cells with confidence that the added numbers of bacteria will be maintained throughout the course of the experiment. Experiments observing number of washes required to remove un-adhered bacteria, revealed that an excess of 4 washes did not change adhered numbers of bacteria significantly and therefore 4 washes will always be used in experiments requiring removal of un-attached bacteria.

All experiments in this chapter have either shaped subsequent methods or have given additional information to support interpretation of results.

CHAPTER 4 Polyunsaturated Fatty Acid treatment affects adhesion of lactobacilli

4.1.0 Introduction

Probiotic bacteria are "*Live microorganisms which when administered in adequate amounts confer a health benefit on the host'[114]."* Lactic acid bacteria are most commonly used as probiotics. They were initially used to convert sugars into lactic acid, which lowers pH and prevents the growth of spoilage organisms. They have been extensively studied for treatment of bacterial infection[115-116] , prevention of colon cancer[117] and reducing inflammation in inflammatory bowel disease[115]. Lactic acid bacteria have been shown to kill pathogenic bacteria through stimulation of the immune system, by increasing IgA plasma cells, T lymphocytes and competition for substrates[115].

Lactobacilli are gram positive bacteria and a member of the lactic acid family. They are extensively used in probiotic research and numbers in the healthy colon can reach approximately 10^9 CFUs [118]. Many lactobacilli are highly resistant to and can produce hydrogen peroxide giving a competitive advantage when this is present. Some have been shown to produce antimicrobial agents that can aid in the destruction of many pathogenic bacteria [119-120]. The adhesion of lactobacillus to intestinal surfaces is an important part of pathogen exclusion since another barrier is formed on the apical surface of the gut blocking invading microbes. Adhesion promotes survival in the harsh gut environment, thus this is one of the first characteristic identified when selecting probiotic strains. Adhesion of bacteria to epithelial cells involves non-specific, i.e. reversible hydrophobic interactions[121] and specific irreversible ligand –receptor adhesion[122]. Adhesion is species

specific in many lactobacilli as proven by the wide range of adhesion abilities of different bacterial strains[123].

N-3 PUFAs have also been shown to have antimicrobial activities[124] along with other beneficial effects on colonic health such as reducing colitis. These n-3 PUFA's, often labelled omega 3's, use the same enzymes in fatty acid metabolism as the n-6 PUFA's (omega 6's) but they result in different end products including opposing inflammatory signals. Omega 6's found in vegetable oils and meats give more pro-inflammatory signals where as omega 3's found in oily fish results in less inflammatory products. This can help in the prevention of atopic disease. Another mechanism in which n-3 PUFA's act is in lipid membranes making them more fluid,[7] this can alter the activity of membrane bound receptors and enzymes. Additionally there is also evidence of PUFAs improving the condition through immunomodulation[68]. However it is known that the chemistry of PUFAs allows them to act as anionic detergents and play a part in denaturing commensal bacteria cell walls[125]. Therefore if we consider foods as part of a normal diet combining PUFAs and probiotics could be potentially counter productive. Despite the negative effects that PUFAs may have on probiotic bacteria, there is good evidence of symbiotic effects in which PUFAs have been shown to increase adhesion of lactobacilli in the intestines of Piglets[75] and Arctic Charr[76], these actions may mean that the foods need to be consumed separately to preserve their benefits to the host.

Bomba et al[75] and Ringo et al[76] analysed lactic acid bacteria following n-3 PUFA diets and have discovered that adhesion of beneficial lactic acid bacteria can be enhanced by omega 3's in animal models. A proposed mechanism is that increased membrane fluidity from higher dietary omega 3's can alter epithelial surface receptors favouring the binding of lactobacilli [76]. Another mechanism in

which n-3 PUFA's can influence adhesion proteins is that many are nuclear receptor ligands hence giving them the potential to affect the gene transcription of adhesive proteins on the host cell surface[126].

There is limited information on the impact of PUFA's on the bacterial adhesion to human colonic cells. I therefore proposed to test the adhesion of three species of lactobacilli with three different intestinal epithelial cell lines: HT29, HT29-MTX and Caco-2 cells pre-treated for 48 hours with n-3's; EPA, DHA and an n-6; AA.

4.2.0 Methods

4.2.1 Uptake of fatty acids into total cell and phospholipid layer

Methods 2.3.1 and 2.3.3-6 were followed to assess the uptake of fatty acids into HT29, HT29-MTX and Caco-2 cells.

4.2.2 Fatty acids concentration in media supplemented with 5% fetal calf serum

5 % fetal calf serum was analysed to assess amount of AA, EPA and DHA in media prior to additional treatments. Fatty acids were extracted (2.3.3), methylated (2.3.5) and run on GC (2.3.6). Results from triplicates were averaged to obtain percentage amounts of AA, EPA and DHA and amounts were adjusted for total lipid content assessed by weight. Amounts for each PUFA were as follows: AA, 7.39 µg; EPA, 1.37 µg; DHA, 5.79 µg, resulting in concentrations of: AA, 30 µM; EPA, 6 µM; DHA, 22 µM.

4.2.3 Adhesion experiments

Cell lines were prepared as stated in 2.5.1. Two methods were then used to assess the adhesion number of bacteria; the bacteria viable count method is stated in 2.5.2 and allows for quantification of living bacteria alone that has adhered to cell lines. The radiation method, shown in 2.5.3 was used to find all bacteria that adhered to the cells and produces a more accurate method of quantification.

4.2.4 Statistical analysis

Data were analysed by ANOVA using the General Linear Model (GLM) with Tukey post-hoc comparisons (Mini tab version 15). Values were considered statistically different when $p<0.05$ or highly significant when $p<0.01$.

4.3.0 Results

4.3.1 Adhesion of lactobacilli strains to HT29, HT29-MTX and Caco-2

Using the viable counts method to analyse percentage adherence of Lactobacillus strains, *L. FI9785*, *L. R1* and *L. 44* gave significantly lower percentage adhesion (figure 18) results when compared with the radiation method (figure 19). Lactobacilli adhere at a higher percentage to cell lines HT29 and HT29-MTX when compared to Caco-2 using the radiation quantification method. Comparison of different lactobacilli strains indicated that *L. FI9785* showed a higher percentage adhesion using the radiation method. Cell lines showed varied affects of PUFAs on adhesion to lactobacilli. Lactobacilli adhesion to HT29 cells increased in the presence of DHA with all bacterial strains, whereas EPA has an affect on adhesion of *L. FI9785* only (figure 19 a). Adhesion of lactobacilli to HT29-MTX cells were largely unaffected by

AA, however EPA and DHA or DHA alone affects *L. FI9785* and *L. 44* respectively (figure 19 b). Additional statistical analysis showed that AA significantly increased percentage adhesion of *L. FI9785* (p=0.003) and L.44 (p=0.000) in mucus secreting HT29-MTX cells when compared to the same lactobacilli to HT29 cells. Caco-2 cells have a varied response to PUFAs in relation to lactobacillus adhesion, as with HT29 cells, DHA gives increased adhesion of *L. FI9785* and *L. 44* (figure 19 c).

Uptake of AA, EPA and DHA in to Colonic cells

The percentage PUFA uptake of AA into total lipids and phospholipids is much lower than for both EPA and DHA (figure 20). Interestingly when AA is added to the HT29 cell line, DHA declines more rapidly than would be expected from percentage shifts caused by the addition of more PUFA (table 3). EPA and DHA both increase after increasing concentrations are added to the media in both total lipids and phospholipids; however the phospholipid increase seems to plateau after 100 µM (figure 20 e-f). Percentage AA in the phospholipid layer decreases on addition of increasing concentrations of AA along with DHA (figure 20 d).

Percentage AA uptake is much higher in HT29-MTX cells compared to HT29 cells; in fact it has the greatest percentage increase of all PUFA treatments in the total lipid samples (figure 21 a). EPA percentage uptake into HT29-MTX cells remains the same as HT29 cells, whilst DHA is lower. However, in the case of DHA pre-treatment EPA percentage significantly increases in media samples (table 3). Caco-2 cells have the greatest percentage uptake of all PUFAs compared to both HT29 and HT29-MTX cells (figure 22).

4.4.0 Discussion

In this study I have shown that adhesion of lactobacilli to three different cell lines is generally increased by pre-treatment with DHA, but AA also increases adhesion of two strains to the mucus secreting cell line, HT29-MTX. I have investigated the possibility that this is associated with difference in the uptake of PUFAs into the cell lines.

Uptake of AA, EPA and DHA varies between cell lines and the differences may be the result of variation in expression of proteins involved in fatty acid uptake in different cell lines [127]. HT29 cells treated with concentrations of AA higher than 50 µM consistently showed up to a 1 % decrease in DHA. The result could be explained by the similar affinities of both of these PUFAs for intestinal fatty acid binding protein (i-FABP). EPA has a 3.5 fold higher kd for i-FABP lessening the chance of competition with AA [12]. Also in the fetal calf serum used there were similar levels of both AA (30 µM) and DHA (22µM); whilst EPA was much lower at 6 µM. Interestingly in the control samples (no PUFA added) HT29 cells have a higher percentage of DHA compared with EPA and AA, although in the control media concentrations of AA and DHA were similar; suggesting that fatty acid uptake and subsequent metabolism favours the retention of DHA. AA pre-treatment of cells decreased percentage AA in the phospholipid bilayer. This could reflect the tight control cells have over their phospholipid membrane, since fluidity is essential for function of many membrane proteins, i.e. the six double bonds in DHA makes the membrane more fluid than the four in AA [128]. The tighter control over percentages of PUFAs in the membrane is seen with increasing concentrations of EPA and DHA added to HT29 media; while total lipid percentage of these fatty acids continue to climb, the phospholipid content plateaus after approximately 100 µM. HT29 media samples show a general increase in PUFA levels after addition of the particular PUFA; however large variation between triplicates is seen, indicating a

problem with sampling from 20 ml media, possibly due to uneven dispersion of fatty acids.

In HT29-MTX cells AA is taken up into total lipids and phospholipids between 13-15 fold more than in HT29 cells. This could be the result of varied expression of fatty acid transport proteins after cells are more differentiated by MTX treatment. There is also evidence of DHA being metabolised to EPA due to an increase in EPA after DHA pre-treatment in total lipid, phospholipid and media samples. The percentage DHA in the media is consistently lower than AA and EPA, suggesting that HT29-MTX cells are actively metabolising DHA more than the other PUFAs. Caco-2 cells consistently have the highest percentage uptake compared to the other cell lines after PUFA treatments. This could be due to reduced control of PUFA uptake and intracellular transport by i-FABP which is expressed at only 10% of that found in rat enterocytes [127]. Unfortunately there is no information available on the levels of i-FABP in the other cell lines to compare.

In this study I have shown that n-3 PUFAs, particularly DHA, can modify adhesion of the lactobacilli to human colonic epithelial cells; an effect which may depend on the concentrations of PUFAs in the cells. Adhesion experiments (figures 18 and 19) show a large variation in results when comparing the two methods of quantification i.e. CFU and radiation methods. The CFU method gives significantly lower percentage adhesion compared to the 3H-thymidine labelling method. This is likely to be as a result of the CFU method only quantifying the viable bacteria attached to the cell line while radio-labelling reflects all bacteria. Interestingly the adhesion of *L. FI9785* shows the greatest percentage adhesion in general using the 3H-thymidine labelling method whilst the CFU method shows a lower percentage adhesion indicating that this bacteria maybe sticking to the cells but it is not surviving. This could have implications for its beneficial affects which require production of

substances from the lactobacilli i.e. antimicrobial production [129]. Caco-2 cells have a lower range of percentage adhesion compared to HT29 and HT29-MTX cells; this could be due the specific nature of adhesion to specific molecules on the cells surface. It is known that confluent Caco-2 cells have a more differentiated phenotype than HT29 cells with a well developed brush-border membrane [101-102]. It maybe that the type of molecules lactobacilli is adhering to is much more abundant on the cell surface of the HT29 and HT29-MTX cells. Another explanation for the lower percentage adhesion is the sensitivity of Caco-2 cell to fatty acids [130] and the ethanol in which they are diluted (chapter 7 figure 33), resulting in potential loss of cells. Analysis of PUFAs affects on adhesion highlights cell line differences, some responses could be explained by the varied amount of PUFAs taken up into the cell. With HT29 cells, DHA gave an increased adhesion of all lactobacilli tested whilst AA had no significant affects; this could be the result of HT29 cells not taking up as much AA as DHA. These effects are similar to those reported by Ringo et al. [76] in arctic charr following fish oil diets. Possible explanations include; increased membrane fluidity from an influx of n-3 PUFAs in the cell walls altering receptor activity that may change binding abilities to lactic acid bacteria. HT29-MTX cells differ in that AA treatment of cell increased bacterial adhesion. This maybe due to AA increased uptake into the total cell allowing it to have a greater impact on mechanisms driven by the presence of PUFAs i.e. PPARs or production of prostaglandins. Since the HT29-MTX cell line differs from the others by secretion of a mucous layer it is possible that AA may exert its affects by modifying this layer. In Katoh et als. review [131] Trefoil factor families (TFFs) mRNA is shown to be up-regulated by AA, these soluble peptides play a role in enhancing mucous secretion, thus AA maybe increasing the secretion of mucus by HT29-MTX cells and therefore trapping more lactobacilli. Caco-2 cells similarly to HT29 showed increased adhesion of *L. FI9785* and *L. 44* following DHA treatment, this is likely to be explained by alterations in adhesion protein/receptors on the epithelial surface by

membrane fluidity or via PUFAs being nuclear receptor ligands that have potential to alter expression of adhesive proteins [75-76, 126]. When considering the sensitivity of Caco-2 cells, increased adhesion maybe due to a combination of a damaging effect of PUFAs on monolayer integrity [130] combined with an effect of DHA on bacterial adhesion to the basolateral surface.

In conclusion, this study suggests that PUFAs can modify bacterial adhesion to human colonocytes. These effects are dependent on the PUFA tested and the cell line used, such that effects are different in the mucus secreting HT29-MTX cell line. The results indicate that a balanced diet of a range of PUFAs may be beneficial to maximise probiotic potential. However, further analysis needs to be conducted using primary cells including mucus secreting cells to observe whether the effects are retained.

Table 3 *Percentage PUFA left in media after 48 h treatments of HT29 cells with 0-200 µM AA, EPA or DHA. HT29-MTX and Caco-2 cells are treated with 50 µM AA, EPA or DHA. *Significance P<0.05 compared with control, ns signifies non-significant.*

	Fatty acid treatment	Percentage			Significance		
		AA	EPA	DHA			
HT29	Control	1.47 +/-0.99	0.69+/-0.21	2.25+/-1.96			
	AA 50 µM	9.53+/-6.46	2.80+/-2.32	2.18+/-1.03	ns	ns	ns
	AA 100 µM	14.53+/-2.42	1.00+/-0.98	2.25+/-0.34	*	ns	ns
	AA 150 µM	24.60+/-5.84	1.47+/-0.80	1.75+/-0.74	*	ns	ns
	AA 200 µM	8.26+/-4.78	1.17+/-1.03	1.45+/-0.56	ns	ns	ns
	EPA 50 µM	1.21+/-0.64	7.02+/-2.77	2.08+/-0.97	ns	ns	ns
	EPA 100 µM	5.02+/-5.31	16.08+/-7.86	2.46+/-1.37	ns	ns	ns
	EPA 150 µM	1.01+/-0.25	30.23+/-1.53	0.71+/-0.36	ns	*	ns
	EPA 200 µM	4.19+/-5.20	25.48+/-20.60	3.65+/-4.83	ns	ns	ns
	DHA 50 µM	2.73+/-2.24	6.42+/-6.41	3.00+/-2.25	ns	ns	ns
	DHA 100 µM	2.18+/-2.88	1.37+/-1.36	18.07+/-11.50	ns	ns	ns
	DHA 150 µM	1.58+/-0.77	1.89+/-1.88	8.03+/-8.34	ns	ns	ns
	DHA 200 µM	1.83+/-0.46	2.18+/-2.18	27.67+/-3.08	ns	ns	*
HT29-MTX	Control	2.98+/-0.01	7.05+/-1.29	0.64+/-0.13			
	AA 50 µM	6.75+/-2.58	0.57+/-0.01	0.54+/-0.52	ns	*	ns
	EPA 50 µM	4.04+/-0.18	5.29+/-0.33	1.09+/-0.06	ns	*	ns
	DHA 50 µM	3.92+/-0.34	9.51+/-0.37	1.53+/-0.36	ns	*	ns
Caco-2	Control	3.87+/-0.26	6.87+/-0.53	1.06+/-0.38			
	AA 50 µM	12.59+/-4.89	6.93+/-2.66	0.53+/-0.49	ns	ns	ns
	EPA 50 µM	2.74+/-1.25	11.94+/-0.54	0.26+/-0.26	ns	ns	ns
	DHA 50 µM	3.56+/-0.48	2.82+/-0.34	13.23+/-3.63	ns	ns	*

ns= not significant

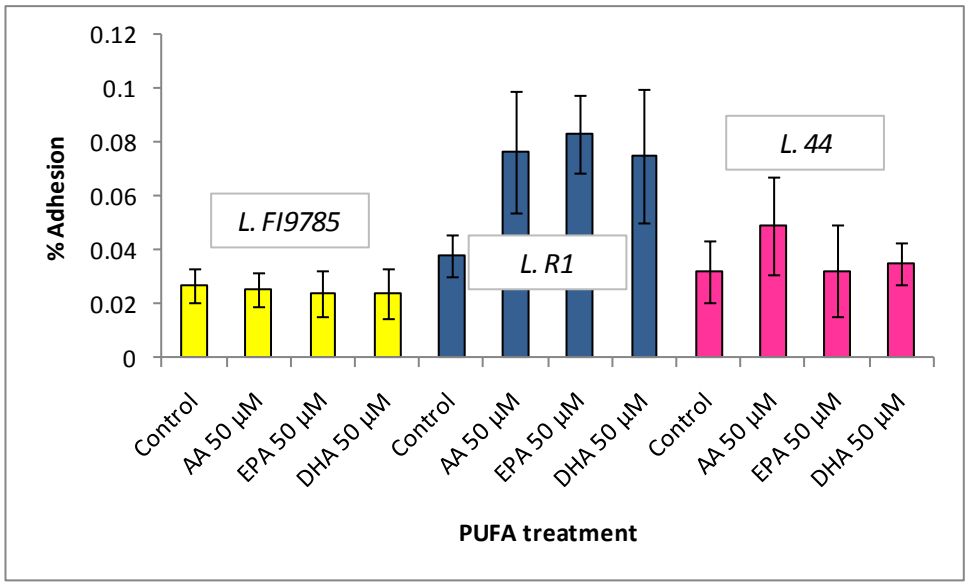

Figure 18 *Percentage adhesion of three lactobacilli strains; L. FI9785, L. R1 and L. 44 to HT29 cells using the bacterial viable method of quantification. No significant differences were found.*

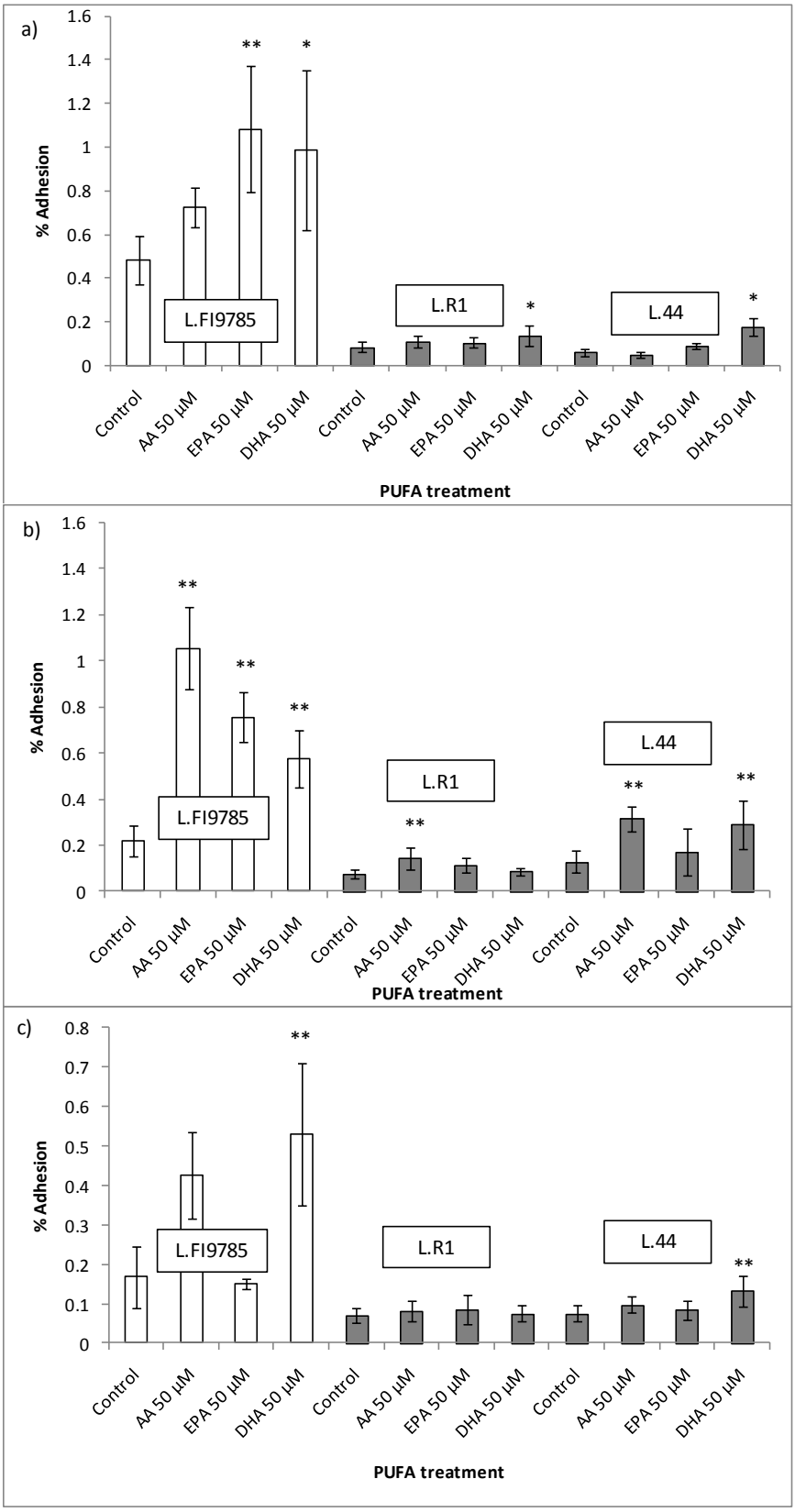

Figure 19 *Percentage adhesion of three lactobacilli strains; L. FI9785, L. R1 and L. 44 to a) HT29 cells b) HT29-MTX cells c) Caco-2 cells, using β counts from H^3 Thymidine treated lactobacilli. *significantly different from Control group P< 0.05 **significantly different from Control group P<0.01.*

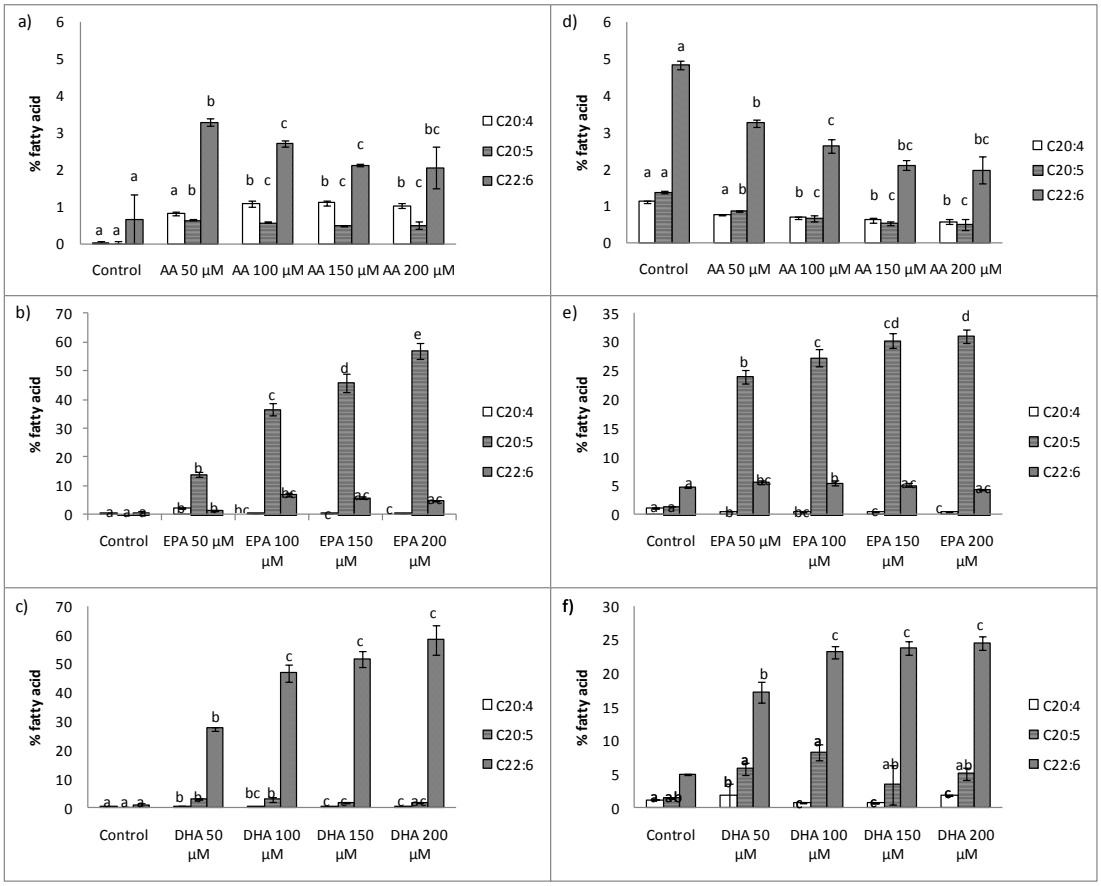

Figure 20 *Percentage uptake of PUFAs in HT29 cells after treatment of 0, 50, 100, 150 and 200 µM AA, EPA or DHA pre-treatment for 48 h. a-c) Total lipids, d-f) Phospholipids only, after 48 h. a and d) show HT29 cells after pre-treatment with AA, b and e) after pre-treatment with EPA, c and f) after pre-treatment of DHA. Each bar represents the mean +/- SD (n=3-5) of each condition. Values for individual fatty acids are compared across treatment groups. Those that do not share a letter are significantly different (P<0.05).*

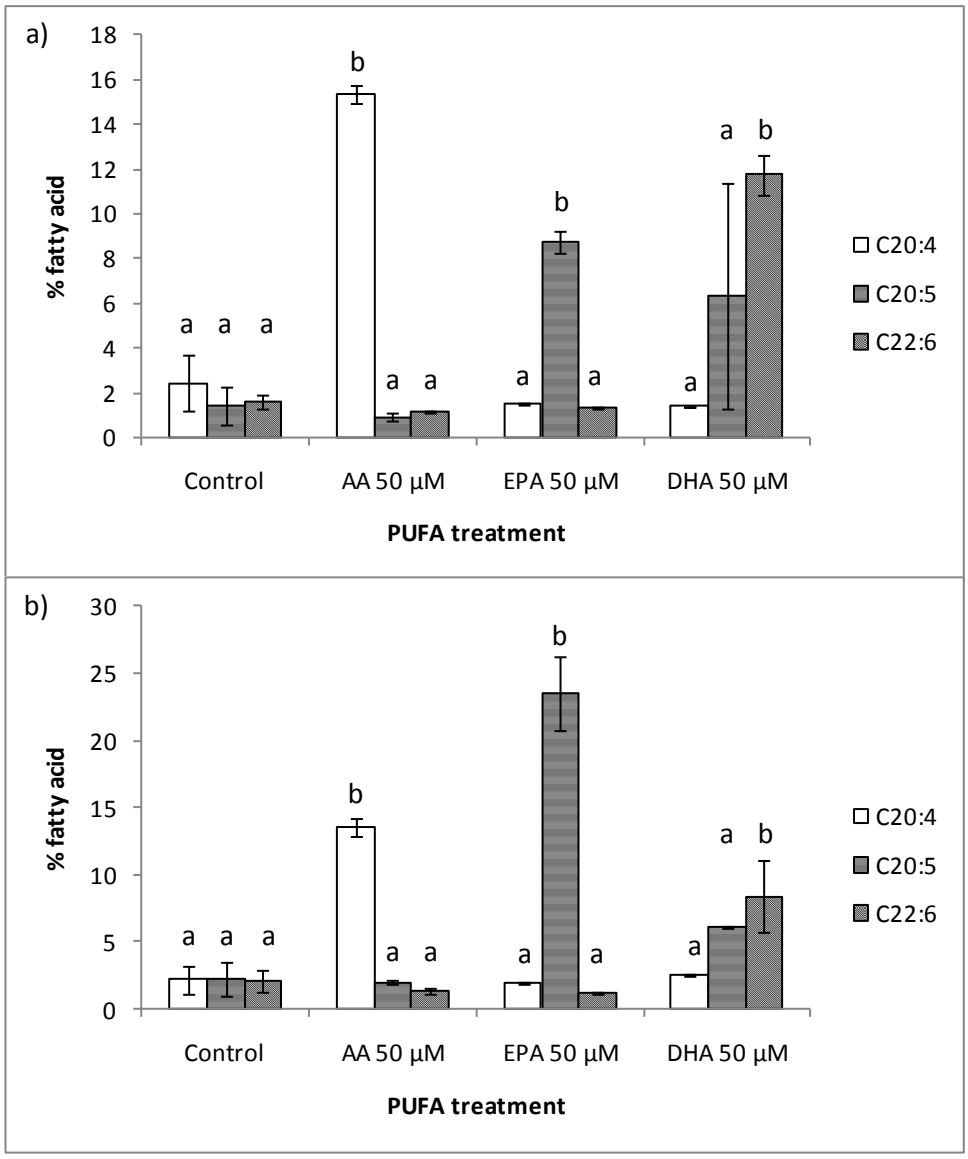

Figure 21 *Percentage uptake of PUFAs in HT29-MTX cells after treatment of 50 µM AA, EPA or DHA pre-treatment for 48 h. a) Total lipids, b) Phospholipids only, after 48 h. Each bar represents the mean +/- SD (n=3-5) of each condition. Values for individual fatty acids are compared across treatment groups. Those that do not share a letter are significantly different (P<0.05).*

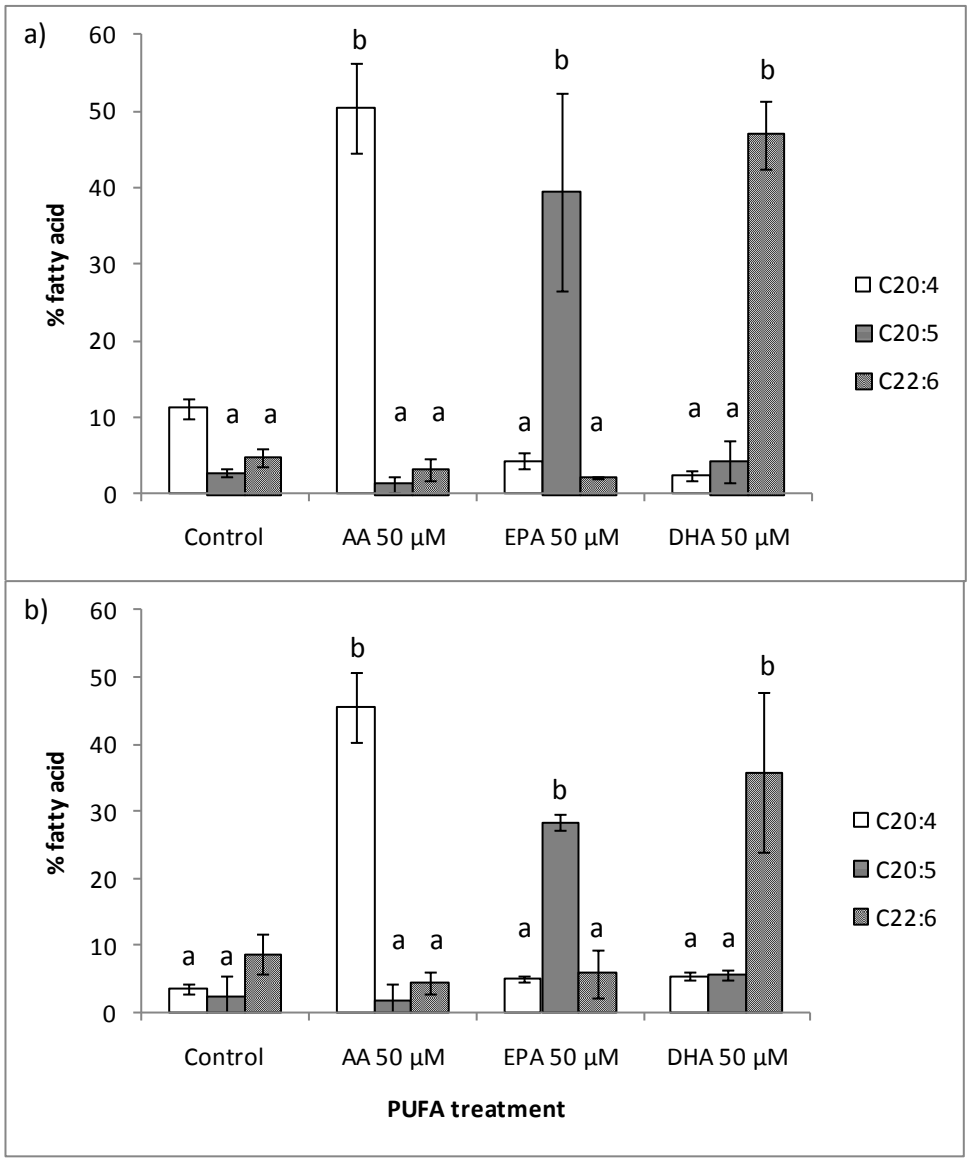

Figure 22 *Percentage uptake of PUFAs in Caco-2 cells after treatment of 50 µM AA, EPA or DHA pre-treatment for 48 h. a) Total lipids, b) Phospholipids only, after 48 h. Each bar represents the mean +/- SD (n=3-5) of each condition. Values for individual fatty acids are compared across treatment groups. Those that do not share a letter are significantly different (P<0.05).*

CHAPTER 5 Impact of polyunsaturated fatty acids on the survival of probiotic bacteria

5.1.0 Introduction

The benefits of both probiotic bacteria and fish oil (n-3 PUFA) consumption on colonic health are well recognized [132-133]. It is possible that these two dietary factors may interact, and indeed two previous studies have shown that PUFAs increase probiotic adhesion in the colon of fish and pigs [75-76] . It is assumed that as most fatty acids are absorbed in the small intestine the observed effects are due to systemic delivery to colonic cells, thus influencing their membrane properties , and that n-3 PUFAs may aid in the adhesion of probiotic lactobacilli. Adhesion of *Lactobacillus sp.* to the colonic surface has been linked with many of their beneficial affects, i.e. exclusion of pathogenic bacteria. However, a few studies have observed that PUFAs directly interact with lactobacilli [77, 134-135], in terms of their growth and death which may be due to modification in hydrophobicity. Previous experiments have shown that PUFAs have bactericidal properties [136] and these may also be important in influencing the survival of beneficial bacteria in the gastrointestinal tract. In contrast to the reported bactericidal effects of polyunsaturated fatty acids at 1660 uM – 4470 uM on a range of pathogenic bacteria [137], the monounsaturated fatty acid, OA has been reported to support lactobacilli growth[135, 138] at concentrations of 18 µM and 76 mM, and is known to modify lipid metabolism in a manner dependant on the lipid composition of the growth media [134]. The two octadecenoic acids found in lactobacillus membranes: OA [*cis*-9-octadecenoic acid; 18:1(9c)] and *cis*-vaccenic acid [*cis*-11-octadecanoic acid; 18:1(11c)] have previously been shown to be methylated into dihydrosterculic acid [9,10-methyleneoctadecanoic acid; *cyc*19:0(9c)] and lactobacillic acid [11,12-methyleneoctadecanoic acid; *cyc*19:0(11c)] respectively [139].

It is known that many species of lactobacilli can survive in the human gastrointestinal tract [140-141]. However, although the bactericidal effect of PUFAs on pathogens has been studied, the susceptibility of commensals to PUFAs has not previously been reported. In the colon survival may depend on a number of selective pressures. For example bacteria from chickens are unlikely to be exposed to high concentrations of fish oils, although plant based feed will contain the shorter chain n-3 fatty acid, linolenic acid. The consumption of fish oils by humans is very variable (0.5 +/- 0.7g/d) [142-143] and that in standard chicken feed 4-8 mg/d[78]. Bacteria are not only exposed to n-3 fatty acids but also to n-6 PUFAs which also have bactericidal properties, in particular AA [136]. The concentrations of these in the chicken and human diets are in the region of 15 mg/d[78] and 130 mg/d. The purpose of this study was to assess whether specific strains of lactobacilli can avoid the bactericidal activities of biologically relevant amounts of PUFA. In addition, bacteria are exposed to bile acids which are also well recognized to have damaging effects. It has been proposed that the effect of deoxycholic acid is counteracted by increases in the percentage of C18:1 which is suggested to increase membrane stabilisation [144].

To test the hypothesis that selection for bile acid resistance in probiotics is associated with tolerance to PUFA exposure I have chosen to use two species selected for their ability to survive in the human digestive tract from an adult human faecal sample. *Lactobacillus casei 44* and *R1* were selected from human faecal sample at the Institute of Food Research, for being both tolerant to low pH and bile acid resistant and compared to two species isolated from the same human faecal samples which showed high susceptibility to bile acids. In addition I have looked at a chicken strain (*Lactobacillus johnsonii FI9785*) selected for probiotic activities in the upper GI tract [145] and has not been selected on the basis of resistance to bile acids [146].

An estimate of the concentration of PUFAs that enter the colon, and thus influence the microbiota directly, can be calculated based on data from ileostomy studies that report the concentrations of PUFAs which escape absorption in the small intestine. Based on values obtained in a study using linolenic acid[79], the percentage of PUFAs not absorbed prior to entry into the colon is estimated to in the region of 2 %. However, values from these studies may be an underestimate as sloughing of epithelial cells from the lining of the gut will also be a source of PUFAs in the luminal. We have therefore chosen to measure susceptibility to fatty acids in De MRS over a range of 0-20uM, reflecting physiological concentrations. Furthermore I have hypothesised that tolerance to PUFA exposure may be dependent on how different species of bacteria take up and metabolise these fatty acids and have therefore analysed lipid composition of the cells and culture media.

5.2.0 Methods

5.2.1 Bioscreen

Bioscreening was used as a method to observe growth/death patterns of bacteria in MRS every 20 mins over 24 hours following treatment of AA, EPA, DHA, OA or equal volumes of ethanol (control). Section 2.8.0 was followed.

5.2.2 Determination of PUFA that escape digestion

The concentrations of PUFAs used in this study were estimated from typical dietary intakes of PUFAs [110-112], the assumption that 2 % escape absorption based on the ileostomy study described in the introduction[79] and an approximate volume of the colon of 700 ml. We estimated the physiological concentrations of AA, EPA and AA reaching the colon to be 16 µM AA, 18 µM EPA and 17 µM DHA as a

consequence of consuming a typical western diet, where as potentially 61 µM EPA and 57 µM DHA could be present if recommended amounts where consumed. Taking into consideration the potential errors relating to these estimates in concentration four different concentrations were used in the study ranging from 0-20 µM to observe how lactobacilli strains would react directly to the PUFAs used.

5.2.3 Uptake of fatty acids into total cell of probiotic bacteria

To investigate the different responses to PUFA treatment in bioscreen experiments between bacterial species methods 2.3.2, 2.3.3, 2.3.5 and 2.3.6 were followed consecutively.

5.2.4Statistical analysis

Data were analysed by ANOVA using the General Linear Model (GLM) with Tukey post-hoc comparisons (Mini tab version 15). Values were considered statistically different when $p<0.05$.

5.3.0 Results

5.3.1 Bioscreen experiments

The survival and growth of lactobacilli strains differed between human (figure 23 b and c) and chicken isolates (figure 23 a). Human strains continued to grow in the presence of 5 µM PUFAs, while the chicken strain failed to increase in number from 4 hours. Initial experiments using higher PUFA concentrations, 10 µM and 20 µM, showed no additional effect on survival (data not included). Analysis over the full

time course of each growth curve, comparing cell number to that of untreated cells at the same time point, shows that all PUFAs slow the growth of human isolated lactobacilli, apart from *L. 10* following EPA and DHA addition (table 4). In the case of *L. 44* the effects of EPA & DHA were less significant than for *L. R1* (figure 23, table 4). In *L. FI9785* bacteria, a chicken isolate, this effect was also observed but to a much greater extent (figure 23a) such that analysis of individual time points shows a significant effect relative to the control at that time point from 12h (table 4). In addition, *L. 2*, human isolate not selected for bile acid resistance, showed significant difference from the control following AA treatment at specific time points 4 and 8 h, demonstrating a more pronounced growth inhibition than other human strains, however after 12 h the strain was able to recover and not differ from the control growth numbers, unlike *L. FI9785*.

5.3.2 PUFAs concentrations in lactobacilli

The percentage of each fatty acid in bacteria was measured at 8h after treatment with no fatty acid added to the MRS or 20µ AA, EPA, DHA, OA (figure 23 and table 5). Fatty acids found to be present in one or more of the samples are as follows: 14:0; myristic acid, 14:1; myristoleic acid, 16:0; palmitic acid,16:1; palmitoleic acid, 18:0; stearic acid, 18:1(9c); OA, 18:2; linolenic acid, cyc19:0(11c); lactobacillic acid, cyc19:0(9c); dihydrosterculic acid and 20:4; AA.

The fatty acid composition of untreated cells was different between different bacteria such that myristoleic, OA and linolenic acid are significantly higher in *L.FI9785* than in *L. R1* and *L. 44*. However *L. R1* and *L. 44* have a higher percentage of lactobacillic acid and stearic acid than the other strain.

Treatment with OA did not increase the percentage of OA in bacteria and in the case of all strains the percentage OA was significantly reduced; although, *L. FI9785* contained the highest percentage OA when compared to other species. Interestingly after OA addition, lactobacillic acid was completely absent, however dihydrosterculic acid was found in its place following a similar pattern in percentage amounts between bacteria as Lactobacillic acid in the control group, i.e. 3.6 % in *L. FI9785*, 11.5 % in *L. R1* and 8.4 % in *L. 44*. Other change when comparing OA and control treatment groups is the absence of myristic and myristoleic acid in *L. FI9785* following OA treatment, however palmitic and stearic acid are significantly higher after treatment in *L. FI9785* and *L. 44* when compared to the control.

Treatment with AA resulted in AA being found in all bacteria when compared to in untreated cells, although AA percentage was significantly higher in *L. FI9785*. *L. R1* and *L. 44* were found to have significantly higher myristic and myristoleic acid than *L. FI9785*, in the later case this opposes the control where myristoleic acid is only found in *L. FI9785.* Similar to the control OA and linoleic acid are present in significantly lower percentages in *L. R1* and *L. 44* compared to *L.FI9785*, however in this case it does not correspond with a higher percentage lactobacillic acid in *L. 44.* Treatment with EPA did not result in EPA being present in lactobacilli. In the majority of fatty acids present the results comparing between bacteria mirrored the control, the exceptions being myristic and myristoleic acid where they are absent in *L. FI9785* following EPA treatment. However comparing percentage amounts with the control EPA treatment is shown to increase stearic and palmitic acid, whilst decreasing oleic and palmitoleic acid in all bacteria.

Treatment with DHA did not result in DHA in any bacteria cells following 8 h treatment, however comparison of percentages of myristic and myristoleic acid with control for each bacteria showed that *L. FI9785* significantly decreased percentages

of these fatty acids, while *L. R1* and *L. 44* increased them. The reverse is apparent for stearic and palmitic acid, i.e. 16.4 % and 24.6 % are found in *L. FI9785* while 4.5-4.9 % and 11-11.9 % respectively.

In addition to fatty acid composition analysis of bacteria, MRS after 8 h was also analysed for its fatty acid composition (table 5). Fatty acids found to be present in one or more of the samples are as follows: 16:0;palmitic acid, 18:0; stearic acid 18:1(11c); *cis*-vaccenic acid, 18:1(9c);OA, 18:2; linolenic acid, 20:4; AA, 22:6; DHA. In untreated MRS these fatty acids previously mentioned fatty acids are present apart from AA and DHA, although there are no significant differences found between bacteria.

Treatment with OA did not result in an increase of OA in the media after 8 h, in fact in the presence of *L. 44* OA decreased in the media when compared with untreated cells, whilst there were no changes in other strains tested. Despite this, the other octadecenoic acid, *cis*-vaccenic acid resulted in a higher percentage left in media when compared to control following exposure to *L. R1* and *L. 44*.
Treatment with AA resulted in AA left in the media of all bacteria; however *L.44* contained the highest percentage followed by *L. FI9785*.

Treatment with EPA resulted in no EPA being found in bacteria or media; however DHA was present in media of all bacteria tested. When comparing significant percentage differences between bacteria species within the EPA treatment group *cis*-vaccenic acid was found in a higher percentage in *L. FI9785* exposed media than *L. R1* and *L. 44*. In contrast the latter two strains of lactobacilli had decrease percentage oleic acid when compared to the control group.
Treatment with DHA resulted in absence of DHA in media of all bacteria. Similar to EPA treatment *cis*-vaccenic acid was found in a higher percentage in *L. FI9785*

media than *L. R1* and *L. 44,* however only *L. FI9785* is significantly different to the control.

MRS media prior to addition of PUFAs contains approximately 85 % OA, whilst AA, EPA and DHA are not present (table 5). Following addition of 20 µM of OA, AA, EPA and DHA all percentages increased, however to differing extents. It must be noted that these percentages do not represent final amounts of each fatty acid and data can be skewed by differing total areas under the peaks, in fact on observation of total areas the increased amount represented by these figures are similar (data not shown). The results confirm that DHA has disappeared from media during experiments with this treatment and more interestingly supports the notion that EPA is converted to DHA by all bacterial strains.

5.4.0 Discussion

When considering the affects of PUFAs on adhesion of lactobacilli, it is important to analyse what affects the PUFAs may have directly on lactobacilli present in the colon. Although a large percentage of fat is absorbed before the colon there is evidence that approximately 2 % can reach colon from the diet [79] and therefore directly influence the bacteria present. Fatty acids have been shown to have bactericidal properties and can aid in protection from pathogenic species [136], however fatty acids will undoubtedly affect some commensal species unless they possess mechanisms to avoid their destructive actions. After examining the growth/death of five lactobacilli in biologically relevant amounts of PUFAs that could reach the colonic contents, I found that four out of five survived throughout the experiment, while one strain showed signs of death after 4 h incubation (figure 23). Interestingly the species that did not survive was isolated from chicken, while the surviving four came from human faeces, suggesting that human strains had

developed the ability to survive in the PUFA concentration found in the colon. Studies have previously shown that fatty acids can induce changes in the lactobacilli populations in broiler chickens [78], however my results for this potential chicken probiotic leads to some concerns about it probiotic potential use in chicken fed high n-3 diets (used for production of high omega 3 eggs). Past studies have shown that EPA and DHA at 7.6 nM have strongly inhibited lactobacilli growth and OA stimulated growth of some strains [135], these high concentrations suggest that lactobacilli are very varied in their response to different PUFAs and death from PUFAs may not be isolated to those derived from chickens. However this highlights an area of concern to be analysed further, since many chicken producers are moving towards the use of probiotic strains (particularly lactobacilli) for the control of bacterial infections, following the ban of many antibiotics in chickens. In addition to Bioscreen results, it must be noted that in my experiments I did not observe growth enhancement of lactobacilli after addition of OA as expected [134-135, 138]. Due to the complex differences between lactobacilli at the level of each strain, it is possible that all strains are not adapted to metabolising oleic acid in a way that promotes growth. Another study supports the variance of PUFA activity on lactobacilli by observing difference in PUFA uptake in bacteria and subsequent variances in hydrophobicity [77]. The human strains selected included two that were selected for bile acid resistance and two that were not, to explore the theory that bile acids act upon certain sensitive strains of lactobacilli in a similar mechanism to fatty acids. This was proved not to be the case in my experiments as they all survived, however bile acid sensitive *L. 2*, showed increased sensitivity to AA compared with other human isolates, but recovered to result in similar numbers of bacteria by stationary phase.

To investigate the different responses to PUFAs, uptake of fatty acids into *L. FI9785, L. R1* and *L. 44* were analysed after 8 h incubation (control samples log phase) with

biological relevant concentrations of PUFAs. The results suggest complex mechanisms in which different lactobacilli strains can convert PUFAs in to shorter/longer more saturated/unsaturated PUFAs. In particular I wanted to analyse differences in species that did survive in PUFAs added and *L. FI9785* that did not. Since a small amount of growth was observed before 4 h incubation of PUFA (figure 23 a) I had expected some uptake of PUFA prior to death, however I did not predict that the *L. FI9785* cells would contain a much higher percentage AA following its addition to media. The possible reasoning behind these results may be a lack of control over PUFA influx, thus resulting in high proportions of these antibacterial agents inside the cell and possible cell death. Another explanation maybe that damage caused to the bacterial cell membrane by PUFAs, in the case of their bactericidal activities can produce holes in the outer membrane [147], leading to the possibility of the influx of PUFAs prior to the analysis of PUFA uptake. More recently it has been suggested that biohydrogenation of PUFAs can occur in *Butyrivobrio fibrisolvens* to enable their survival from PUFAs toxic effects, however suggest the cause of toxicity as being mediated through a metabolic effect rather than disruption of membrane integrity [148].

Following addition of EPA and DHA to MRS media it was interesting to find that the PUFAs added were not found in the media of cells or any bacteria, suggesting its prompt metabolism to another compound, in the case of *L. FI9785* before its death. Interestingly in all lactobacilli tested following addition of EPA, a significant increase in DHA was found, showing the potential for EPA's elongation and saturation to DHA. This is of major significance since humans convert EPA to DHA at a very low efficiency although it is of major importance to body function[149], and it may be that enzymes involved in the conversion in lactobacilli could be isolated and used as an additional supplement to efficiently convert EPA to DHA from natural sources, such as fish.

OA was added into Bioscreen experiments as it has been shown to stimulate lactobacilli growth [138]. Johnsson et al. [134] studied its presence in the culture medium during lactobacilli growth and deduced that low levels in culture medium resulted in more lactobacillic acid and high levels resulting in higher amounts of dihydrosterculic acid, meaning that changes in amount of one PUFA can have an affect of the mechanisms in which lactobacilli can convert/metabolise the fatty acid. A similar observation is present when analysing survival of *L. FI9785* following a small increase in percentage of OA present in the media. An increase of 0.4 % results in cell death after 4 h incubation compared to the control growth in normal media that continues to grow normally. The results shows that this *Lactobacillus strain* is particularly sensitive to slight changes to the standard culture conditions and possible explanations may be due to a tight control on the amount of PUFA that can be converted/metabolised and in the presence of additional amount a build up of PUFA inside the cell maybe the cause of cell death. Interestingly there is a variation in the percentages of OA found in the bacteria of the control, OA, AA and EPA treatments of *L. R1* and *L. 44* compared with *L. FI9785*, since OA percentage is significantly higher in *L. FI9785* cells. This corresponds with the increase in either lactobacillic or dihydrosterculic acid, that is found at higher percentages in both human strains; *L. R1* and *L. 44* compared to chicken; *L. FI9785*, suggesting an increased methylation of the octadecenoic acids to their respective cyclopropane forms in human strains, however this affect is observed in control samples eliminating *L. FI9785*'s early demise as an explanation.

In conclusion, experiments have revealed clear differences in the way PUFAs are taken inside lactobacilli and are metabolised/ synthesised in species that can survive in small concentrations of PUFAs and those that cannot. Some bacteria have known enzymes that can synthesis PUFAs, however there are mainly marine species[150]. Therefore due to lack of studies looking into biosynthetic routes in

lactobacilli there is an opportunity to discover how the species may avoid antibacterial actions of PUFAs leading to increased selection criteria when looking for potential probiotics expected to be used when PUFAs will be present in the diet.

Table 4 Lactobacilli *following treatment with 5 µM PUFA: AA, EPA, DHA and OA shown as bacterial number relative to control over a 20 h growth peroid. Values are expressed as mean relative to control (n=3) ±SD at 4 hourly time points throughout the experiment for each treatment group. Data where there is a significant difference between treatment and control at that time point are shown in the main table as *p<0.05 and **p<0.01. Comparison between control and PUFA treatment over the whole time course (n=12) was analysed by two-way ANOVA and the results of the TUKEY post-hoc test for effect of treatment is shown at the bottom of the table(*p<0.05 and **p<0.01).*

PUFA	Time	*L. 44*	*L. R1*	*L. FI9785*	*L. 2*	*L. 10*
AA	0h	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00
	4h	0.70 ±0.02	0.46 ±0.24	0.79 ±0.17	0.85 ±0.21	0.76 ±0.10
	8h	0.52 ±0.24	0.29 ±0.29	0.54 ±0.25	0.82 ±0.16	0.69 ±0.15
	12h	0.58 ±0.32	0.35 ±0.37	0.36 ±0.21*	0.86 ±0.02	0.77 ±0.15
	16h	0.67 ±0.29	0.41 ±0.37	0.28 ±0.16**	0.90 ±0.02	0.84 ±0.15
	20h	0.60 ±0.30	0.46 ±0.37	0.28 ±0.20**	0.92 ±0.05	0.86 ±0.10
EPA	0h	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00
	4h	0.75 ±0.15	0.51 ±0.30	0.78 ±0.16	0.85 ±0.19	0.85 ±0.10
	8h	0.61 ±0.30	0.38 ±0.30	0.54 ±0.25	0.76 ±0.11	0.79 ±0.13
	12h	0.68 ±0.40	0.41 ±0.33	0.36 ±0.21*	0.84 ±0.03	0.80 ±0.04
	16h	0.75 ±0.45	0.37 ±0.37	0.27 ±0.16**	0.92 ±0.05	0.87 ±0.05
	20h	0.64 ±0.39	0.38 ±0.32	0.27 ±0.16**	0.97 ±0.03	0.95 ±0.06
DHA	0h	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00
	4h	0.77 ±0.12	0.51 ±0.24	0.77 ±0.14	0.79 ±0.12*	0.87 ±0.05
	8h	0.63 ±0.29	0.37 ±0.25	0.55 ±0.23	0.78 ±0.08*	0.87 ±0.15
	12h	0.64 ±0.34	0.37 ±0.33	0.38 ±0.22*	0.89 ±0.08	0.93 ±0.09
	16h	0.73 ±0.39	0.40 ±0.38	0.30 ±0.15**	0.90 ±0.11	0.97 ±0.08
	20h	0.66 ±0.36	0.43 ±0.40	0.30 ±0.18**	0.92 ±0.08	0.99 ±0.18
OA	0h	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	nd	nd
	4h	0.72 ±0.08	0.47 ±0.13	0.83 ±0.22	nd	nd
	8h	0.52 ±0.02	0.28 ±0.16	0.55 ±0.24	nd	nd
	12h	0.60 ±0.30	0.37 ±0.31	0.36 ±0.20*	nd	nd
	16h	0.67 ±0.34	0.45 ±0.35	0.28 ±0.28*	nd	nd
	20h	0.61 ±0.33	0.58 ±0.46	0.29 ±0.19*	nd	nd
Control						
AA		**	**	**	*	**
EPA		*	**	**	*	ns
DHA		*	**	**	**	ns
OA		**	**	**	nd	nd

nd= no data

Table 5 *Percentage PUFAs: 16:0=palmitic acid, 18:0=stearic acid , 18:1(9c[a])=OA, 18:2=linolenic acid, 20:4= AA, 20:5= EPA, 22:6= DHA additon of OA, AA, EPA or DHA into MRS media prior to introduction of bacteria.*

PUFA	Control	OA	AA	EPA	DHA
16:0	3.9	5.3	21.0	6.2	4.5
18:0	3.0	2.9	14.6	3.5	3.3
18:1 (9c)[a]	85.0	86.1	37.1	80.6	83.7
18:2	8.0	5.7	17.7	5.8	5.9
AA	0.0	0.0	9.6	0.0	0.0
EPA	0.0	0.0	0.0	3.9	0.0
DHA	0.0	0.0	0.0	0.0	2.5

[a]9c-double bond in position 9 and *cis* (c) configuration

Table 6 *Percentage values for individual fatty acids: 14:0, 14:1, 16:0, 16:1, 18:0), 18:1(9c), 18:2,cyc19:0(11c), cyc19:0(9c) and 20:4 expressed as mean+/-SD, present in lactobacilli: L. FI9785, L.R1 or L.44 cells following 8 h incubation with a variety of PUFAs (20 μM). Within the table significance between treatment groups and control, for each fatty acid within a bacterial species is shown and significance differences are labelled *P <0.05 and **P<0.01.*

Bacteria	Fatty acid	Percentage				
		Control	OA	AA	EPA	DHA
L. FI9785	14:0	0.78 ± 0.30	BDL**	0.33 ± 0.08*	BDL**	BDL**
	14:1	0.15 ± 0.03	BDL**	BDL**	BDL**	BDL**
	16:0	14.47 ± 0.66	27.13 ± 2.25**	12.08 ± 1.73	25.35 ± 0.53**	24.61 ± 0.77**
	18:0	4.25 ± 0.27	14.09 ± 0.93**	5.68 ± 0.42	13.86 ± 1.01**	16.38 ± 2.14**
	16:1	0.27 ± 0.03	BDL**	BDL**	BDL**	BDL**
	18:1(9c)[a]	76.03 ± 0.06	54.51 ± 2.62**	75.06 ± 1.07	55.78 ± 0.76**	54.05 ± 1.59**
	18:2	0.86 ± 0.03	0.65 ± 0.02**	0.34 ± 0.02**	0.77 ± 0.01*	0.73 ± 0.04**
	*cyc*19:0(11c)	3.19 ± 0.27	BDL**	2.67 ± 0.50	4.25 ± 0.22*	4.23 ± 0.28*
	*cyc*19:0(9c)	BDL	3.61 ± 0.41**	BDL	BDL	BDL
	20:4	BDL	BDL	3.33 ± 0.22**	BDL	BDL
L. R1	14:0	0.52 ± 0.16	0.54 ± 0.05	2.52 ± 0.29**	BDL*	2.85 ± 0.21**
	14:1	BDL	BDL	0.09 ± 0.01	BDL	0.21 ± 0.07**
	16:0	13.45 ± 1.97	29.40 ± 1.45**	12.28 ± 0.59	21.72 ± 0.43**	11.92 ± 0.13
	18:0	14.48 ± 1.04	20.31 ± 0.33**	5.78 ± 1.01**	20.05 ± 0.69**	4.92 ± 1.08**
	16:1	2.54 ± 0.43	BDL**	16.06 ± 2.74**	BDL	22.89 ± 1.06**
	18:1(9c)	58.18 ± 2.05	38.14 ± 2.45**	54.06 ± 0.96	44.40 ± 1.10**	48.00 ± 4.71**
	18:2	0.49 ± 0.11	0.11 ± 0.02	0.18 ± 0.02	0.43 ± 0.19	0.34 ± 0.31
	*cyc*19:0(11c)	10.34 ± 0.77	BDL**	8.67 ± 0.89	13.39 ± 1.25	8.87 ± 4.99
	*cyc*19:0(9c)	BDL	11.49 ± 3.60**	BDL	BDL	BDL
	20:4	BDL	BDL	0.36 ± 0.05**	BDL	BDL
L. 44	14:0	1.16 ± 0.85	0.40 ± 0.21	1.62 ± 0.65	0.30 ± 0.08	2.83 ± 0.34*
	14:1	BDL	BDL	0.07 ± 0.01**	BDL	0.07 ± 0.02**
	16:0	10.93 ± 2.58	34.53 ± 4.88**	9.91 ± 0.98	26.36 ± 2.07**	10.96 ± 3.25
	18:0	8.69 ± 1.85	21.66 ± 3.07**	4.38 ± 1.43	19.32 ± 1.61**	4.50 ± 0.77
	16:1	8.79 ± 2.47	BDL**	33.77 ± 1.75**	BDL**	24.07 ± 2.73**
	18:1(9c)	60.04 ± 0.89	39.66 ± 5.07**	49.54 ± 0.80*	40.88 ± 1.94**	48.42 ± 4.45*
	18:2	0.28 ± 0.22	0.09 ± 0.06	0.14 ± 0.04	0.06 ± 0.01	0.18 ± 0.05
	*cyc*19:0(11c)	10.11 ± 4.80	BDL**	0.12 ± 0.03**	13.08 ± 3.48	8.97 ± 0.23
	*cyc*19:0(9c)	BDL	8.38 ± 4.89**	BDL	BDL	BDL
	20:4	BDL	BDL	0.43 ± 0.07**	BDL	BDL

[a]9c-double bond in position 9 and *cis* (c) configuration, BDL= below detection limit

Table 7 *Percentage fatty acids expressed at means +/-SD (16:0=palmitic acid, 18:0=stearic acid 18:1(11c)=cis-vaccenic acid, 18:1(9c)=OA, 18:2=linolenic acid, 20:4= AA, 22:6= DHA found in media following L. FI9785, L. R1 or L. 44 cells 8 h incubation with a variety of PUFAs (20 µM). Significant difference between PUFA treatment and control for each of the following: 16:0, 18:0, 18:1(11c), 18:1(9c[a]), 18:2, 20:4 and 22:6 is shown within the table, split by bacteria type and significant difference between L. FI9785 and L. R1 or L. 44 are shown for each PUFA: 16:0, 18:0, 18:1(11c), 18:1(9c), 18:2, 20:4 and 22:6 in the bottom 2 rows. Significant results are labelled *P <0.05 and **P<0.01*

Bacteria	Fatty acid	Percentage															
		Control			OA			AA			EPA			DHA				
L. FI9785	16:0	4.51	±	0.51	5.47	±	0.99	4.32	±	0.44	5.59	±	0.16	5.31	±	0.56	
	18:0	4.93	±	0.22	8.75	±	0.81*	3.75	±	0.29	7.25	±	0.75	7.06	±	2.26	
	18:1(11c)	0.87	±	0.09	1.86	±	0.49	0.65	±	0.24	2.55	±	0.50**	2.60	±	0.39**	
	18:1(9c)	81.52	±	0.57	76.84	±	1.60	82.85	±	0.46	78.29	±	1.65	78.54	±	4.15	
	18:2	8.17	±	1.25	7.08	±	0.81	7.60	±	1.08	5.86	±	0.46	6.49	±	1.79	
	20:4	BDL			BDL			0.83	±	0.06**	BDL			BDL			
	22:6	BDL			BDL			BDL			0.47	±	0.07**	BDL			
L. R1	16:0	3.42	±	0.47	5.63	±	0.67*	3.73	±	0.66	5.85	±	0.12**	4.72	±	0.92	
	18:0	5.34	±	0.80	6.44	±	0.84	4.30	±	0.31	8.35	±	1.47*	5.87	±	0.44	
	18:1(11c)	0.56	±	0.43	2.62	±	0.35**	1.05	±	0.01	1.37	±	0.33*	0.59	±	0.06	
	18:1(9c)	82.37	±	1.00	78.75	±	2.12	82.45	±	1.79	77.27	±	0.92*	80.32	±	1.21	
	18:2	8.29	±	1.03	6.56	±	0.43	7.96	±	2.61	6.65	±	2.33	8.50	±	2.53	
	20:4	BDL			BDL			0.50	±	0.08**	BDL			BDL			
	22:6	BDL			BDL			BDL			0.51	±	0.09**	BDL			
L. 44	16:0	3.49	±	0.55	5.59	±	0.49	3.78	±	0.97	5.81	±	1.14	5.39	±	1.04	
	18:0	4.76	±	0.55	8.22	±	0.18*	4.96	±	0.24	9.73	±	2.35**	5.17	±	0.13	
	18:1(11c)	0.72	±	0.10	2.29	±	0.95*	1.02	±	0.58	1.49	±	0.25	0.54	±	0.06	
	18:1(9c)	81.64	±	0.60	77.29	±	0.67*	80.75	±	1.16	75.96	±	2.55*	79.02	±	2.10	
	18:2	9.39	±	0.80	6.62	±	0.39*	8.44	±	0.62	6.43	±	1.30*	9.87	±	0.96	
	20:4	BDL			BDL			1.05	±	0.15**	BDL			BDL			
	22:6	BDL			BDL			BDL			0.59	±	0.02**	BDL			
L.R1	16:0	ns			ns			ns			ns			ns			
	18:0	ns			*			ns			ns			ns			
	18:1(11c)	ns			ns			ns			*			**			
	18:1(9c)	ns			ns			ns			ns			ns			
	18:2	ns			ns			ns			ns			ns			
	20:4	ns			ns			ns			ns			ns			
	22:6	ns			ns			ns			ns			ns			
L. 44	16:0	ns			ns			ns			ns			ns			
	18:0	ns			ns			*			ns			ns			
	18:1(11c)	ns			ns			ns			*			**			
	18:1(9c)	ns			ns			ns			ns			ns			
	18:2	ns			ns			ns			ns			ns			
	20:4	ns			ns			*			ns			ns			
	22:6	ns			ns			ns			ns			ns			

ns= non significant, BDL= below detection limit

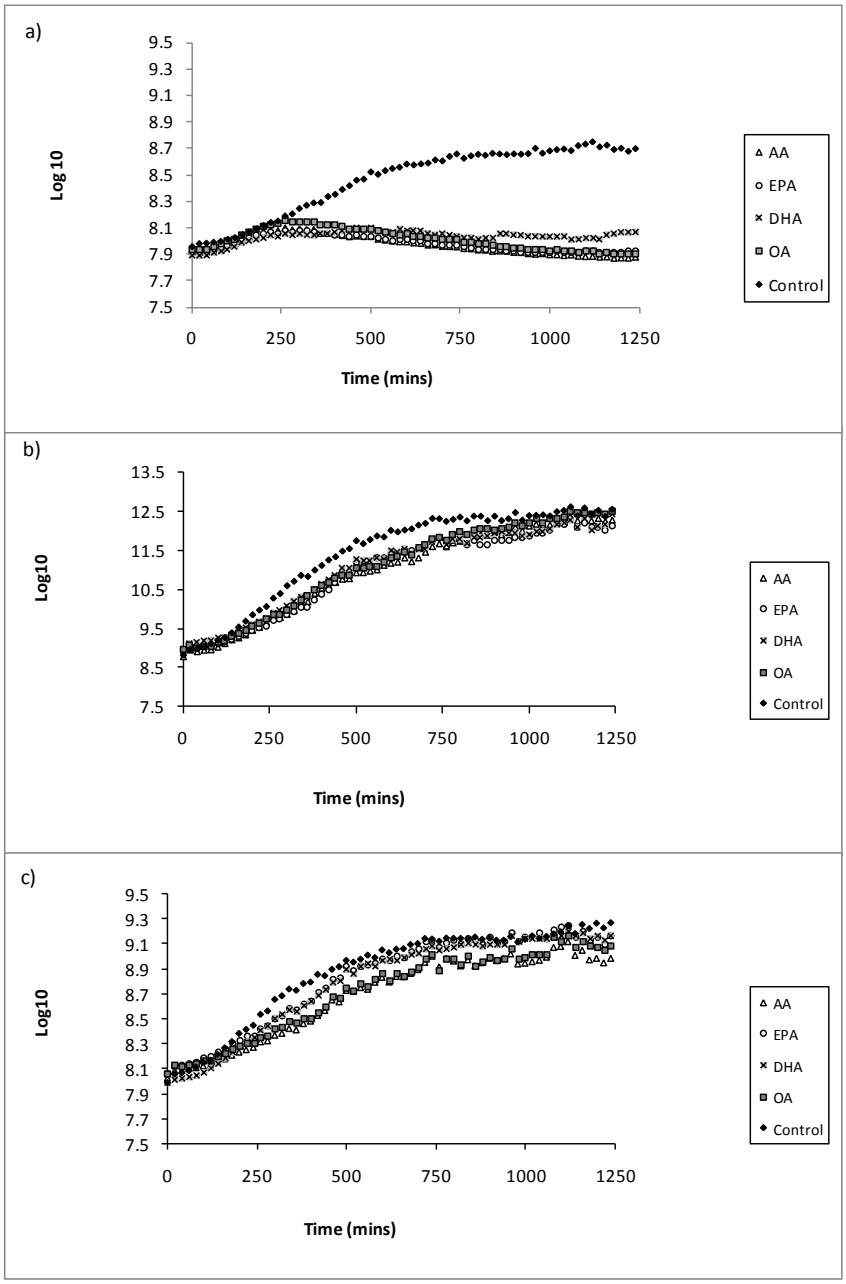

Figure 23 *Numbers of bacteria (Log10) CFU/ml of 10 % (approximately 10^8-10^9 CFU/ml) innoculated in MRS media at 0 mins every 20 mins until stationary phase. a) L. FI9785 b) L. R1 and c) L. 44, after treatment of 20 µM AA, EPA, DHA, OA or equal volumes of ethanol (Control).*

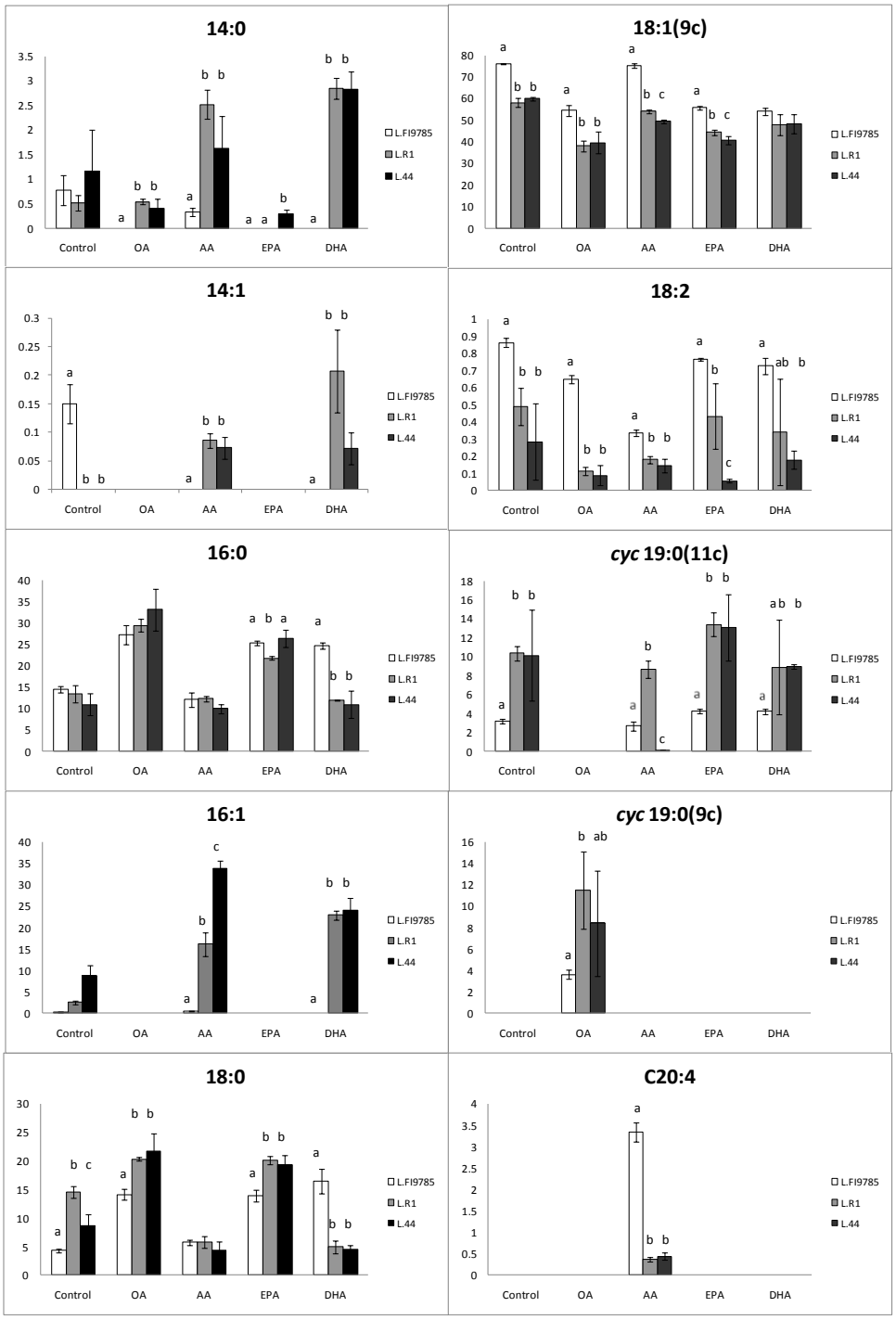

Figure 24 *Percentage values for individual fatty acids:* 14:0, 14:1, *16:0, 16:1, 18:0), 18:1(9c), 18:2,cyc19:0(11c), cyc19:0(9c) and 20:4 taken up by Lactobacillus species* ☐ *L. FI9785* ▒ *L. R1* ■ *L. 44 after 8 hour incubations with 20 μM AA (20:4), EPA (20:5), DHA (22:6), OA (18:1) or ethanol (control). Each bar represents the mean +/- SD (n=3) of each condition. Treatment groups are compared for individual fatty acids and between bacterial types. Those that do not share a letter are significantly different (P<0.05).*

CHAPTER 6 Immunomodulatory effects of PUFAs on colorectal cell lines (HT29 and HT29-MTX) in response to commensal microbes versus pathogenic bacteria

6.1.0 Introduction

It is now well recognised that probiotic bacteria can modulate immune responses and can improve conditions such as inflammatory bowel disease (IBD)[151]. There is also evidence PUFAs may modify the risk and impact of IBD through their immunomodulatory effects [152-154].

Furthermore, there is good evidence of a symbiotic effect between dietary PUFAs and probiotic bacteria in which n-3 PUFAs have been shown to increase adhesion of lactobacilli in the intestines of piglets and arctic charr [75-76]. Both pro- and anti-inflammatory effects of lactobacilli and PUFAs have previously been reported [155-162]. These studies suggest that these food groups function in an immunomodulatory homeostatic manner rather than completely inhibiting or increasing a particular type of inflammatory response. In a healthy individual, a compromise between pro- and anti-inflammatory responses is needed to maintain appropriate immune surveillance to prevent invading pathogens, while at the same time allowing the survival of beneficial commensal bacteria through the avoidance of an excessive immunological response, and removal of foreign organisms. Therefore, the human epithelial cells lining the colon must be able to distinguish between potential threats from pathogenic bacteria and benefit from resident commensals, although much of the components recognised by the innate immune responses are similar in both cases.

Immunomodulatory effects may be mediated through TLR signalling, allowing recognition of specific bacterial antigens. TLR 2 can recognise peptidoglycan present in gram positive bacteria, such as *Lactobacillus sp [163]*. The result of activation can lead to transcription of NF-κB regulated genes that produce many pro-inflammatory signals that aid in the clearance of the bacteria by attracting immune cells to the site of activation. However, TLRs have also been implicated in mediating control of tissue homeostasis and commensal binding to surface epithelia. Additionally, they can up-regulate HSPs that are well established to play a cytoprotective role in intestinal epithelial cells [63].

The immunomodulatory effects of PUFAs may arise as a result of a number of potential mechanisms [154]. For example PUFAs will tend to increase membrane fluidity and thus the function and activity of cell surface proteins [164-165]. Alternatively they may modify gene expression. For example, they and their eicosanoid and docasanoid metabolites are recognised ligands for PPARs. For example, DHA binding to PPAR γ can lead to activation which in turn has been reported to signal increased expression of anti-inflammatory cytokines, such as IL-10, that can dampen the pro-inflammatory response [24]. Of particular relevance to the present study, are the reports that PPAR δ activation up-regulates TGF- β [22] which is involved in oral tolerance of bacteria, thus aiding the survival of bacteria [166-167].

To observe immunomodulation of food groups using a model system, it is necessary to have components of the immune system present, such as the white blood cells, since IECs can regulate innate and immune responses by directly interacting with DC, lamina propria lymphocytes and intra-epithelial lymphocytes. To achieve this, Haller et al. [168] previously employed a co-culture transwell system with intestinal epithelial cell lines to represent the gut epithelium in the top compartment and

PBMC to represent immune cells present in the lamina propria in the bottom compartment of the transwell plate [168]. This system allows cross-talk between the two compartments, theoretically modelling cross-talk between gut epithelium and immune cells close by. As I am particularly interested in the immunomodulatory effects of commensals and PUFAs in relation to colonic health, I selected to use HT29 cells that do not differentiate into small intestinal cells after reaching confluence. However, I was also interested in how the presence of mucin might impact on the epithelial cell response to bacteria as this may modify adhesion or access to the apical surface [169].

Since both PUFAs and lactobacilli have strong immunomodulatory effects I wanted to observe if PUFAs could modify the immune responses to lactobacilli and if there was potentially a common pathway by which these two components might play a role in physiological immune homeostasis and potentially also improve IBD pathogenesis. To allow the comparison with a more pathological response, I also included potentially pathogenic strains (gram positive and gram negative) in the transwell system. Therefore the aim of this study was to observe changes in immunomodulatory signalling in colorectal cells treated with combinations of lactobacilli or pathogenic bacteria and PUFA incorporation [170] in the presence of an immune cell compartment. Furthermore, I wished to explore the possibility that differences in gene expression could be linked to differences in PPAR expression in different cell lines and in response to different PUFAs.

6.2.0 Methods

6.2.1 Gene and protein expression analysis

Methods in section 2.6.0 were followed to explore release of immunomodulatory genes and proteins that maybe be modified by bacterial exposure and/or PUFA treatment.

6.2.2 PPAR Transcription Factor Assay

Methods described in 2.7.1 were used to determine PPAR expression in HT29 and HT29-MTX cells.

6.2.3 Statistical analysis

qRT-PCR results were analysed using inbuilt relative quantification software (Light –Cycler 480 software version 1.0), using the standard curve for both target and reference (GAPDH) gene, the software then determines the target to reference ratio.
Triplicate ratios of each sample were analysed by Two-Way ANOVA using the General Linear Model with Tukey comparisons (Mini tab version 15). Difference with $P<0.05$ (*) and $P<0.01$ (**) were considered statistically different.

6.3.0 Results

6.3.1 Gene expression in response to *different bacteria (figure 25)*

Responses to *L. gasseri* exposure resulted in an increase in TGF-β1 mRNA compared with control (no bacteria) in HT29 and HT29-MTX cells. Exposure to *E. coli LF82* had no effect on TGF-β1 expression in either cell line while *S. aureus* reduced expression only in HT29 cells.

L. gasseri exposure consistently increased cytokine IL-8 mRNA in both HT29 and HT29-MTX cells, however the gram negative pathogen *E. coli LF82* failed to up regulate IL-8 in HT29 cells, whilst there was an increase in mRNA levels of this cytokine after exposure to the gram positive *S. aureus.* Exposure of the more differentiated cell line HT29-MTX *to E. coli LF82* and *S. aureus* resulted in contrasting effects; where *E. coli LF82* led to an elevated mRNA for IL-8, *S. aureus* exposure resulted in no change compared to control levels in absence of bacterial exposure. Furthermore the expression of IL-8 relative to GAPDH in HT29 cells exposed to *S. aureus* was considerably higher than in cells exposed to *L. gasseri.*

L. gasseri exposure resulted in no change in HSP72 in either cell line, while both *E. coli LF82* and *S. aureus* down regulated HSP72 in HT29-MTX cells and *E. coli* reduced HSP72 mRNA in HT29 cells. In addition, TNF-α mRNA was produced by HT29-MTX cells following *E. coli* and *S. aureus*, although higher levels were obtained in *E. coli* exposed cells (table 8). However *L. gasseri* exposure failed to induce TNF-α gene expression to detectable levels (standard curve fewer than 30 cycles) in both HT29 and HT29-MTX cells (data not shown).

6.3.2 Polyunsaturated fatty acids modify immunological responses

In HT29 cells pre-treatment with 50 μM AA increased HSP72 mRNA expression in control group (no bacteria) and *L. gasseri* exposed cells (table 8). TGF-β1 mRNA was raised by all PUFAs in the control group (no bacteria), however after *L. gasseri* exposure only EPA gave a significant increase above the effect of *L. gasseri* (figure 26). AA treatment resulted in increased HSP72 mRNA in the control group (no bacteria) but PUFA pre-treatment did not modify the effect of *L. gasseri* ($p<0.02$, table 9). No affects of PUFAs were found on HSP25 mRNA (table 8). EPA and DHA treatment of HT29 cells resulted in an increase level of HSP72 mRNA after *E. coli LF82* exposure (table 9).

In HT29-MTX cells EPA significantly increased TGF-β1 mRNA in the control group, whereas EPA increased TGF-β1 mRNA compared with AA with *L. gasseri* treated cells, but not the control (figure 26). PUFAs did not affect HSP25, HSP72 or IL-8 mRNA production (table 8). PUFAs did not affect immune protein mRNA tested following *S. aureus* exposure similarly to HT29 cells. However, following *E. coli LF82* exposure, AA treatment resulted in a small but significant increase in HSP72 in HT29-MTX (table 9).

6.3.3 Protein expression

L. gasseri exposure led to a three-fold increase in IL-8 (1.11 +/- 0.02 ng/ml) compared to control HT29 cells (0.38 +/- 0.09 ng/ml) while data could not be obtained from HT29 cells exposed *E. coli LF82* and *S. aureus* due to lack of protein. However, expression in HT29-MTX was increased relative to the control following exposure to gram negative *E. coli LF82,* whilst there was no change after gram positive *L. gasseri* or *S. aureus* exposure (figure 27). Protein expression of TGF-β1 was raised relative to the control (no bacteria) by *L.gasseri* in both HT29 and HT29-MTX cell lines, whilst *E. coli LF82* did not significantly change TGF-β1 from control levels. *S. aureus* increased TGF-β1 protein in HT29 cells, however failed to do so in the more differentiated HT29-MTX (figure 28). Differences in TGF-β1 protein between PUFA treatment and control with no PUFA were tested in *L. gasseri* exposed cells, although no significant difference was found, similar patterns to those for mRNA expression following PUFA pre-treatment were observed. For example EPA increased TGF-β1 expression but not significantly in both cell lines following *L. gasseri* exposure (table 10).

6.3.4 PPAR expression

HT29-MTX cells expressed high levels of PPAR δ, and PPAR γ compared to HT29 cells (relative ratio 3.08, 2.94 respectively). However, there was no difference in response to PUFA treatments in HT29 cells and in HT29-MTX.

6.4.0 Discussion

The gut surface is constantly exposed to commensal bacteria that elicit no adverse immune responses in healthy, tolerant individuals. However, to control the levels of pathogenic bacteria entering the host on a daily basis a certain degree of immune response is required to remove them efficiently. Therefore it has been proposed that an active immune system is required, although this must be tolerant to non-pathogenic bacteria that can confer many benefits to the host. A single *in vitro* system cannot effectively represent the complexity of the human gastrointestinal tract, therefore to obtain more physiologically relevant results; separate cell lines isolated from the human colon were used in a co-culture system with PBMC. This model, developed from that described by Haller et al. [168] allows for cell to cell cross talk that is an important part of many cytokine responses.

Results show that colonic epithelial cells can differentially respond to commensal and pathogenic bacteria. However, the responses to bacteria can be cell line specific, for example HT29 cells exposed to *S. aureus* expressed higher levels of IL-8 mRNA than those exposed to *L. gasseri* while this effect was not observed in the more differentiated HT29-MTX. Responses to *E. coli* showed a contrasting pattern, in that IL-8 mRNA was up-regulated in HT29-MTX but not HT29 cells. The results for IL-8 protein level in response to *E. coli* and *S. aureus* reflected gene expression patterns in HT29-MTX. TGF-β1 expression of mRNA and protein level was increased in both cell lines in response to *L. gasseri*, but not in response to *E. coli*. In relation to TGF-β1 expression, the only difference between the cell lines was following exposure to *S. aureus* where only HT29 cells showed increased expression. The differences in response between the cell lines maybe the result of HT29-MTX being a more differentiated cell line that produces mucin as well as other proteins involved in immune responses, for example TLRs [103]. Additionally, it could be the result of differences in adhesion of bacteria to different cell types [171].

In HT29-MTX cells, IL-8 protein was not up-regulated compared with the control following exposure to either gram positive bacteria; *L. gasseri* or the opportunistic pathogen *S. aureus*, supporting the concept that neither causes damage to the intact epithelial layer. Tolerance to non-invasive bacteria is proposed to be due to the lack of apical TLR-2, therefore to stimulate an immune response bacteria would need to enter or bypass the epithelial cell to elicit a response via TLR-2 at the basolateral membrane or on macrophages [105]. The lack of response in HT29 cells when exposed to gram negative *E. coli LF82* could be explained by a down-regulation of apically expressed TLR-4 that would recognise the LPS in a gram negative bacterial membrane and initiate a local immune response as shown by Furrie et al. using *E. coli* co-culture with HT29 cells [103]. In addition, more differentiated cell lines maybe more adept at detecting differences in pathogenic capabilities. However, HT29 cells did respond to gram positive bacteria *L. gasseri* and *S. aureus* with increased production of IL-8 mRNA, again contrasting with the negative responses in HT29-MTX cells. This result is consistent with the suggestion by Furrie et al.(2005) that undifferentiated HT29 cell are similar to newly formed crypt cells, both showing an up regulation of TLR-2 in response to gram positive bacteria.

Consistent with the increase in IL-8 expression, I also observed an increase in TNF-α mRNA expression in HT29-MTX cells when exposed to *E. coli*. However, whilst *L. gasseri* showed a high level of IL-8 mRNA, a significantly lower amount of TNF-α mRNA was produced. These results may explain lack of protein production from lactobacilli treated cells as reported by McCraken et al. [172], and also maintenance of HT29-MTX IL-8 protein compared to the control, since TNF-α enhances production of IL-8 protein [173].
The increase in TGF-β1 expression in colonic cells exposed to *L.gasseri* is consistent with a previous mouse study in which increased TGF-β1 in BALB/c mice was attributed to an increase in CD4+differentiation into T regulatory cells (Tr1) in response to secreted lactobacilli bacterial components [174]. Firstly, Tr1 cells are known to secrete large amounts of TGF-β1 and have been proposed to have a role in oral tolerance to bacteria in the gut via

an inhibitory feedback effect on IL-12 that drives CD4+ differentiation to TH_1 cells. Secondly, TGF-β1 may facilitate tolerance, through Smad signalling leading to degradation of TLR-2. The inclusion of leukocytes below the epithelial layer as used in the current study allows for the first possibility and also suggests a reason why Vizoso Pinto et al. [175] did not observe expression of TGF-β1 from HT29 cells alone.

Another potential mechanism by which commensal bacteria may modify TGF-β signalling is by modifying PPAR regulated pathways. There is evidence that TGF-β1 is a molecular target for PPAR δ [22] and PPAR γ shares a down stream target gene with TSC-22 (transforming growth factor stimulated clone 22) [176]. This mechanism could result in a negative feedback response that down-regulates pro-inflammatory cytokines, such as IL-8, through inhibition of NF-κB [177-178]. This mechanism would help to distinguish between pathogenic and non-pathogenic gram positive bacteria where-by lack of TGF-β1 production would lead to an enhanced inflammatory response to clear the invading pathogen. This hypothesis is supported in the current study by the observation that TGF-β1 mRNA is undetectable following pathogen exposure and no increase of TGF-β1 protein from *E. coli* and opportunistic pathogen *S. aureus* in HT29-MTX cells. Furthermore, this may explain why we observe an increase in IL-8 gene expression exposed to *L. gasseri*, but not protein expression, in HT29-MTX. Although in the current study an increase in IL-8 gene expression is reported, several authors have previously suggested that lactobacilli can down-regulate IL-8 in TNF-α or LPS sensitized cell lines [159, 179]. This difference is likely to be due to the presence of white blood cells in the transwell model, where stimulation of immune cells such as the differentiation of CD4+ cells to a TH_1 phenotype could lead to an increase in IL-8 production [168]. Additional modulation in gene expression by bacterial type is shown for HSP72, where *E. coli LF82* and *S. aureus* both down-regulated mRNA levels in HT29-MTX, while *E. coli* alone decreased levels in HT29 cells. HSP72 production can be stimulated by TLR-2 activation and NF-κβ signalling [180] and in HT29 cells *E. coli* has been shown to down regulate TLR-2, resulting in possible HSP down-regulation in HT29s [103].

Modulations of immunological responses to PUFAs showed cell lines increasing TGF-β1 mRNA after pre-treatment with EPA. EPA is well recognised to be a ligand for PPARs and although these receptors tend to bind most efficiently to the most unsaturated fatty acids, (in this experiment DHA), in the case of PPAR δ EPA has a conformation that binds more efficiently than DHA [23]. Fish oil diets, high in EPA and DHA, have been shown to up-regulate the PPAR δ responsive gene UCP3, resulting in increased wound healing after dextran-sodium sulphate induced colitis in pigs [21], supporting a mechanism in which TGF-β1, involved in colitis wound healing [181], may be up-regulated by EPA via PPAR δ[22]. A similar, but non-significant, increase in TGF-β1 protein was observed in EPA loaded cells exposed to *L. gasseri*.

Another immunomodulatory gene that PUFAs significantly changed was HSP72. HT29 and HT29-MTX cells, exposed to *L. gasseri* and *E. coli* respectively showed a significant increase in HSP72 after AA treatment (table 8 and 9). This may arise as a result of increased production of prostaglandin 15-deoxy- $\Delta^{12/14}$ (PGJ2) from its substrate AA . PGJ2 has previously been shown to induce HSP72 expression , possibly through a mechanism involving PPAR γ [182]. Interestingly, in HT29 cells, EPA and DHA treatment results in HSP72 mRNA increase following *E. coli* exposure, and it is possible that the mechanism in which this effect is caused is also via PPAR γ as a result of the ability of PUFAs to act as ligands for this receptor [183]. However, individual PUFAs differentially affect HSP expression from HT29 cells following gram positive and gram negative bacteria, for example AA increases HSP72 in cells exposed to *L. gasseri*, while EPA and DHA increase HSP in cells exposed to *E. coli*. A possible explanation maybe through TLR signalling, since ligand activation can inhibit PPAR γ through the NF-κB pathway [184-185] and since TLR-4 is down-regulated by *E. coli* in HT29 cells [103] this inhibition is reduced, thus allowing subsequent downstream targets to be produced, such as HSP72. In addition, *E. coli* damage may lead to disruption of PGJ2 formation from AA, and as AA itself is a relatively weak ligand for PPAR γ the effects of EPA and DHA, as stronger PPAR γ ligands, may then

dominate. This hypothesis would suggest a complex interaction between PUFAs and bacteria, with respect to HSP72, as shown in table 8 and 9.

This study builds on the observation that bacterial signalling at the mucosal surface is dependant on the presence of the underlying leukocyte population by examining the effects of pathogenic bacteria. I have demonstrated that the previously recognised complex mechanisms that exist to distinguish between pathogen and commensals are likely to involve TGF-β1, IL-8 and TNF-α. Secondly I show that these responses are differentially modified by n-3 and n-6 PUFAs, suggesting a subtle effect of these dietary components on colonic mucosal immune responses.

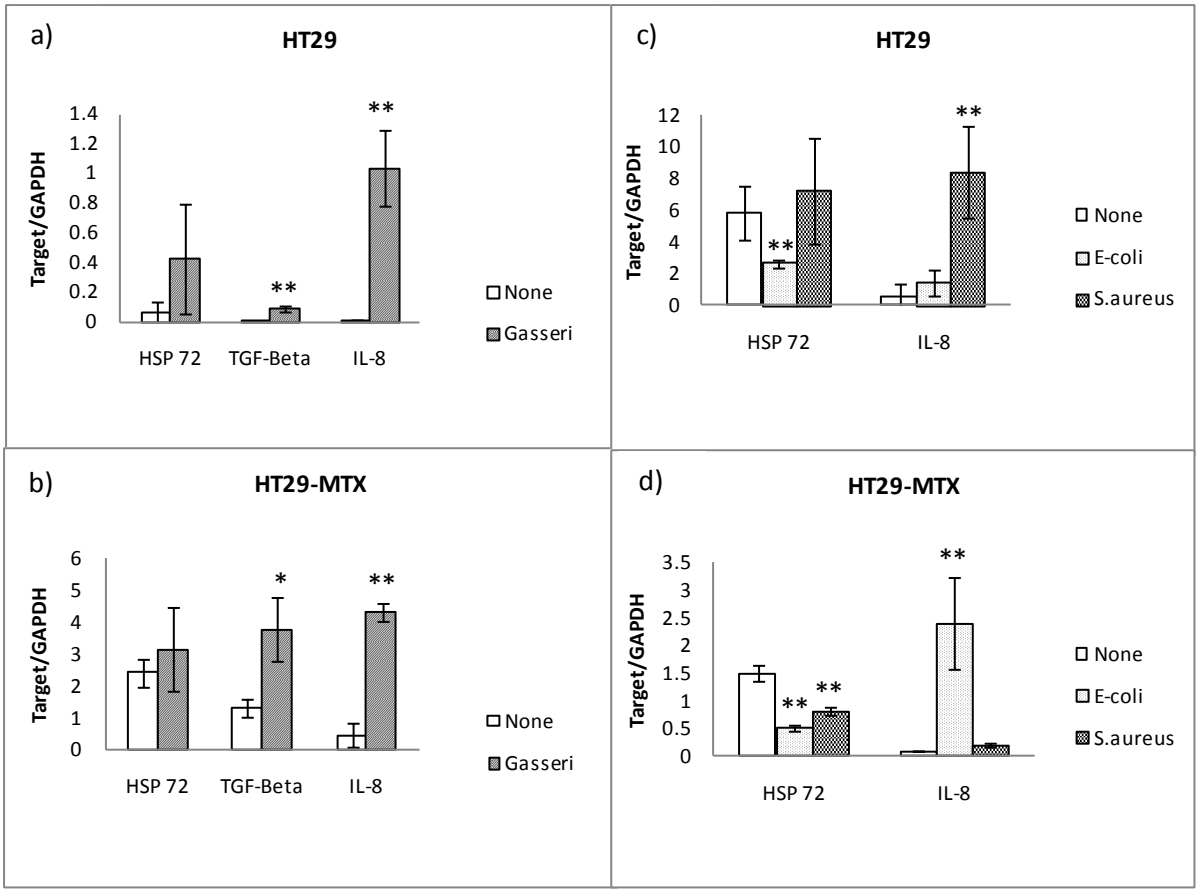

Figure 25 *HT29 and HT29-MTX cells were exposed to; L. gasseri (a and b) or control with no bacteria (none); E. coli, S. aureus (c and d) or control with no bacteria (none). PUFA treated cells were omitted to observe bacteria effects only. Each bar represents the mean relative to GAPDH (fold change) +/- SD (n=3) of each bacterial treatment split for mRNA of each target/reference gene. Significance is relative to control (none) *P<0.05 **P<0.01*

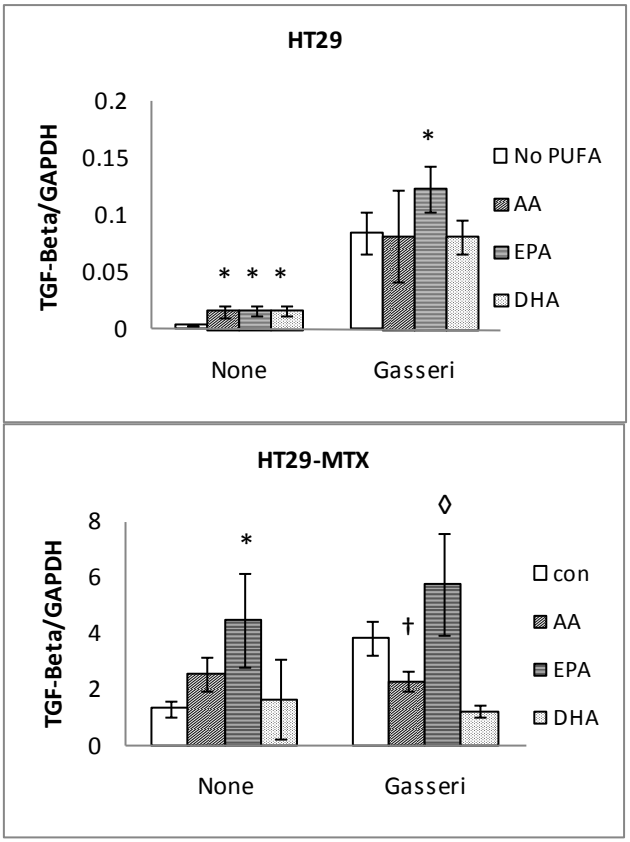

Figure 26 *Cell lines (HT29 and HT29-MTX) production of TGF-β/reference gene were compared between treatment groups of PUFAs; AA, EPA, DHA or Con (no PUFA). Data was split further into L. gasseri exposed and none bacteria (none) exposed groups. Each bar represents the mean relative to GAPDH (fold change) +/- SD (n=3). Significance relative to control *P <0.05 and different symbols represent significance between the two bars labelled †◊ P<0.05.*

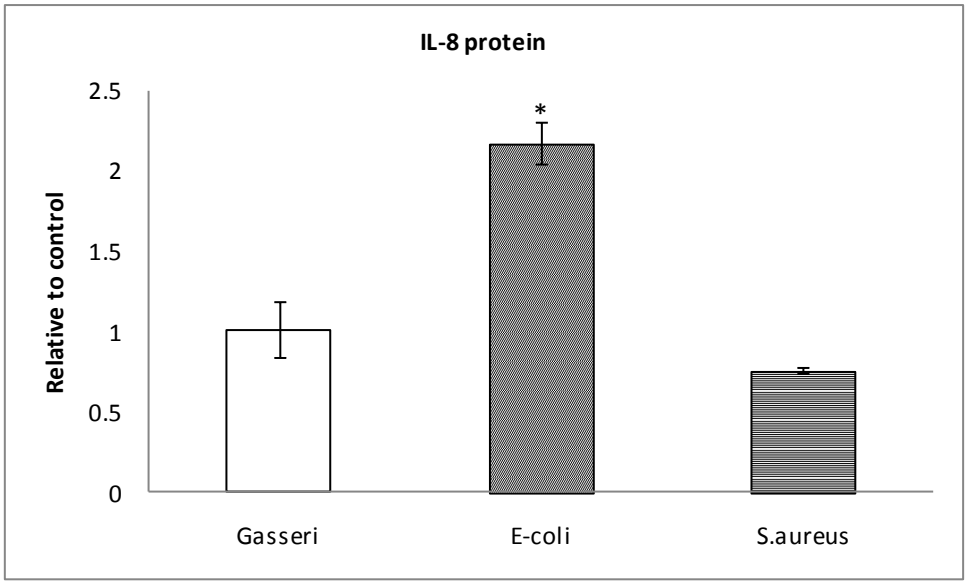

Figure 27 *HT29-MTX cells production of IL-8 protein (pg/ml) relative to experimental controls (control=1). Each bar represents the mean of fold change when compared to the control +/- SD (n-3). Significance is shown between the control and each bacterial treatment (*P<0.05).*

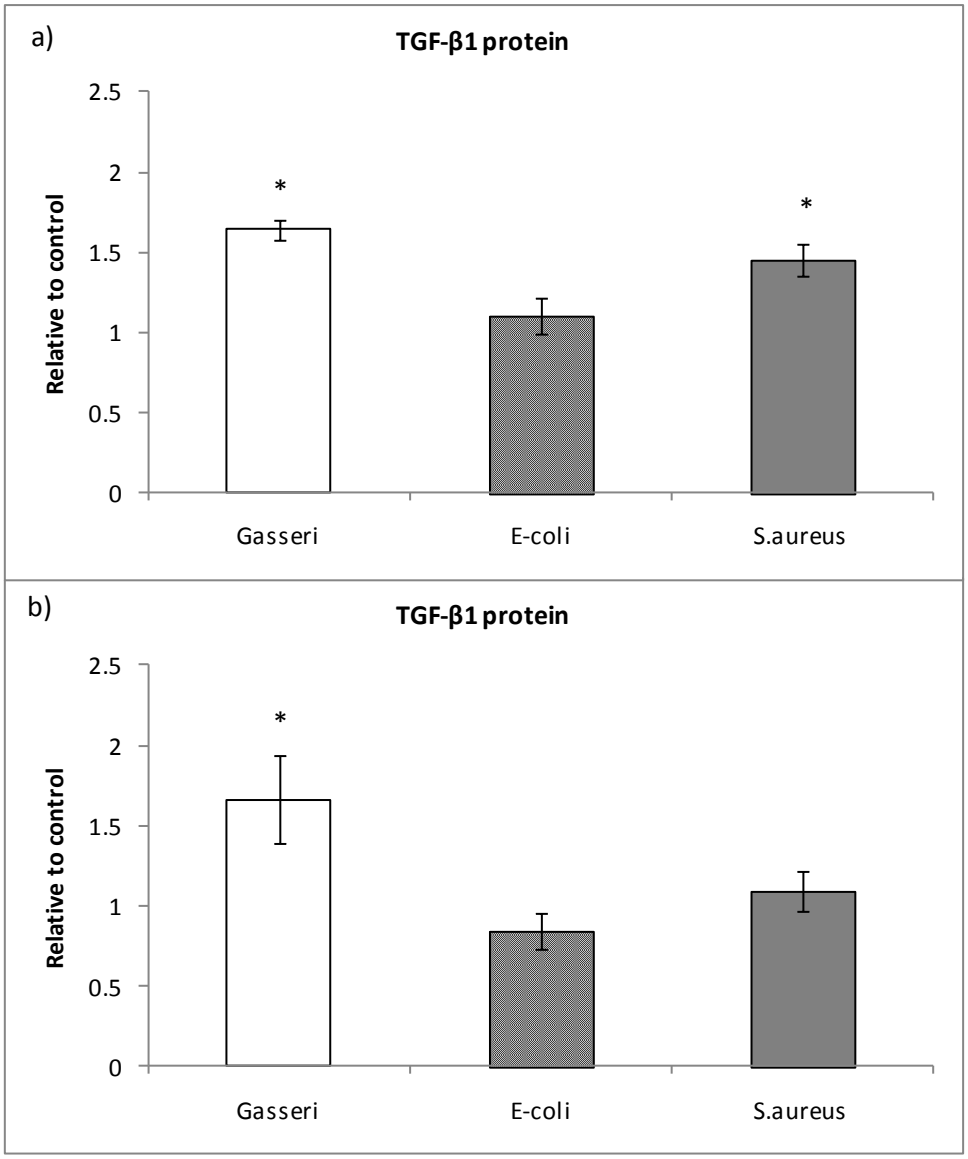

Figure 28 *Cell lines a) HT29 b) HT29-MTX production of TGF-β1 protein (pg/ml) relative to experimental controls (control=1). Each bar represents the mean of fold change relative to control +/- SD (n-3). Significance is shown between the control and each bacterial treatment (*P<0.05).*

Table 8 *Immunomodulatory protein gene expression that was modified by PUFAs/L. gasseri and not shown as a figure. Results were expressed as means of fold change compared to house keeping gene GAPDH (+/- SD). Significant difference between PUFAs and control for each gene are shown on the right column of the table excluding the bottom quadrant and differences between L. gasseri being present or not are shown in 4th column bottom row. The bottom right quadrant shows significance of an interaction between bacteria and PUFAs. Significant results are labelled * <0.05 and highly significant **<0.01*

Cell lines	Fatty acid	No bacteria (none)			*L. gasseri*			Significance		
		HSP 25	HSP 72	IL-8	HSP 25	HSP 72	IL-8	HSP 25	HSP 72	IL-8
HT29	**Control**	0.143	0.059	0.006	0.097	0.428	1.035			
		(0.03)	(0.07)	(0.01)	(0.03)	(0.37)	(0.26)			
	AA	0.115	1.390	0.232	0.188	2.296	1.162	ns	**	ns
		(0.04)	(0.57)	(0.14)	(0.05)	(1.33)	(0.22)			
	EPA	0.149	0.887	0.007	0.160	0.592	1.439	ns	ns	ns
		(0.09)	(0.26)	(0.01)	(0.08)	(0.43)	(0.22)			
	DHA	0.073	0.764	0.027	0.104	1.561	0.857	ns	ns	ns
		(0.06)	(0.40)	(0.03)	(0.03)	(0.74)	(0.04)			
HT29-MTX	**Control**	1.167	2.396	0.434	1.676	3.152	4.326			
		(0.46)	(0.45)	(0.38)	(0.59)	(1.75)	(1.14)			
	AA	1.266	2.804	0.071	1.107	2.046	1.361	ns	ns	ns
		(0.08)	(0.26)	(0.03)	(0.22)	(0.94)	(1.03)			
	EPA	1.920	1.575	1.654	1.535	2.599	4.183	ns	ns	ns
		(0.57)	(0.56)	(1.40)	(0.20)	(1.33)	(2.19)			
	DHA	0.624	0.341	0.043	0.000	8.802	0.364	ns	ns	ns
		(0.01)	(0.12)	(0.06)	(0.01)	(6.71)	(0.01)			
HT29					ns	ns	**	ns	ns	ns
HT29-MTX					ns	ns	**	ns	*	ns

ns= non significant

Table 9 *Immunomodulatory protein gene expression from cell lines that were modified by PUFAs/E. coli or S. aureus and not shown as figure 25. Results were expressed as means of fold change compared to housekeeping gene GAPDH (+/- SD). Significant difference between PUFAs and control for each gene are shown on the right column of the table for E. coli and S. aureus bacterial exposures (excluding the bottom quadrant). Differences in protein gene expression between E.coli being present or not are shown in 4th column bottom row, while differences occurring after S. aureus exposure compared to no bacteria control (none) are shown in 5th column bottom row. The bottom right quadrant shows significance of an interaction between bacteria and PUFAs. Significant results are labelled *P <0.05 and highly significant **<0.01*

Cell lines	Fatty acid	No bacteria (none)			*E. coli LF82*			*S.aureus*			Significance - *E. coliS.aureus*		
		HSP 72	IL-8	TNF-α	HSP 72	IL-8	TNF-α	HSP 72	IL-8	TNF-α	HSP 72	IL-8	TNF-α
HT29	**Control**	5.863 (1.71)	0.610 (0.72)	nd	2.664 (0.26)	1.416 (0.81)	nd	7.262 (3.40)	8.426 (2.87)	nd			
	AA	8.426 (0.94)	0.246 (0.05)	nd	1.513 (1.05)	1.187 (0.11)	nd	2.689 (0.530)	2.027 (1.33)	nd	ns/ns	ns/ns	nd/nd
	EPA	11.40 (1.63)	2.517 (0.19)	nd	8.258 (1.50)	7.610 (1.13)	nd	5.368 (0.65)	3.242 (1.93)	nd	*/ns	ns/ns	nd/nd
	DHA	18.40 (4.06)	0.204 (0.03)	nd	4.498 (1.69)	4.515 (0.18)	nd	3.171 (0.39)	2.944 (1.19)	nd	**/ns	ns/ns	nd/nd
HT29-MTX	**Control**	1.492 (0.13)	0.073 (0.01)	0.073 (0.02)	0.502 (0.04)	2.398 (0.84)	2.419 (0.95)	0.809 (0.07)	0.187 (0.03)	0.189 (0.04)			
	AA	1.692 (0.13)	0.046 (0.01)	0.046 (0.01)	0.597 (0.06)	3.469 (0.54)	2.809 (0.57)	0.845 (0.03)	0.221 (0.09)	0.142 (0.04)	*/ns	ns/ns	ns
	EPA	1.425 (0.04)	0.010 (0.01)	0.083 (0.02)	0.578 (0.03)	2.428 (0.41)	2.701 (0.58)	0.897 (0.17)	0.331 (0.08)	0.324 (0.09)	ns/ns	ns/ns	ns
	DHA	1.479 (0.03)	0.058 (0.01)	0.059 (0.02)	0.560 (0.08)	1.915 (0.33)	1.932 (0.37)	1.003 (0.16)	0.239 (0.10)	0.229 (0.09)	ns/ns	ns/ns	ns
HT29					**	ns	nd	ns	*	nd	**/**	ns/ns	nd/nd
HT29-MTX					**	**	**	**	ns	ns	ns/*	* /ns	ns/ns

ns= non significant, nd= no data

Table 10 *TGF-β1 protein expression (pg/ml) by cell lines treated with PUFAs: AA, EPA and DHA with and without L. gasseri exposure. Results are shown as means (pg/ml) (+/- SD).*

Cell lines	Fatty acid	No bacteria (none)	*L. gasseri*
HT29	**Control**	62.09	58.03
		(10.1)	(0.75)
	AA	56.24	59.81
		(8.93)	(5.10)
	EPA	42.48	71.62
		(3.81)	(22.04)
	DHA	nd	52.59
			(14.68)
HT29-MTX	**Control**	100.86	99.95
		(5.96)	(20.2)
	AA	74.98	109.87
		(12.1)	(3.68)
	EPA	80.51	120.54
		(12.2)	(33.0)
	DHA	89.53	67.96
		(12.3)	(40.4)

nd= no data

CHAPTER 7 Immunomodulatory effects of PUFAs on colorectal cell line (Caco-2) in response to commensal microbes versus pathogenic bacteria

7.1.0 Introduction

The diet comprises of many different components, many of which have been show to modify the immune system, i.e. PUFAs [5] , lactobacilli[186] and sulphoraphane[187]. Studies often focus on one food-component's interaction with the immune system; however in our complex diets it is inevitable that the components will interact or oppose each other by influencing immune regulation differently. For example, commensal lactobacilli can up-regulate inflammatory TH_1 cell responses[188] , while PUFAs have been shown to up-regulate anti inflammatory mediators, such as resolvins[189]. The results of these opposing affects show immune regulation, rather than completely inhibiting or elevating one type of response. In healthy individuals a balance between both anti and pro inflammatory mechanisms are necessary for non-recognition of beneficial components entering the gastrointestinal tract and appropriate immune surveillance to prevent pathogen invasion.

Detection of pathogens often starts with innate immune cells, which can initiate a pro-inflammatory cascade to aid removal, via activation of TLRs. These can be present on epithelial cells, for example in the colon TLR-4s (LPS recognition) among others are present [105]. In contrast down regulation of pro-inflammatory signalling can be linked to mechanisms involving PPAR signalling, shown to reduce levels of pro-inflammatory products, such as TNF-α [21].

PUFAs and lactobacilli have both been linked with immunomodulatory mechanisms [155-162]. Previous studies have demonstrated that PUFAs interact with lactobacilli

by increasing their adhesion in intestines of piglets [75] and arctic charr [76], therefore this study proposes to use intestinal epithelial cells to explore immunomodulation of both PUFAs and lactobacilli by employing a co-culture system, described by Haller et al. [168]. This system comprises of immune cells isolated from human blood incubated below a monolayer of epithelial cells to allow cross-talk between the two cells types and theoretically model cross-talk between the gut and immune cells of the lamina propria. This work supplements similar studies using HT29 and HT29-MTX cells to analyse difference in colonic epithelial cell models (chapter 6).

7.2.0 Methods

7.2.1 Gene and protein expression analysis

Methods in section 2.6.0 were followed to explore release of immunomodulatory genes and proteins that maybe be modified by bacterial exposure and/or PUFA treatment.

7.2.2 Exploring theories behind immunomodulation results

Methods in 2.7.0 were followed with the omission of HT29 and HT29-MTX cells in section 2.7.1.

7.2.3 Statistical analysis

qRT-PCR results were analysed using inbuilt relative quantification software, using the standard curve for both target and reference (GAPDH) genes, the software then determines the target to reference ratio.

Triplicate ratios of each sample were analysed by General linear model with Tukey comparisons (Mini tab version 15). Difference with $P<0.05$ (*) where considered statistically different $P<0.01$ (**) very significant for qRT-PCR and PPAR ELISAs. Difference with $P<0.05$ in LDH assays are indicated by a different letter.

7.3.0 Results

7.3.1 Immunological response to *Lactobacillus gasseri*, *Escherichia coli LF82* and *Staphylococcus aureus*

Following exposure to *E. coli LF82,* IL-8 mRNA levels rose 5 fold compared to the no bacteria control (figure 29 b), whilst *S. aureus* and *L .gasseri* showed no significant changes in IL-8 expression compared to control levels. Heat shock proteins; HSP 25 and HSP 72; were up regulated compared to controls following *L. gasseri* exposure (figure 29 a), however *S. aureus* and *E. coli LF82* caused no changes in HSP 72.

7.3.2 Polyunsaturated fatty acids modify immunological responses

EPA modulated the response of Caco-2 cells to *S.aureus* by down-regulating HSP 72 mRNA expression (table 12), whilst there were no effects of PUFAs following exposure of cells to either *L. gasseri* or *E. coli* LF82 (table 11 and 12).

Both EPA and DHA caused down regulation of TGF-β1 mRNA expression by Caco-2 cells compared with no PUFA controls without bacterial exposure (figure 30). However following incubation with bacteria these PUFA effects were lost. None of the PUFAs tested were found to affect expression of HSP 25 or IL-8 either alone or following exposure to any of the bacteria species tested.

7.3.3 Protein expression

Contrary to the mRNA expression patterns seen for IL-8 in Caco-2 cells, IL-8 protein production was significantly increased following exposure to *L. gasseri* (figure 31). However, no significant differences were observed in TGF-β1 protein levels from Caco-2 cells following incubation with any of the bacteria compared with controls either with or without PUFA treatment.

7.3.4 Transepithelial resistance and fatty acid analysis of media

Caco-2 cells had the highest TEER reading resulting in 74 Ω.cm2, followed by HT29-MTX (43 $\Omega.cm^2$) and HT29 (29 $\Omega.cm^2$) in control treatment groups. Interestingly this result did not represent transfer of PUFAs to the basolateral side of cells since Caco-2 cell were the only cells that transported PUFAs across the cell layer into the baso-lateral compartment of the transwell; 3.5 % AA, 15.6 % EPA and 18.4 % DHA were detected .

7.3.5 PPAR expression

PUFAs significantly affect expression of PPAR δ, whereby they tend to decrease the expression relative to the control. A similar trend is seen for both PPAR α and γ whilst not significant (figure 32).

7.3.6 LDH assay

Both ethanol (control) and AA treatment groups induce significantly higher percentage cytotoxicity in Caco-2 cells, when compared with other colonic cell lines (figure 33).

7.4.0 Discussion

The gut surface is continuously being exposed to foreign components many of which are beneficial to gut health, for example lactobacilli that can aid pathogen exclusion [190]. However often bacteria can pose a threat and therefore complex mechanisms are required to give an active immune response to remove pathogens, but maintain tolerance toward commensals. My previous experiments conducted in HT29 and HT29-MTX cells have shown that responses to cytokines can be cell specific (chapter 6) and this could be the result of varied adhesion of *L. gasseri* to different cell types or different expression of certain proteins involved in immune responses. The elevation of genes compared to GAPDH is also likely to be explained by varying adhesion abilities or sensitivity of cells, for example high expression from Caco-2's maybe the result of increased sensitivity [130] to the PUFA treatment and even the small quantity of ethanol solution they are diluted in (figure 33).

Caco-2 cells respond to *E. coli LF82* with a large increase in IL-8 mRNA compared with control (none), however this does not result in an increase of IL-8 protein. The response to *E. coli LF82* could be due to a small change in levels of TLR-4 compared to HT29 cells (no response to *E. coli LF82*-chapter 6) as shown for Caco-2 cells previously [103] . The lack of protein production maybe the result of a delay in its production or alternative signalling that blocks its transcription. However, it

must be noted that Caco-2 cells do produce a large amount of IL-8 protein after commensal *L. gasseri* exposure, this could be explained by the higher numbers of *L. gasseri* (10^9 CFUs) used in incubations compared with *E. coli LF82* (10^6 CFUs).

In my experiments Caco-2 cells exposed to *L. gasseri* showed increased HSP 72 and 25 production relative to control, these proteins are shown to be linked with repair therefore increased production from commensal bacteria alone suggests a symbiotic mechanism between lactobacilli and gut epithelial cells. Tao et al. [191] suggested one possible mechanism is that protein kinases from lactobacilli are released and bind to HSF-1, which up regulated HSP 72 between 4-6 h. Johnson et al. [192] reviews HSPs and highlights that they can be released even if only minor stressors have occurred. Thus the documented evidence that Caco-2 cells are particularly sensitive to stress either via ethanol or PUFAs [130] (figure 33), could explain the increase in HSPs, which in not seen in HT29 and HT29-MTX cells (chapter 6). Another mechanism in which lactobacilli may up-regulate HSPs is through TLR-2 activation and stimulation of NF-κβ, that results in HSP 25 and 72 production [180] supported by the higher levels of TLR-2 mRNA found in Caco-2 cell compared to HT29 cells [103]. In comparison, *E. coli LF82* down regulated mRNA levels of HSP 72 in HT29 and HT29-MTX (chapter 6), while no change was observed in Caco-2 cells, these results could be explained by decreases in TLR-2 following co-culture with *E. coli* in HT29 cells but not Caco-2 cells [103].

Modulations of immunological responses to PUFAs are cell specific. The biggest variation in results is from Caco-2 cells after *L. gasseri* exposure, where mRNA TGF-β1 and protein is not up-regulated by PUFAs, unlike in HT29 and HT29-MTX cells (chapter 6). The reason could be attributed to the affects small quantities of ethanol or PUFAs have on this cell line in terms of membrane leakage (figure 33), whereby movement of PPARs in to the nuclear fraction on addition of RXR and for

example a fatty acid, would not occur if the cell was responding to damage as a priority. This movement of PUFAs through the cells is supported by the results from fatty acid extraction indicating that Caco-2 cells are the only cell line tested to result in added PUFA being found in the bottom compartment, maybe as a result of effective transepithelial transport mechanisms. Alternatively, this may arise due to cell damage as indicated by LDH release, even though in control conditions these cells maintain the highest TEER reading indicating preservation of tight junction integrity. Unfortunately TEER readings were not completed in the PUFA treated cells to make comparisons. As a cell model for the colon TEER results suggest that under normal growth conditions and the time allowed following confluence that HT29 and HT29-MTX cells are within the normal range for human colon 12-69 $\Omega.cm^2$ [100], whilst Caco-2 cells gave a higher resistance possibly causing discrepancies between the cell lines and must be taken into account on analysis of other data presented within this study.

Caco-2 cells exposed to EPA and *S. aureus* resulted in a decreased HSP 72 mRNA level, possible reasoning behind the change in PUFA affects after this opportunistic pathogen exposure may be the additional damage caused by the bacteria resulting in disruption in the cells protective mechanism. Another explanation may be due to the high evolutionary conservation of HSPs across species [193], therefore since the pathogens are most likely to be able to invade the colonic cell, the RNA samples may include bacterial HSP 72. In Caco-2 cells a much larger percentage of PUFAs are found inside the cell following PUFA treatment (chapter 4), therefore due to PUFAs anionic detergent actions these can aid in killing bacteria. After the addition of EPA more of the pathogenic bacteria could have been destroyed, resulting in less HSP 72 in the RNA samples.

Experiments looking at expression of PPARs show a lowering of PPAR δ following AA treatment in Caco-2 cells. This maybe due to the sensitivity of the cell line towards AA or ethanol, causing leakage [130] (figure 32) and thus preventing the translocation of PPARs into the nuclear fraction of the cell. Another mechanism could be due to the presence of cyclooxygenase-2 (COX-2), that converts PUFAs into a variety of prostaglandins. Caco-2 cells express a large amount of COX-2 [194-195], hence plenty would be available for conversion of PUFAs into prostaglandins and thus less PUFAs may be available to bind the PPAR-RXR complex and subsequent translocation into the nucleus. That is, there would be competition for PUFAs between COX-2 and PPAR activity. However, prostaglandins has also been shown to bind the PPAR-RXR complex and this theory would only stand if the PUFAs were more efficient at binding to PPARs affecting translocation rate to the nucleus.

When summarising all suggested possible mechanisms in which PUFAs and bacteria may act it shows that their immunomodulatory affects are likely to work through different mechanisms. However, resulting affects can oppose or enhance each others interaction. The experiments suggest that PUFAs and lactobacilli can either up or down regulate pro or anti-inflammatory immune responses, suggesting that foods containing these constituents can play a part in normal immune regulation. That is, a balance is needed between pro and anti-inflammatory responses to allow immune surveillance to recognise threats from pathogens, but support tolerance to commensal microbes.

Table 11 *Immunomodulatory protein gene expression that was modified by PUFAs/L .gasseri and not shown as a figure. Results were expressed as means of fold change compared to housekeeping gene GAPDH (+/- SD). Significant difference between PUFAs and control for each gene are shown on the right column of the table excluding the bottom quadrant and differences between L. gasseri being present or not are shown in 4th column bottom row. The bottom right quadrant shows significance of an interaction between bacteria and PUFAs. Significant results are labelled * P<0.05 and highly significant **P<0.01.*

Fatty acid	**No bacteria (none)**						***L. gasseri***						**Significance**		
	HSP 25		**HSP 72**		**IL-8**		**HSP 25**		**HSP 72**		**IL-8**		**HSP 25**	**HSP 72**	**IL-8**
	mean	SD	mean	SD	mean	SD	mean	SD	Mean	SD	mean	SD			
Control	1.01	0.19	0.25	0.06	0.27	0.17	2.72	0.10	3.28	0.76	5.39	3.87			
AA	1.29	0.01	0.42	0.12	0.35	0.01	2.54	1.03	2.15	1.50	3.24	3.58	ns	ns	ns
EPA	1.36	0.10	0.37	0.02	0.29	0.21	2.87	0.47	2.41	0.74	5.16	5.24	ns	ns	ns
DHA	1.02	0.20	0.35	0.10	0.29	0.11	3.54	0.71	3.64	1.44	11.66	0.37	ns	ns	ns
Significance (p =)							**		**		**		ns	ns	ns

ns= non significant

Table 12 *Immunomodulatory protein gene expression that was modified by PUFAs/E. coli or S. aureus and not shown as a figure. Results were expressed as means of fold change compared to housekeeping gene GAPDH (+/- SD). Significant difference between PUFAs and control for each gene are shown on the right column of the table combining all bacterial exposures (excluding the bottom quadrant). Differences in protein gene expression between E. coli being present or not are shown in 4th column bottom row, while differences occurring after S. aureus exposure compared to no bacteria control (none) are shown in 5th column bottom row. The bottom right quadrant shows significance of an interaction between bacteria and PUFAs. Significant results are labelled *P <0.05.*

Fatty acid	**No bacteria (none)**				***E. coli LF82***				***S. aureus***				**Significance *E. coli/S. aureus***	
	HSP 72		**IL-8**		**HSP 72**		**IL-8**		**HSP 72**		**IL-8**		**HSP 72**	**IL-8**
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD		
Control	1.29	0.39	0.42	0.16	1.52	0.19	2.42	0.81	1.32	0.07	0.68	0.26		
AA	0.90	0.15	0.26	0.03	1.24	0.13	2.17	0.41	0.85	0.13	0.77	0.04	ns/ns	ns/ns
EPA	0.81	0.12	0.24	0.03	1.08	0.30	2.66	0.56	0.66	0.22	0.49	0.06	ns/*	ns/ns
DHA	1.44	0.47	0.14	0.13	1.01	0.24	1.38	0.33	1.30	0.29	0.65	0.29	ns/ns	ns/ns
Significance (p =0)					ns		*		ns		ns		ns/ns	ns/ns

ns= non significant

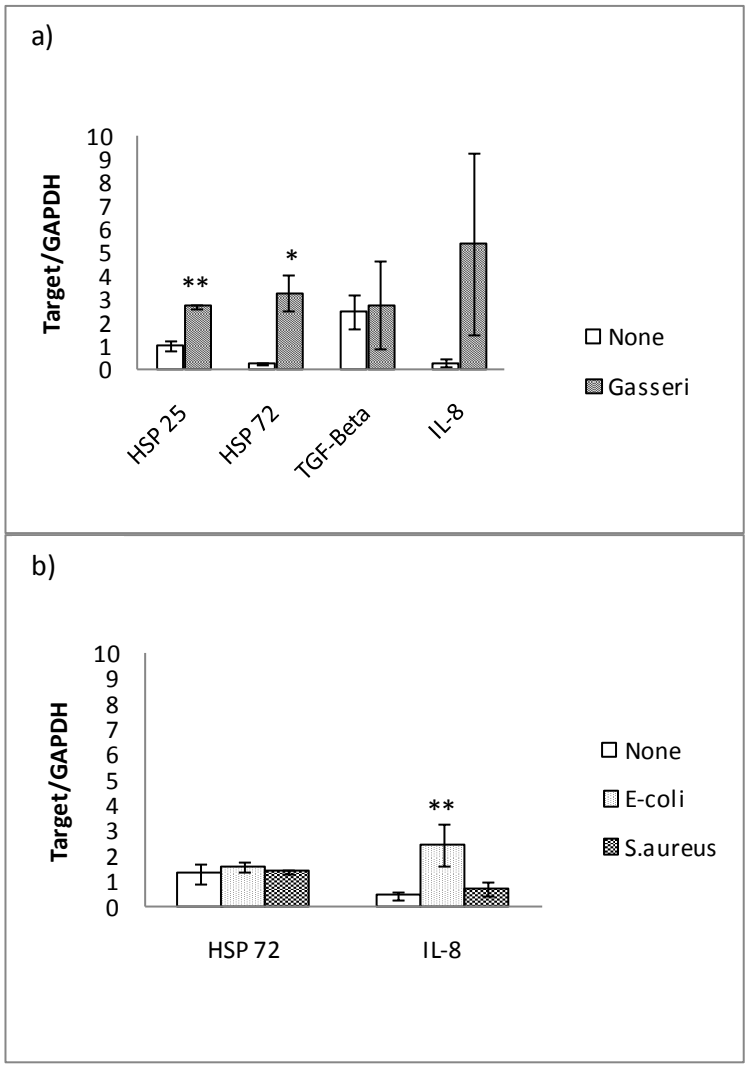

Figure 29 *Caco-2 cells exposed to; (a) L. gasseri or control with no bacteria (none);(b) E. coli, S. aureus or control with no bacteria (none). PUFA treated cells were omitted to observe bacteria effects only. Four immunological protein mRNA were found to be significantly altered by the presence of L.gasseri, whilst pathogenic E. coli and S. aureus were shown to alter HSP 72 and IL-8 in all cell lines. Each bar represents the mean of fold change compared to housekeeping gene GAPDH +/- SD (n=3) of each bacterial treatment split for mRNA of each target/reference gene. Significance is relative to control (none) *P <0.05 **P<0.01*

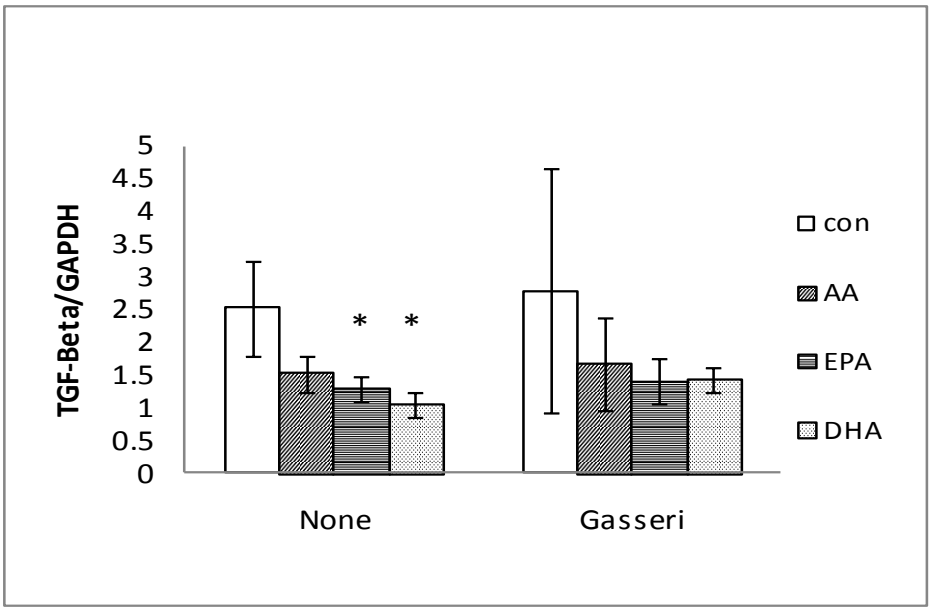

Figure 30 *Caco-2 cell production of TGF-β/reference gene compared between treatment groups of PUFAs; AA, EPA, DHA or Con (no PUFA). Data was split further into L.gasseri exposed and none bacteria (none) exposed groups. Each bar represents the mean of fold change compared to housekeeping gene GAPDH +/- SD (n=3). Significance relative to control *P <0.05 and different symbols represent significance between the two bars labelled †◊ P<0.05.*

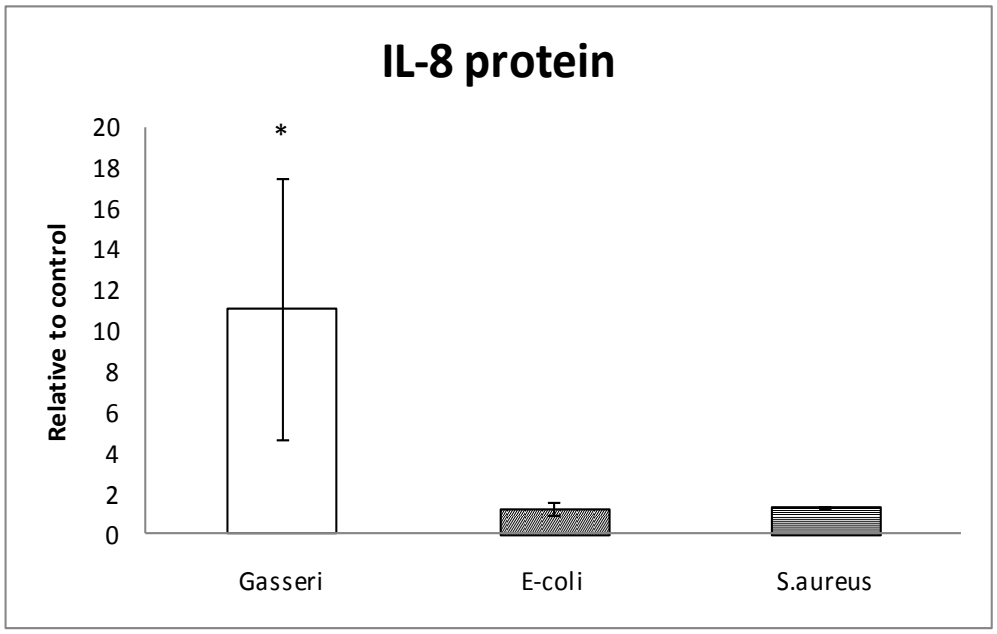

Figure 31 *Caco-2 cell production of IL-8 protein, shown at relative to control (no bacteria), following exposure of L. gasseri, E. coli* or *S. aureus. Each bar represents the mean of fold change compared to control treatment with no bacteria +/- SD (n=3). Significance between bacteria are shown as *P <0.05*

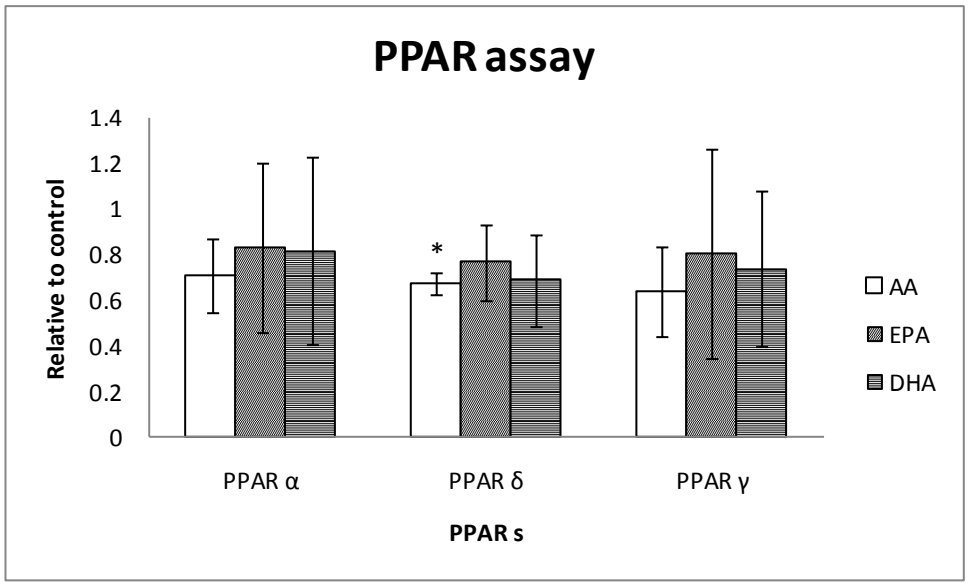

Figure 32 *Caco-2 cell expression of PPAR α, δ and γ following AA, EPA or DHA treatment. Each bar represents the mean of fold change relative to control with no PUFA treatment +/- SD (n-3). Significance is shown for differences between the control and each PUFA treatment within each cell line (* P < 0.05).*

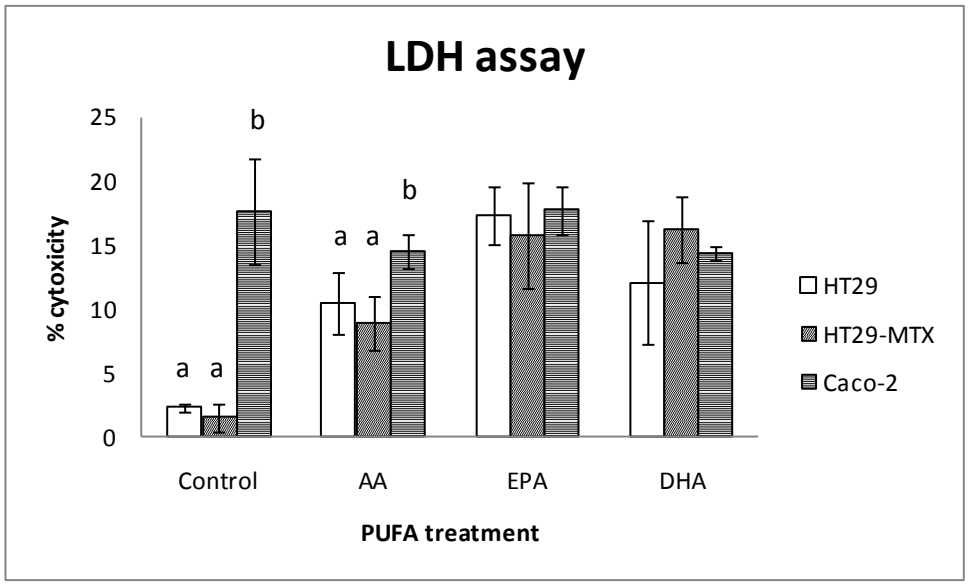

Figure 33 *Percentage cytotoxicity of HT29, HT29-MTX and Caco-2 cells, when treated with different PUFAs or ethanol (control). Each bar represents the mean +/- SD (n=6-8) of each condition and cell line. Values for cell lines are compared across treatment groups. Those that do not share a letter are significantly different (P<0.01). Caco-2 cell line results are significantly different to both HT29 and HT29-MTX results in that more LDH is present after addition of ethanol or/and PUFAs.*

CHAPTER 8: Final discussion

To summarise; I have studied the effects that PUFAs can have on lactobacilli, via direct contact and systemic delivery into the colonic epithelia. I have observed uptake of PUFAs into the phospholipid membrane and found that their influence is limited due to a tight control of the percentage range taken up of each PUFA, even at concentrations as high as 200 µM. I have studied adhesion of three strains of lactobacilli in the presence of PUFA and my data supported results by Bomba et al. [75] in piglets and Ringo et al. [76] in arctic charr; that is, my findings demonstrate that generally n-3 PUFAs increase adhesion to human colonic cell lines. I have explored PUFAs effects when immune cells are present to model the epithelial cells of the colonic tissue and their cross-talk with the lamina propria. I have been able to explore some of my own ideas into the ability of epithelial cells, with immune cells present, to differentiate between a pathogen and commensal bacteria. With each study completed more questions rather than answers have emerged and in this chapter I aim to summarise my findings and suggest further experiments to give a more detailed understanding.

Consistently throughout the studies there has been evidence of Caco-2 cells inability to tolerate PUFAs and ethanol that PUFAs are dispersed in. This work has supported findings from Dommels et al. [130] and poses issues for the use of this cell line in studies that may involve excess PUFA or ethanol, for example drug development analysis of uptake[196]. Results may not accurately model true *in vivo* intestinal epithelial cells, since Caco-2 sensitivity to PUFAs and probable cell membrane damage would allow a greater influx of drug into the cell.

Low adhesion number of bacteria to Caco-2 cells when compared to HT29 and HT29-MTX cells is most likely caused by cell damage from the PUFA and/or ethanol

pre-treatment of the cells. However a positive effect on adhesion was found for some lactobacilli compared to control treatments. There is no clear evidence from my LDH assay that DHA is less damaging to the cell compared with other PUFA types, thus even if some damage arises following PUFA treatment some beneficial effects on adhesion can still be observed.

With the Caco-2 cell line being a poor model for PUFA research, I have focused on comparing the HT29 and HT29-MTX cells results to predict outcomes from *in vivo* colonic epithelial cells. The HT29-MTX cell line is more differentiated than HT29 cells as would be seen in the *in vivo* adult colon. I believe the model that most accurately represents the *in vivo* results is using HT29-MTX cells with the Haller et al. [168] model with PBMC representing additional cross-talk between different cell types present *in vivo*. However, additional results from the HT29 cell line suggests this is a good model for the effects seen in newly formed crypt cells [103], particularly when looking at mechanisms involving apical TLRs. In view of this HT29 cell comparison I have interpreted results as equivalent to bacteria adhering to neonatal colonic cells.

When modelling the human colon most PUFAs will have been absorbed in the small intestine, thus are most likely to enter the colonic epithelial cells by systemic delivery. This has been modelled in the current study by pre-treating cell lines with PUFAs and allowing 48 h of uptake prior to addition of any bacteria, however while my confluent monolayers take PUFAs through their apical surfaces *in vivo* this would most likely be through the basolateral side of cells.

The highest adhesion of bacteria found in my experiments was approximately 1 % of the total added bacteria. In the human colon many lactobacilli pass straight through to faecal matter although some must adhere if only for a short time to produce

beneficial effects on human health as is widely documented. Thus adhesion of 5.2 x 10^6 per cm^2 would be predicted to have a beneficial effect. In addition the increased 0.1 % or 5.2 x 10^5 per cm^2 of *L. FI9785* adhesion following DHA pre-treatment of cells should result in additional beneficial effects of these bacteria. In conclusion, the adhesion studies suggest that systemically delivered DHA can improve the effectiveness of probiotic lactobacilli in humans. The mechanisms involved in DHAs ability to increase lactobacilli adhesion, has been previously suggested to be due to modification in membrane fluidity. Membrane fluidity controls the position of adhesive apical surface molecules in the lipid bilayer and thus exposure to bacteria may be increased [76]. The results support this theory by showing that DHA is actively taken up into the phospholipid layer of human cell lines. However, I now believe that there are additional influences on adhesion that may act in addition to modulation of cell membrane fluidity, for example modification the immune system. Previously well studied mechanisms of prostaglandin production from PUFAs, show that fish oils: DHA and EPA, produce less inflammatory product when compared to n-6 AA[197]. This may promote maintenance of lactobacilli, since fewer numbers of immune cells will be activated and therefore it is less likely for bacteria to be removed by immune cells from the epithelial surface. In addition, my studies suggest that the role of TGF-β up-regulation is important in lactobacilli survival at the colonic mucosal surface and it may form part of a critical mechanism that can distinguish commensal bacteria from pathogens. Literature review has allowed me to summarise pathways that involve inhibition of inflammatory processes by up-regulating TGF-β, in particular, focusing on the inhibition of IL-8 protein as found in my transwell co-culture study, with PBMC present and following lactobacilli exposure (figure 34).

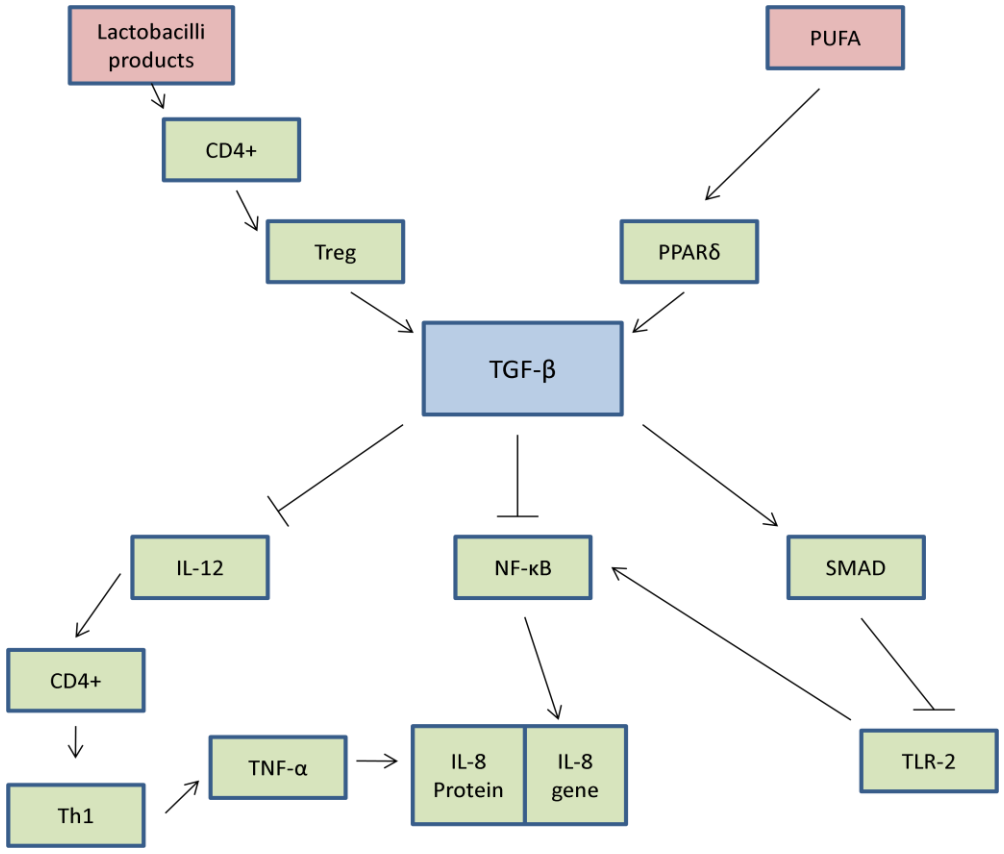

Figure 34 *The complex interactions between polyunsaturated fatty acids (PUFAs), lactobacilli products and the up-regulation of TGF-β. It also shows how downstream targets, associated with inflammatory pathways can be inhibited by TGF-β.*

During initial literature reviews I came across a study that suggested 2 % of PUFA can enter the colon directly, thus the idea of studying direct interaction of small amounts of PUFA on lactobacilli arose. Interestingly, my results suggest that human isolates that would regularly be exposed to PUFA in the diet survived, whilst a chicken isolate did not. It is unlikely that standard chicken feed would include many PUFAs, particularly fish oils, EPA and DHA, thus the potential chicken probiotic [145] could be introduced to a chicken feed without a problem. However, the current trend towards production of eggs high in n-3 fatty acids may add an additional problem for the survival of probiotic lactobacilli in chickens. Therefore, dependant on the foreseeable use of chicken probiotics, I would suggest that screening for survival in small concentrations of PUFA, with the possibility of mutant development

for lactobacilli that survive, in the view that beneficial probiotic mechanisms remain intact.

A time dependant aim of my studies was to produce a mutant form of the chicken *Lactobacillus johnsonii FI9785*, which can survive in the presence of small concentrations of PUFAs. During an experiment using bioscreen (chapter 5) one well including lactobacilli and DHA showed a small amount of growth toward the end of the 24 h incubation period. This demonstrated the possibility that mutant development was feasible and I could therefore develop a mutant by slowly increasing small concentrations of PUFA and select strains that survived. Following development I would have liked to complete microarray analysis in mutant versus wild type *L. FI9785* and look for pathways which may be involved in tolerance towards PUFAs. Unfortunately I was unable to pursue this line of investigation.

Additional ideas of mine were to knockout cell surface molecules in human cells and assess adhesion capabilities. However, with the high number of possible candidate surface molecules I believe the task to be outside the scope of a PhD, possibly resulting in few positive results. An idea that developed and would produce novel results would be to assess adhesion capabilities of a number of lactobacilli to normal colonic tissue obtained from surgical procedures. This tissue would represent more accurately the normal surface molecules in the colon and my have also presented the opportunity to collect some colonic mucus for additional adhesion experiments. This experiment was well in development stage with ethics preparations and surgeons at the Norfolk and Norwich Hospital had been contacted to be involved with tissue harvesting. However, during this time the opportunity to complete experiments at Plant and Food, New Zealand arose using the method by Haller et al. [168] that they had used previously worked with. Although I took the latter opportunity to work in a different country, I believe the colonic tissue experiments

would give a true incite of adhesion capabilities to normal colonic tissue and hopefully in the future I can get the opportunity to complete this study.

I have depicted an overview of mechanisms that I have considered to affect maintenance of lactobacilli at the colonic mucosa, in attempt to present my results in relation to the wider literature (figure 35). Direct contact with unabsorbed PUFA should not have an affect on human isolates of lactobacilli, since they possess a mechanism to survive PUFA's anionic detergent affects. Therefore systemic delivery of PUFAs to colonic cells has the predominate effect on survival of human lactobacilli present to the colonic mucosa. One mechanism may involve up regulation of TGF-β, involved in tolerance, possibly via PPAR signalling. My experiments predict that EPA has the largest impact via this mechanism when compared with AA and DHA. However, it is important to mention that PPAR signalling can be through PUFA's prostaglandin products, in particular PGJ2 [182], although I found no evidence of this in my studies. An additional mechanism, reviewed in the literature, is immune modulation directly by prostaglandin products of n-3 and n-6 PUFAs, the latter being most inflammatory [197]. As discussed previously higher inflammatory signalling may increase immune surveillance and subsequent removal of bacteria from the colonic mucosa. Thus EPA and DHA production of prostaglandins that result in fewer inflammatory signals may have a positive affect on survival of lactobacilli present on colonic cells [197].

AA (n-6) can induce inflammatory signalling via its prostaglandin products, however, as mentioned previously these products can bind PPARs, leading to inhibition of inflammatory responses [198], thus exhibiting a negative feedback mechanism. In my study I found that AA can increase adhesion to mucus secreting cells. As the literature suggests opposing effects of AA through prostaglandin and PPARs, I

suggest an alternative mechanism of action through alterations in mucus composition or production. This idea is highly speculative and would involve analysis of mucin type and number following AA pre-treatment to assess this mechanisms viability.

In addition to immunomodulatory mechanisms that may increase lactobacilli survival and adhesion, I have suggested that PUFAs function by modulating the exposure of colonic cell surface molecules to which lactobacilli adhere. In my study I found DHA to be highly incorporated into the phospholipid layer and that this PUFA increased adhesion of many lactobacilli strains tested to a range of cell lines. Indicating that this mechanism had the biggest impact on adhesion and therefore promoted survival at the colonic mucosa.

Interestingly, my studies have indicated that all PUFAs tested had a positive impact on adhesion of certain strains of lactobacilli to at least one cell line tested. Although it is likely that each predominately acts through a different mechanism.

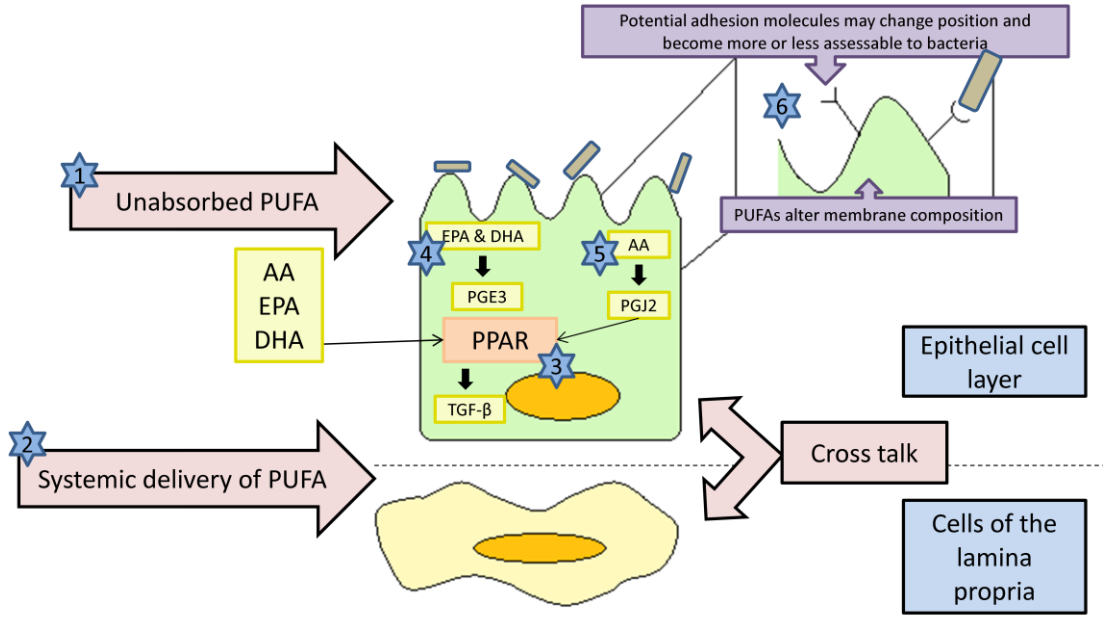

Figure 35 *Overview of PUFAs potential effects on bacteria adhesion. 1. PUFAs not absorbed in the small intestine can come into direct contact with bacteria in the colon. 2. Systemic delivery of PUFAs following absorption in the small intestine. 3. PUFAs and their prostaglandin products bind PPARs leading production of TGF-β and subsequent inhibition of the NF-κB pathway, resulting in less inflammatory responses and thus aid in bacteria maintenance at the colonic surface. 4. N-3 PUFAs; EPA and DHA, are precursors for series 3 prostaglandins that lead to lower inflammatory responses than the series 2 type. 5. n-6; AA, lead to series 2 prostaglandins, these can enhance inflammatory products, however these products can bind PPARs and result in inhibition of inflammatory products also. 6. unsaturated PUFAs lead to a more fluidic membrane composition, which can affect the position of proteins etc. on the cell surface, these can lead to potential adhesion molecules being more or less available for binding.*

The area of lactobacilli adhesion research is not fully understood and with the complexity of lactobacilli adhesion specificity even between strains there is a lot of future work to fully elucidate mechanisms involved in binding to human cell membranes or mucus. I hope that my work has highlighted some additional ideas of mechanisms involved in binding of lactobacilli and has highlighted a potential concern of probiotic use in chickens. I also believe my ideas for additional studies into this area can one day be completed to advance knowledge.

CHAPTER 9: CONCLUSION

- **DHA up-regulates lactobacilli adhesion to human epithelial cells and part of the mechanism may involve modification of host cell surface receptors.**

- **AA up regulates adhesion of some lactobacilli to mucus secreting HT29-MTX cells, possibility involving modification of mucin production or composition to favour binding.**

- **Lactobacilli can up-regulate TGF-β that maybe involved in survival by down-regulation of inflammatory signalling, thus avoiding recognition and removal from the host cell surface.**

- **EPA can enhance the production of TGF-β and therefore may aid in lactobacilli survival.**

- **Results suggest that AA, EPA and DHA act through different mechanisms to support the beneficial effects of lactobacilli in the colon.**

- **Lactobacilli isolated from chickens may not survive in small concentrations of PUFA. This may be due to lack of adaptation to PUFAs through a lack of exposure in chicken gut, since a standard feed is low in fish oils. Could this pose a problem when lactobacilli probiotics are added to feed, high in fish oils, as currently a trend?**

List of Abbreviations

PUFA	Polyunsaturated fatty acid
AA	Arachidonic acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
PKC	Protein kinase C
LCFA	Long-chain fatty acid
FABP	Fatty acid binding protein
FAT	Fatty acid translocase
FATP	Fatty acid transport protein
FACS	Fatty acyl CoA synthease
PPAR	Peroxisome-proliferator activated receptors
PPRE	Peroxisome proliferator response element
CLA	Conjugated linoleic acid
TLR	Toll-like receptor
PAMPs	Pathogen-associated molecular patterns
LPS	Lipopolysaccharide
IRAK	Interleukin 1 receptor activated kinase
IECs	Intestinal epithelial cells
APC	Antigen presenting cell
PGN	Peptidoglycan
DAP	Diaminopilemicacid
MDP	Muramyl dipeptide
DC	Dendritic cell

LCPUFA	Long chain polyunsaturated fatty acid
FCS	Fetal calf serum
L. FI9785	*Lactobacillus johnsonii FI9785*
L. gasseri	Lactobacillus gasseri ATCC 33323
L. R1	*Lactobacillus casei sp.R1*
L. 44	*Lactobacillus casei sp.44*
L. 2	*Lactobacillus casei sp.L2*
L. 10	*Lactobacillus casei sp.L10*
MRS	De mann, Rogosa and Sharpe media
PBS	Phosphate buffer saline
OA	Oleic acid
FAMES	Fatty acid methyl esters
GC	Gas Chromatography
CFU	Colony forming units
PBMC	Peripheral blood mononuclear cells
E. coli	*Escherichia coli LF82*
S. aureus	*Staphylococcus aureus*
TSB	Trypticase soy broth
BHI	Brain heart infusion broth
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ACTB	β actin
HSP 25	Heat shock protein 25
HSP 72	Heat shock protein 72
TGF-β	Transforming growth factor β
IL-8	Interleukin 8

TNF-α	Tumour necrosis factor α
IL-10	Interleukin 10
IL-2	Interleukin 2
NOD-2	Nucleotide oligomerization domain 2
TLR-4	Toll-like receptor 4
IFN-γ	Interferon γ
GATA-3	GATA binding protein 3
ELISA	Enzyme-linked immunosorbant assay
LDH	Lactate dehydrogenase
TEER	Transepithelial electrical resistance
OD	Optical density
SEM	Scanning electron microscopy
iFABP	Intestinal fatty acid binding protein
TFFs	Trefoil factor family
GLM	General liner model
IBD	Inflammatory bowel disease

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