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The Effect of Multi-Strain Probiotics on Liver Disease

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Abstract

The microbiome in the human gastrointestinal tract (GIT) is the largest body community of bacteria. In conjunction within an appropriate internal milieu, the microbiome induces the development of regulated pro– and anti–inflammatory signals that promote immunological tolerance. In addition, microbial interactions provide cues for upholding metabolic regulations and controlling and regulating GIT inflammation. Failure to regulate inflammatory responses can increase the risk of developing inflammatory conditions such as Inflammatory Bowel Diseases (IBD) or Irritable Bowel Syndrome (IBS). Disruption to the microbiome homeostasis can also affect other end–organs (e.g., liver, kidneys). For example, the liver receives 70% of its blood supply from the GIT, making regulation of the gut-liver-axis vital. Inflammation of the GIT may lead to inflammatory conditions of the liver and the development of diseases such as non-alcoholic fatty liver disease (NAFLD).

The first study in this thesis examined the effects of a multi–strain probiotics supplement that was administered to mice prone to tumour development ($atm^{-/-}$) and housed in a 'dirty' environment. This study proved unsuccessful due to uncontrollable changes in animal housing conditions. The changes limited the exposure of mice to pathogens that have previously been reported to accelerate tumour development. The second study investigated the effects of a multi–strain probiotics supplement that was administered to mice fed a high fat diet (HFD). The results suggested that the multi–strain probiotic investigated may assist with reducing HFD induced steatosis and lipid disposal by reducing the accumulation of fat deposits in the liver and preventing reductions in tight junction proteins ZO-1 and ZO-2. The third study investigated the effectiveness of a multi–strain probiotics supplement in mice on a HFD that were prone to iron overload (hemochromatosis). This study showed that a multi-strain probiotic supplement had reduced efficacy in the presence of high iron concentrations. Probiotics fed to $hfe^{-/-}$ mice partly rescued genes involved in lipid metabolism (Cpt1, Lfabp, AdipoR2), hepatic iron concentration, proteins involved in iron uptake (Tfr2), serum ALT, AST and triglycerides. However, probiotics did not alter serum cholesterol, hepatic lipid peroxidation, triglycerides, genes involved in lipid metabolism (PPAR-α, PPAR-γ, LDLr, CD36) and proteins involved in iron uptake (Tfr1). The fourth study investigated the effectiveness of curcumin, vitamin E or a combination of the two in both wild type and mice prone to iron overload (hemochromatosis). The combination of curcumin (CU) and vitamin E (VE) proved to be the most effective for reducing the effects of a HFD. Curcumin plus vitamin E (CUVE) reduced total body and liver weight and reduced the severity of steatosis and liver injury. Treatment groups CU or VE alone showed reduced fat deposits; however, the combination treatment, CUVE, resulted in a

greater reduction of both macro- and micro-vesicular fat deposits and the degree of change was similar to the chow group. However, nothing compared with simply returning the animals to a healthy, balanced diet. Removal of the HFD resulted in a reduction of body and liver weight and a return to normal liver pathology in 80-90% of mice.

These findings propose that neither probiotics, curcumin, vitamin E or a combination treatment is a panacea for over consumption of calories through a high saturated fat diet in the hope of down–regulating GIT inflammation and or liver fatty acid metabolism. However, they may provide a therapeutic measure whereby there is a significant reduction in risk for NAFLD progression.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications During Candidature

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Phillips LK, Peake JM, Zhang X, Hickman IJ, **Briskey DR**, et al. (2013) Postprandial total and HMW adiponectin following a high-fat meal in lean, obese and diabetic men. European journal of clinical nutrition 67: 377-384.

Mullins AL, van Rosendal SP, **Briskey DR**, Fassett RG, Wilson GR, et al. (2013) Variability in oxidative stress biomarkers following a maximal exercise test. Biomarkers 18: 446-454.

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Shing CM, Peake JM, Lim CL, **Briskey D**, Walsh NP, et al. (2014) Effects of probiotics supplementation on gastrointestinal permeability, inflammation and exercise performance in the heat. European Journal of Applied Physiology 114: 93-103.

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Conference abstracts

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Keywords

Inflammation, fatty-liver disease, lipid peroxidation, cell signalling, high fat diet, high iron, probiotics

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Table of Contents

Abbreviations

AA – arachidonic acid

AdipoR2 – adiponectin receptor 2

ALT – alanine transaminase

AST – aspartate transaminase

Atm – ataxia telangiectasia

B. – bifidobacterium

B-IBS – irritable bowel syndrome with bloating

BTF-3 – basic transcription factor 3

CCL – cytokine and chemokine (C-C motif) ligand

CD – Crohn's disease

CFTR – cystic fibrosis transmembrane conductance regulator

cfu – colony forming units

CH – chow

Col1a1 – Collagen 1A1

COX – cyclooxygenase

Cpt1A – carnitine palmitoyl transferase 1A

CRP – C-reactive protein

CU – curcumin

CUVE – curcumin + vitamin E

DC – dendritic cells

D-IBS – diarrhea-predominant irritable bowel syndrome

DPI – diphenyliodonium

DSS – dextran sodium sulphate

E. – Escherichia

EcN – Escherichia coli Nissle 1917

EIEC – enteroinvasive *Escherichia coli*

ELISA – enzyme-linked immuno sorbent assay

EPEC – enteropathogenic *Escherichia coli*

Gapdh – Glyceraldehyde-3-phosphate dehydrogenase

GI – gastrointestinal

GIT – gastrointestinal tract

GPx – glutathione peroxidase

GSH – reduced glutathione

GSSG – oxidized glutathione

H&E – haematoxylin and eosin

Hamp – hepcidin anti-microbial peptide

HFD – high fat diet

Hfe – hemochromatosis

HH – hereditary hemochromatosis

HIC – hepatic iron concentration

HSC – hepatic stellate cells

HSP – heat-shock protein

IBD – inflammatory bowel disease

IBS – irritable bowel syndrome

IL – interleukin

INF – interferon

iNOS – inducible nitric oxide synthase

KO – knock out

L. – lactobacillus

LFABP – liver fatty acid binding protein

LPMC – Lamina propria mononuclear

LPS – lipopolysaccharide

LTB_4 – Leukotriene B4

MCP-1 – monocyte chemotactic protein-1

MDA – Malondialdehyde

MIP-2 – Macrophage inflammatory protein 2

MLN – mesenteric lymph node

MnSOD – manganese superoxide dismutase

MPO – myeloperoxidase

mRNA – messenger ribonucleic acid

MyD88 – Myeloid differentiation primary response gene (88)

NAC – N-acetylcysteine

NAFLD – non-alcoholic fatty liver disease

NAS – NAFLD activity score

NASH – non-alcoholic steatohepatitis

NF-κB – nuclear factor kappa B

PBMC – peripheral blood mononuclear cells

PGE_2 – prostaglandin E2

PKC – protein kinase C

PPAR – Peroxisome proliferator-activated receptor

PUFA – polyunsaturated fatty acids

QC – quality control

ROS – reactive oxygen species

RT-PCR – reverse transcriptase-polymerase chain reaction

S. – streptococcus

SEM – standard error of the mean

siRNA – small interfering ribonucleic acid

SOCS – suppressor of cytokine signalling

TER – transepithelial resistance

TGF – transforming growth factor

TH – T-helper

Timp – tissue inhibitor of metalloproteinase

TJP – tight junction protein

TLR – toll-like receptor

TNBS – trinitrobenzene sulfonic acid

TNF – tumour necrosis factor

Trx1 – thioredoxin-1

UC – ulcerative colitis

VAT – Visceral adipose tissue

VE – vitamin E.

WT – wild type

β2-m – β2-microglobulin

Chapter 1

Literature review

Probiotics as a prophylactic and therapeutic intervention for intestinal inflammation

1.1. ABSTRACT

Probiotic bacterial species can modify the milieu of the gastrointestinal tract, by reducing the triggers that can induce inflammatory episodes in conditions such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). The microbiota acts in an immuno-surveillance capacity, detecting pathogenic bacteria with resulting stimulation of local innate immune inflammatory responses that clear the pathogenic cells. I have conducted a systematic literature review to evaluate the efficacy of probiotics treatment in modulating the inflammatory responses and cellular signalling pathways that regulate the pathophysiology of intestinal inflammation specifically in IBD and IBS. Evidence exists that probiotics can enhance intestinal permeability, control the synthesis of pro-inflammatory and anti-inflammatory mediators. Almost all probiotic strains tested induced a beneficial effect on inflammation. The weight of the evidence supports that treatment with probiotics benefit patients with both IBD and IBS, in part by reducing the synthesis of pro-inflammatory cytokines (e.g., IL-17, IFN-γ, TNF-α and IL-12) and increasing the synthesis of anti-inflammatory cytokines (e.g., IL-27 and IL-10). This effect, I postulate is a rescuing of pathological inflammation toward normal, which the gastrointestinal tract (GIT) is subjected to throughout life. The magnitude of the effect, and the most effective species and strains remain equivocal. Probiotics are effective as either a prophylactic or therapeutic intervention, but they appear more effective when used prophylactically, and with a mixture of strains. Further research is required to investigate and understand the metabolic and signalling mechanisms of probiotics in greater detail, particularly in the IBD model. Such effort will help to develop better efficacy of treatment with probiotics, and to establish the optimal dose and duration of treatment.

1.2. INTRODUCTION

The human GIT contains more than 1,000 different commensal bacterial species.[1-4] Commensal bacteria perform a number of functions such as (i) regulating the normal development and function of the mucosal barrier function[5]; (ii) assisting the maturation of immunological tissues, which in turn promotes immunological tolerance to antigens from foods or pathogenic organisms[6]; (iii) controlling nutrient uptake and metabolism,[7, 8] and (iv) preventing the propagation of pathogenic micro-organisms.[9] Changes in the number of commensal bacteria may reduce their function, and alter the GI immune response.

Immune responses are necessary to reduce the GIT concentration of pathogenic cells. The immune system does this by initiating a pro-inflammatory response. The microbiota acts in part in an immuno-surveillance role detecting pathogenic bacteria, stimulating the immune system, and subsequently initiating an appropriate regulated inflammatory response. Once the overload of pathogenic cells have been cleared, anti-inflammatory signals are switched on to restore the pro-inflammatory response back to normal. Accordingly, the gut is in a constant state of 'regulated inflammation'. The role that microbiota play in triggering the anti-inflammatory response is still unclear. Failure to 're-regulate' inflammatory responses increases the risk of developing inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS).

IBD comprises a group of inflammatory conditions of the colon and small intestine. The etiology of IBD is not fully understood, but it is considered to be a T-cell-driven inflammation resulting from a persistent preponderance of pro- over anti-inflammatory cytokine production.[10] Crohn's disease and ulcerative colitis are the two main types of IBD.[11] Crohn's disease and ulcerative colitis differ in the nature and location of the affected part of the GIT. Crohn's disease is driven by T-helper 1 (TH1) immune responses,[12, 13] and can affect any part of the GIT, from the mouth to the anus. In contrast, ulcerative colitis is T-helper 2 (TH2) driven, and is restricted to the mucosa of the colon and rectum.[12, 14] Alternatively, IBS is a functional bowel disorder affecting mostly the large intestine. While the cause remains unknown, symptoms including abdominal pain, diarrhea and constipation. It has however been presented that disruption to the gut microbiome may result in altered bowel function.[15]

Accumulating evidence indicates that the balance of commensal bacteria within the GIT may be associated with the development of some GI disorders[16]. Patients with IBD or IBS present with increased numbers of the pro-inflammatory bacteroids,[16] Escherichia coli,[17, 18] enterococci and decreased *Bifidobacterium* and *Lactobacillus* species.[19, 20] IBD is one of the more prevalent GI

disorders, with a prevalence of 206 cases per 100,000 in Switzerland,[7, 21, 22] 61,000 affected in Australia [23] (about 290 per 100,000), and 388 per 100,000 in the USA.[24]

The past 50 years has witnessed an increase in the prevalence of autoimmune diseases.[25] The hygiene hypothesis provides a biologically plausible explanation for this trend that implicates a loss or diminished early childhood infectivity with an enhanced risk for later life GIT inflammatory problems such as autoimmune diseases.[26] With the increasing prevalence of GI diseases, and bacteria resistant to antibiotic medications,[27] multi-strain probiotics may significantly reduce the risk of developing GI diseases by promoting restoration of GIT commensal bacteria balance Probiotics are live microorganisms that when administered in adequate amounts confer health benefits to the host.[28] The mechanisms through which probiotics reduce inflammation are complex and diverse, and depend on a number of factors that include strain specificity, dosage, the presence and concentration of other bacteria, and the specific disease state of the individual. One mechanism through which probiotics act is by regulating cell signalling pathways that mediate the production of pro- and anti-inflammatory cytokines.

Probiotic research is still in its infancy; interpreting the results of currently available publications can be equivocal. Some of the problems associated with probiotic research include a great deal of variability regarding: (a) the specific strains or combination of strains used; (b) the dosages administered; (c) the models used (cell, animal or human); (d) the number, age and sex of participants; (e) the timing of when the dose is administered, and the study design (e.g., blinded vs. non-blinded).

The aim of this review is to review the literature investigating the effects of probiotics on inflammatory and cellular signalling pathways, specifically in the gastrointestinal tract of IBD and IBS models. IBD and IBS are two distinct disorders with clinical and biological variations; however, the main focus is on the common underlying mechanisms of each disease, as opposed to the disease symptoms.

In the first section I discuss the in vivo response to probiotics with no inflammation present. The second section discusses in vitro and in vivo response when probiotics are administered simultaneously as inflammation occurs. The third section discusses the prophylactic effects of probiotics, and the fourth section details the therapeutic effects of probiotics. Each section is further divided into sub-sections covering probiotics applied to cells, animals and humans.

1.3. PROBIOTICS ADMINISTERED IN THE ABSENCE OF INFLAMMATION

To understand the mechanisms by which probiotics function, it is necessary to evaluate the effects of probiotics in the absence of inflammation. The following section discusses the response to probiotics, including the cytokine and signalling response and GI tissue morphology in cell culture models or animals without inflammation. The results of studies using no inflammatory stimulation are summarised in Table 1.

1.3.1. Probiotics in cell cultures

Cell culture studies incorporate a diverse range of experimental conditions. The cell lines presented in this section are: Caco-2, HeLa, T84 (human) and IEC-6 (rat) cells. These cell lines were used to investigate the effects of probiotics on the Nedd8 pathway.

Kumar and colleagues[29] investigated the effects of *L. rhamnosus*, *Bacteroides thetaiotaomicron*, and *E. coli* stimulation on Caco-2, HeLa, T84 (human) and IEC-6 (rat) cells. Bacterial stimulation oxidized both thioredoxin-1 (Trx1) and glutathione (GSH) (two major antioxidant systems), with *L. rhamnosus* inducing the greatest change. Further supporting a bacterial induced increase in reactive oxygen species (ROS), *L. rhamnosus* rapidly increased luminol chemiluminescence in these cells. An increase in ROS causes oxidative inactivation of the Nedd8-conjugating enzyme (catalytic cysteine residue of Ubc12), leading to a loss of Cul-1 neddylation. Neddylation is the process that conjugates Nedd8 to the conserved lysines of cullins (Cul-1 and Cul-3).[30] Co-culture of the epithelial cells with *L. rhamnosus* reduced Cul-1 neddylation within 30 minutes compared with control cells. Reduced Cul-1 neddylation blocks the NF-κB pathway,[31] and is therefore an important process that regulates inflammation. Pre-treating epithelial cells with the antioxidant N-acetylcysteine (NAC) or diphenyliodonium (DPI) maintained Cul-1 neddylation when the cells were colonised with commensal bacteria (Figure 1.1).

These results indicate that if the inflammatory response is dysregulated, as is the case in IBD and IBS, bacterial stimulation may help to re-regulate the inflammation by reducing Cul-1 neddylation and blocking the NF-κB pathway. ROS appear to inactivate the Nedd8-conjugating enzyme, thereby preventing Cul-1 neddylation and subsequent activation of NF-κB. Unregulated production of ROS may also create a state of oxidative stress, however. Oxidative stress can increase inflammation by activating various inflammatory pathways, including the NF-κB pathway. [32]

1.3.2. Probiotics in healthy animals

Animal models provide much of the current understanding of how probiotics influence intestinal inflammation. The animal models presented below include: BALB/c, germ free wild type (WT) and germ free IL-10 knockout (KO) mice. These models were used to test the effects of probiotics on the Nedd8 pathway, cytokine producing cells and the histology of the intestinal tract.
Supporting their *in vitro* findings, Kumar and colleagues[29] investigated the effects of *L. rhamnosus, Bacteroides thetaiotaomicron,* and *E. coli* stimulation in female BALB/c mice. *L. rhamnosus,* and to a lesser extent *B. thetaiotaomicron,* increased ROS in the mucosa of the small bowel 30 minutes after oral or rectal inoculation of bacteria. *L. rhamnosus* administered into the ileal loops of mice attenuated Cul-1 neddylation in mucosal lysates within 30 minutes.

Demonstrating the disparity in the effects between differing strains of bacteria, Maassen and colleagues[33] administered eight individual strains of *Lactobacillus* to female BALB/c mice resulting in a number of immunomodulatory responses. Immunohistochemical analysis of cytokine-producing cells in the gut villi showed that all eight probiotics did not significantly alter the concentration of IL-1α, IL-4, IL-10 and IFN-γ producing cells. In contrast, with the exception of *L. fermentum,* all probiotic strains increased the concentration of TNF-α producing cells. *L. reuteri* and *L. brevis* increased the concentration of IL-2 cytokine-producing cells, and *L. reuteri* alone increased the concentration of IL-1β cytokine-producing cells. These strains therefore appeared to promote a TH1 cytokine response, which favours cell-mediated immune reactions.

Investigating the effects of administering probiotics in an environment lacking in natural microbiota, Moran and colleagues[34] applied *B. animalis, B. infantis* or *B. bifidum* to germ free WT and germ free IL-10 KO mice. Compared with WT mice, probiotics caused considerable thickening of the duodena, with massive cellular infiltration of mononuclear cells into the lamina propria (including the villi) and significant crypt hyperplasia in IL-10 KO mice. The mucosa was altered by the formation of abnormal crypt and villus structures consisting of branched and fused villi. Mucosal ulceration was localised to the duodenum. Colonic explants from IL-10 KO mice also spontaneously released higher amounts of IL-12/IL-23 p40.

These findings highlight the importance of IL-10 in preventing and regulating inflammatory responses to bacteria as well as demonstrating that in a susceptible host, some probiotic bacteria strains may be potentially pathogenic. Taken together, these results indicate that depending on the model, probiotic administration have very diverse effects, even when strains from the same genus are administered.

1.4. PROBIOTICS APPLIED SIMULTANEOUSLY WITH INFLAMMATORY STIMULANTS

The following section discusses probiotics applied simultaneously with inflammatory stimuli. The probiotics assessed in this section include: *L. casei* , Escherichia coli Nissle 1917, *B. breve, B. adolescentis, B. longum, L. paracasei, L. plantarum, L. rhamnosus GG, S. Boulardii* and a combination of *S. thermophilus, L. acidophilus* and *B. lactis*. The experimental models used include T84, Caco-2, RAW264.7 and $CD4^+$ T cells, male Sprague-Dawley and Wistar rats and IL-10 KO mice. The inflammatory stimulants used in these studies included trinitrobenzene sulfonic acid (TNBS), TNF-α, IL-6, lipopolysaccharide (LPS) and dextran sodium sulphate (DSS). The efficacy of probiotics was assessed by measuring in vitro and in vivo cytokine production (IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α, INF-γ and SOCS proteins), intestinal permeability, junctional complex properties (ZO-1, PKC-ζ) and histology scoring of the intestinal tract for inflammation. The results are summarised in Table 2.

1.4.1. Probiotics in cell cultures

A significant problem for treating IBD and IBS is that the precise aetiology of these diseases remains unclear. A number of mechanisms are proposed to contribute to the development and progression of the disease. One such mechanism is degradation of the intestinal epithelial barrier leading to an increase in intestinal permeability. Enteropathogenic *Escherichia coli* (EPEC) has been shown to reduce transepithelial resistance by down-regulating and redistributing the tight junction proteins ZO-1[35] and ZO-2[36] away from the cell membrane and translocating the protein kinase C - ζ (PKC-ζ) away from the cytosol.[36] Regardless of the timing of application, in T84 cells infected with EPEC and treated with 1×10^7 and 1×10^8 cfu/mL (colony forming units) of *L. casei*, ZO-1 staining was well-defined at the cell membrane and transepithelial resistance values were similar to non-stimulated cells. In contrast, in T84 cells stimulated with EPEC, and a lower dose (1×10^6 cfu/mL) of *L. casei*, ZO-1 staining and transepithelial resistance was similar to cells stimulated with EPEC alone. Incubation with 1×10^6 or 1×10^7 cfu/mL of *L. casei* did not inhibit EPEC adhesion.[35] Addition of the probiotic *Escherichia coli* Nissle 1917 (EcN) with EPEC blocked PKC-ζ from translocating to the membrane, and maintained transepithelial resistance.[36]

The work of Parassol et al.[35] and Zyrek et al.[36] support the notion that increased intestinal permeability resulting from the translocation of PKC-ζ, ZO-1 and ZO-2 may initiate or promote dysregulated inflammation. Translocation of the proteins away from the cell membrane or cytosol reduces the cell membrane integrity, thereby allowing pathogens to move freely through the epithelial barrier causing dysregulated inflammation. Maintaining PKC-ζ in the cytosol protects

tight junctions, and maintains barrier function by preventing the removal of tight junction protein complexes.[37, 38] Treatment of T84 and Caco-2 cells with probiotics helped to restore or maintain PKC-ζ within the cytosol and ZO-1 and ZO-2 to the tight junction complexes, thereby restoring the epithelial barrier function in EPEC-stimulated cells. Regardless of the timing, incubation with EcN or *L. casei* following or during EPEC infection restored the integrity of the epithelial cell barrier and redistributed ZO-2 to the cell boundary (figure 1.2).[35, 36]

Okada and colleagues[39] stimulated RAW264.7 cells with LPS, *Enterococcus faecalis* or LPS + *E. faecalis*. Stimulation significantly increased IL-1β, IL-12p40 and TNF-α synthesis, and reduced IL-10 production. Simultaneous addition of *B. breve* or *B. adolescentis* blocked the production of IL-1β in response to LPS, but increased TNF-α production. *B. breve* and *B. longum* attenuated the production of IL-12p40. When cells were co-cultured with *B. breve* plus LPS, IL-10 production increased significantly above the untreated cells, or cells treated with LPS, or LPS + *E. faecalis*. All *Bifidobacterium* strains suppressed IκB-α phosphorylation in response to LPS, whereas *B. breve* and *B. longum* suppressed IκB-α phosphorylation induced by LPS, *E. faecalis* or LPS + *E. faecalis*. Exposure to LPS alone increased mRNA levels of SOCS1 and SOCS3. Exposure to *Bifidobacterium* species further increased the expression of SOCS1 and SOCS3. *B. longum* has also been shown to reduce tissue supernatant production of TNF-α and IL-8 and NF-κB p65 expression in Lamina propria mononuclear (LPMC) cells derived from mucosal biopsies collected from patients diagnosed with ulcerative colitis[40].

Demonstrating that the effects of probiotics on cytokine production may rely on enhancement of additional signalling molecules, Reilly and colleagues[41] investigated the effects of *L. paracasei subsp. paracasei F19* or *L. plantarum* 2362 on Caco-2 cells treated with IL-1β or TNF-α. Treatment with IL-1β increased spontaneous IL-6 and IL-8 production. Co-culture of live or heat-inactivated *L. paracasei* or *L. plantarum* with IL-1β substantially increased IL-6 production above IL-1β treatment alone, but did not alter IL-8 synthesis. Further analysis showed that treatment with *L. paracasei* increased cellular levels of heat-shock protein 70 (HSP70) and HSP27. IL-1β treatment either alone or in combination with *L. paracasei* did not alter HSP expression. Silencing of the HSP genes reduced or blocked the *L. paracasei*-stimulated production of IL-6.

Isolating intestinal $CD4^+$ T cells from healthy individuals and patients with Crohn's disease, Hvas and colleagues[10] showed an imbalance in the production of pro- and anti-inflammatory cytokines. Following co-culture with autologous dendritic cells, intestinal T cells from patients with Crohn's disease produced high levels of IFN-γ and low levels of IL-10. Conversely, T cells from healthy

individuals produced less IFN-γ but more IL-10. Subsequent incubation of T cells with *L. rhamnosus GG* alone or *L. rhamnosus GG* combined with *L. acidophilus* only reduced IFN-γ production in cells from the healthy individuals. These divergent cytokine responses indicate an imbalance in the pro- and anti-inflammatory cytokine response in patients with Crohn's disease. A higher dose or different combination of probiotics may be required to normalise the cytokine response in cells from patients with Crohn's disease.

These results indicate that applying probiotics in adequate doses appears to prevent the detrimental effects of pathogens. Probiotics restore the integrity of the epithelial membrane in several ways by: (i) helping to retain restore ZO-1 and ZO-2 at the cell membrane, (ii) preventing PKC-ζ translocation to the membrane, (iii) increasing anti-inflammatory and reducing pro-inflammatory cytokines, (iv) reducing IκB-α phosphorylation induced by pathogens, and (v) increasing HSP (Figures 1.1 and 1.2).

1.4.2. Probiotics in animal models of intestinal inflammation

Simultaneously initiating inflammation and administering probiotics, Lee and colleagues[42] investigated the effects of *S. Boulardii* in male Sprague-Dawley rats. Compared with rats receiving TNBS alone, *S. Boulardii* treatment restored the histological appearance and the mass/length of the colon. *S. Boulardii* administered simultaneously with TNBS suppressed or abolished the down-regulation of PPAR-γ mRNA, and up-regulation of IL-8, IL-1β, IL-6, IL-8R, TNF-α and iNOS mRNA in the colon tissue that occurred in response to TNBS. Similarly, Schultz and colleagues[43] found that compared with untreated IL-10 KO mice, simultaneous or pre-treatment with *L. plantarum* 299v reduced diarrhoea, improved total colonic, rectal and cecal histology scores, and attenuated spontaneous colonic mucosal production of IL-12 and IFN-γ by anti-CD3 stimulated mesenteric lymph node (MLN) cells.

Demonstrating the importance of bacteria colonisation, Amit-Romach and colleagues[44] investigated *L. GG* and a combination of *S. thermophilus, L. acidophilus* and *B. lactis* (YO-MIX™ Y 109 FRO 1000) in male Wistar rats. Experimental colitis (using TNBS) resulted in macroscopic damage, with diarrhoea, shortening and thickening of the colon and severe disruption of the normal architecture of the colon, extensive ulceration and inflammation of all of its layers. YO-MIX™ only minimally improved the pathophysiology of colonic tissue, whereas *L. GG* significantly improved colonic tissue architecture. The degree of colonic inflammation was shown to be related to the colonisation of certain bacteria. Following TNBS induction the faecal concentration of *Bifidobacteria* and *Lactobacillus* bacteria did not significantly change relative to the controls. The appearance of *E.*

coli strains and *Aeromonas* was increased however. *L. GG* treatment slightly increased the amount of *Lactobacillus* bacteria but tended to reduce the propagation of *E. coli* and *Aeromonas*. Whereas YO-MIX™ raised *Bifidobacteria* bacteria compared with all other groups but had variability in the colonization of *E. coli* and *Aeromonas*. These findings illustrate the relationship between changes in microbiota and pathogenic bacteria populations and inflammation of the intestinal tract, and the importance of restoring the microbiota populations.

Applying probiotics with an inflammatory agent showed that probiotics may prevent the detrimental effects of TNBS and DSS in mice and rats. This is achieved by improving both the appearance and histology score of the colon, preventing weight loss and the severity/onset of diarrhoea, increasing the expression of tight junction proteins such as ZO-1, reducing the production of pro-inflammatory cytokines IL-12 and INF-γ, and changing the bacterial composition of the faecal matter.

1.5. PROBIOTICS APPLIED PROPHYLACTICALLY

The issue of whether probiotics work better as a prophylactic or therapeutic intervention in patients with IBD is important to consider. Prophylactic treatment with probiotics is important for individuals with a genetic predisposition for IBD, or individuals in remission from IBS or IBD. Probiotics can act in a prophylactic fashion by colonising the GIT and counteracting inflammation caused by invading pathogens by regulating pro- and anti-inflammatory cytokine production, and improving the integrity of the intestinal barrier. The results of prophylactic studies are presented in Table 3.

1.5.1. Prophylactic effects of probiotics in cell cultures

Cell lines presented in the following section include HT-29, Caco-2, T84, colo329, SW480, RAW264.7 and young adult mouse colon cells. These cells were stimulated using gastrin or pro-inflammatory cytokines INF-γ, TNF-α or IL-1β. The efficacy of probiotics was established by measuring changes in IL-8 synthesis, cytoskeletal and tight junction proteins, transepithelial resistance, PPAR-γ, COX-2 promoter activity and protein, PGE_2 and NF-κB activity. In two separate studies, Resta-Lenert and colleagues[45, 46] investigated the effects of *S. thermophilus, L. acidophilus,* a commensal bacterium or *Bacteroides thetaiotaomicron* on IFN-γ[45], TNF-α[45] or enteroinvasive *Escherichia coli* (EIEC)[46] treated HT29[45, 46] and Caco-2[45] cells. IFN-γ stimulation alone reduced forskolin-stimulated chloride secretion, cystic fibrosis transmembrane conductance regulator (CFTR) expression, and Na-K-Cl cotransporter expression. IFN-γ or TNF-α stimulation significantly reduced transepithelial resistance. Simultaneous treatment of cells with live bacteria

plus IFN-γ or TNF-α, or pre-treatment with heat-inactivated bacteria failed to modify the effects of IFN-γ or TNF-α. Pre-treatment with live *S. thermophilus* or *L. acidophilus*, however, protected against the effects of IFN-γ or TNF-α stimulation.[45] Treatment with *S. thermophilus* and *L. acidophilus* maintained or increased the phosphorylation of cytoskeletal and tight junction proteins, and helped to maintain transepithelial resistance following EIEC stimulation. By contrast, pre-treatment using *S. thermophilus* + *L. acidophilus* was superior. *S. thermophilus* + *L. acidophilus* also significantly limited adhesion, and physiological dysfunction induced by EIEC.[46]

Together with the findings of Resta-Lenert and Barrett[46], Otte and colleagues[47] show the benefits of multi-strain probiotics compared with a single strain of probiotics. Stimulating the human colon cancer cell lines Colo329 and SW480 showed an increased COX-2 promoter activity when exposed to gastrin (up to 8-fold) or TNF-α (up to 3-fold). In a dose- and time-dependent manner, *L. acidophilus applied* alone increased COX-2 promoter activity, COX-2 protein expression and PGE_2 secretion. *L. acidophilus* applied prior to gastrin or TNF-α further enhanced COX-2 promoter activity and PGE_2 secretion compared with gastrin or TNF-α alone. Conversely, the probiotic EcN and VSL#3 (a combination of 3 strains of *Bifidobacterium*, 4 strains of *Lactobacillus* and *Streptococcus thermophilus*) reduced COX-2 expression and PGE_2 secretion.

Lee and colleagues[42] administered *S. Boulardii* to HT-29 cells treated with TNF-α or IL-1β. Compared with control cells, pre-treating HT-29 cells with *S. Boulardii* increased mRNA expression of PPAR-γ, decreased IL-8 expression and suppressed nuclear translocation of NF-κB in response to TNF-α stimulation. siRNA-mediated knockdown of PPAR-γ abolished the effects of *S. Boulardii*, indicating that PPAR-γ plays a crucial role in suppressing pro-inflammatory cytokine synthesis. PPAR-γ is primarily found in adipose tissue. However, it is also expressed in high levels in the colon, stomach and small intestine mainly by epithelial cells, stellate cells, monocytes/macrophages, Kupffer cells, dendritic cells (DCs), and B and T cells.[48] The molecular mechanisms mediating the anti-inflammatory action of the PPAR-γ/RXR heterodimer are not fully understood at present. Activation of PPAR-γ may interfere with several signalling pathways regulating the expression of pro-inflammatory genes, such as those controlled by NF-κB, activating protein 1 (AP-1) and signal transducers and activators of transcription (STATs).[49, 50] PPAR-γ therefore may modulate the production of inflammatory cytokines, chemokines and cell-adhesion molecules limiting the recruitment of inflammatory cells and promoting an anti-inflammatory state.[49]

The probiotic strain *L. reuteri* has also been shown to reduce IL-8 production in various cell lines. Ma and colleagues[51] stimulated T84, HT-29 and Caco-2 cell lines with TNF-α. Pre-incubation of cells with 1×10^7 cells/mL *L. reuteri* significantly inhibited but did not completely block IL-8 production in response to TNF-α stimulation. When T84 cells were incubated with *L. reuteri* (1×10^7 cells/mL) and TNF-α simultaneously, IL-8 synthesis was similar to that of TNF-α stimulation alone.

Investigating the mechanisms by which probiotics reduce NF-κB activation, Petrof and colleagues[52] administered *L. plantarum, L. paracasei* or *L. acidophilus* to murine RAW 264.7 macrophage and young adult mouse colon cells. Medium conditioned with *L. plantarum* inhibited NF-κB binding activity and degradation of IκBα in response to stimulation with TNF-α. Further analysis showed that this effect was due to less binding of the p50/p65 subunits of NF-κB (Figure 1.2). In turn, this effect attenuated the synthesis of monocyte chemotactic protein-1 (MCP-1).

Together, the results from applying probiotics prophylactically to cell lines indicate the importance of timing and dose of probiotic therapy. The results indicated that probiotic treatment of cells was more effective when applied prophylactically compared with the simultaneous application with an inflammatory agent. Multi-strain probiotics also proved superior in reducing inflammation compared with single strains.

1.5.2. Prophylactic effects of probiotics in animals

The following section provides a summary of the prophylactic effects of various probiotic strains. The models include BALB/c, ICR, IL-10 KO, C57BL/6, C57BL/10J mice and female Wistar rats. Analysis included body mass, colon appearance, inflammatory scores, changes to dendritic cell migration, cytokine production (IL- 1β, IL-2, IL-4, IL-10, IL-12, TNF-α), PGE_2, MyD88, myeloperoxidase (MPO), iNOS, COX-2 and glycosaminoglycan production.

Kamada and colleagues[53] investigated the effects of EcN in pathogen-free C57BL/6 and IL-10 KO mice. Administration of DSS to C57BL/6 mice caused loss of body mass, the shortening and thickening of the colon, increased disease activity index scores and colon mass, severe ulceration and inflammatory cell infiltration over the proximal and distal region of the colon. Treatment with EcN attenuated these effects of DSS. These results may indicate an increase in epithelial permeability following DSS administration allowing inflammatory cells to infiltrate the colon. This will lead to dysregulated inflammation and the observed effects on the colon. EcN has been shown to alter tight junction proteins to maintain transepithelial resistance.[36] In IL-10 KO mice, treatment

with *EcN* reduced colon mass and spontaneous production of IFN-γ and macrophage inflammatory protein (MIP)-2 from lamina propria mononuclear cells. *L. salivarius* subspecies *salivarius* 433118 and *B. infantis* 35624 showed similar effects in IL-10 KO mice. McCarthy et al.[22] showed that both probiotic strains reduced intestinal inflammation scores, and in vitro stimulation with *B. infantis* significantly reduced the production of IFN-γ, TNF-α and IL-12 in response to stimulation with *Salmonella typhimurium*. *B. infantis* was more effective for modulating cytokine production compared with *L. salivarius*, even though a lower dose was administered.

Extending on from the DSS model of colitis, results from Lee and colleagues[54] and Mane and colleagues[55] show the ability of probiotics to confer similar effects over a variety of colitis models. Lee and colleagues[54] administered 14 lactic acid bacteria (eight *Lactobacillus* and six *Bifidobacterium* strains) with *Bacteroides stercoris* — a bacterium that produces glycosaminoglycan degradation enzymes in male ICR mice. They showed most bacteria inhibited glycosaminoglycan degradation. Subsequent research showed that *B. longum* HY8004 and *L. plantarum* AK8-4 prevented or inhibited TNBS-induced colon shortening, inflammation, glycosaminoglycan degradation, IL-1β and TNF-α expression, MPO activity and activation of NF-κB in colon homogenate samples.

Mane and colleagues[55] fed *L. fermentum CECT 5716* to BALB/c mice prior to administering TNBS. Mice fed *L. fermentum* maintained greater body mass and developed more mild colitis (as indicated by a lower histology score) compared with control mice. The improvement was associated with an increase in PGE_2, IL-2 and IL-4, in addition to lower nitrotyrosine staining (for protein oxidation). *L.* fermentum did not alter immunohistochemistry staining for MyD88 in colon explant cultures. Increased PGE_2, IL-2 and IL-4 synthesis may improve IBD by initiating an appropriate early immune response, and by repairing and improving the integrity of the intestinal barrier through the activation of toll-like receptors (TLRs)[56], COX-2[56] and proliferation of regulatory T cells.[57] MyD88 is an adaptive protein that may aid in the reduction of TNBS induced inflammation due to its role in TLR-2, TLR-4 and TLR-9 signalling and subsequent NF-κB activation.[58]

Peran and colleagues[59, 60] conducted consecutive studies investigating the prophylactic effects of probiotics [*L. fermentum* or *L. reuteri*[60] and *L. casei, L. acidophilus* or *B. lactis*[59]] in female Wistar rats. Following TNBS administration, intestinal inflammation was evident as loss of body mass, anorexia and diarrhea in both studies. Probiotics did not prevent these effects, with the exception that diarrhoea decreased in response to treatment with *L. fermentum*[60] and *B. lactis.*[59] Rats administered probiotics presented fewer signs of mucosal inflammation, but only the rats

administered *L. fermentum* or *L. acidophilus* showed significantly lower colonic damage scores (as indicated by a reduction in colonic necrosis and/or inflammation). Colonic MPO activity and adhesion of the colon to neighbouring organs were lower in rats treated with *L. fermentum* or *L. acidophilus*. TNBS increased colonic TNF-α, LTB_4 and IL-1β[60] expression, increased expression of iNOS and COX-2,[59] and reduced IL-10 production.[60] *L. reuteri* and *L. fermentum* administration reduced TNF-α production, *B. lactis* significantly reduced colonic TNF-α, iNOS and COX-2 expression, whereas *L. acidophilus* significantly reduced colonic LTB_4 production and expression of iNOS.

Dendritic cells are a vital component for initiating an early immune response to foreign antigens. The interaction between probiotics and DC's is largely unknown. Wang and colleagues[61] investigated the effects of VSL#3 on the distribution and phenotype of DCs in C57BL/10J mice. Administration of VSL#3 reduced the number of plasmacytoid DCs within the lamina, and increased the number of plasmacytoid DCs in the lymph nodes compared with the control group. In contrast, myeloid DC infiltration was greater in the control animals for all intestinal lymphoid tissue compartments compared with mice treated with VSL#3. These findings suggest that probiotics modulate the distribution of DCs, thus altering the immune response. How these responses might influence inflammation within the GIT is unknown. An extension to this research may include investigating the effects of probiotics and changes in DCs following DSS- or TNBS-induced inflammation.

Extending the work of Resta-Lenert and Barrett,[46] Roselli and colleagues[62] investigated whether one combination of probiotics is more effective over another combination. One of two combinations of probiotics [(1) *L. acidophilus* Bar 13 and *B. longum* Bar 33 (2×10^9 cfu/mL), and (2) *L. plantarum* Bar 10, *Streptococcus thermophilus* Bar 20 and *B. animalis* subspecies *lactis* Bar 30 (3×10^9 cfu/mL)] was applied to female BALB/c mice prior to administering TNBS. Both probiotic combinations reduced or prevented inflammation and loss of body mass following exposure to TNBS. The combination of *L. acidophilus* Bar 13 and *B. longum* Bar 33 prevented the systemic release of cytokines (IL-12, IFN-γ, TNF-α and MCP-1) and $CD4^+$ T cells in the colon. In contrast, the combination of *L. plantarum*, *S. thermophilus* and *B. animalis* reduced the serum concentration of TNF-α and MCP-1. Both combinations of probiotics increased serum IL-10 concentration. Similar to the work of McCarthy and colleagues,[22] the findings from this study also indicated that higher doses of probiotics were not more effective for modulating tissue inflammation compared with lower doses.

The prophylactic effect of probiotics in animals appears effective over a number of colitis models, but is strain-dependent. Various single probiotic strains prevent inflammation through a variety of cell signalling pathways. However, combining different single strains into a multi-strain probiotic is more effective, even at lower doses. The beneficial effects include altering the distribution of DC's, improving the intestinal barrier via activation of TLR's and decreasing pro-inflammatory cytokines (IFN-γ, TNF-α and IL-12) and increasing anti-inflammatory cytokines (IL-10).

1.5.3. Prophylactic effects of probiotics in humans affected by IBD

Guslandi and colleagues[63] administered *S. boulardii* or a placebo to patients in remission with previously diagnosed Crohn's disease in the past year. Following a 6-month course of probiotics, significantly fewer patients clinically relapsed compared with the control group. In contrast, supplementing patients with Crohn's disease with *L. rhamnosus* strain GG or a placebo[64, 65] resulted in no significant changes in the severity of Crohn's disease. Patients relapsed in both the supplement and placebo groups.[65]

The findings from these studies were limited by small sample size, and poor completion rates (~50%). Further investigation into the effects of probiotics in patients with IBD in remission is needed. Future research would benefit from an increased sample size and a more detailed analysis of inflammation variables from collection of blood and faecal matter, or where possible, intestinal tissue.

1.6. **PROBIOTICS APPLIED THERAPEUTICALLY**

Probiotics may function differently when inflammation is already present. The following section discusses the therapeutic effects of probiotics on monocyte-derived DCs, splenocytes and segments of distal colons from BALB/c mice. The efficacy of various probiotic strains was assessed by monitoring changes in existing inflammation, focusing on cell signalling, cytokine responses (IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12, IL-27 and TNF-α), symptoms and quality of life. The results of studies involving therapeutic interventions are summarised in Table 4.

1.6.1. Therapeutic effects of probiotics in cell cultures

The beneficial effects of probiotics on inflammation are fairly consistent when applied prophylactically. The therapeutic effects however appear to be more variable. Showing the variability between single strains of probiotics, Latvala and colleagues[66] investigated the effects of *L. rhamnosus* GG and *B. animalis* subspecies *lactis* Bb12, in addition to seven bacterial strains with

potential probiotic characteristics. In monocyte-derived DCs from healthy individuals, stimulation with *Streptococcus thermophilus* (THS) increased the synthesis of TNF-α, IL-6, IL-12 and cytokine and chemokine (C-C motif) ligand 20 (CCL20). Incubation of monocyte-derived DCs with *B. animalis* or *B. breve* increased the production of TNF-α, IL-1β, IL-6, IL-10, IL-12 and IFN-γ. In contrast, *L. rhamnosus* and *L. mesenteroides* did not significantly alter cytokine production compared with control cells. All of the bacterial strains above altered CCL20 concentrations in a dose-dependent manner. Furthermore, all of the strains induced CCL20, whereas no strain induced CCL19. CCL19 and CCL20 play a unique role in the regulation of epithelial immunity. CCL19 and CCL20 are cytokines belonging to the CC chemokine family that attract cells of the immune system, particularly DCs, toward sites of inflammation. CCL20 is capable of inducing the most potent response, and is found in abundance in gut mucosa.[67] CCL20 is up-regulated following inflammatory stimuli IL-1β and TNF-α or T cell signals.[68]

Showing similar variability in the effectiveness of various probiotic strains, Tanabe and colleagues[69] administered *B. bifidum, B. catenulatum, B. infantis, L. acidophilus* or *L. bulgaricus* to splenocytes isolated from female BALB/c mice, and segments of the distal colon tissue. Addition of TGF-β + IL-6 to splenocytes significantly increased IL-17 production, suppressed IL-4, IL-5, IL-12 and IL-27 production and had no effect on IL-10. *B. infantis* suppressed IL-17 production, increased IL-10 production and partially restored IL-5, IL-12 and IL-27 synthesis. In contrast, *B. bifidum, B. catenulatum, L. acidophilus* and *L. bulgaricus* did not influence IL-17 production. DSS applied to segments of distal colon tissue enhanced IL-17 and eotaxin production in a dose-dependent manner, whereas production of IL-27 decreased slightly. All of the *Bifidobacterium* strains suppressed IL-17 and eotaxin production in response to DSS. DSS markedly reduced IL-10 production, but *B. infantis* prevented this effect.

These findings clearly illustrate the variable effects of different strains of probiotics. Some strains showed little or no effect when applied therapeutically to certain cell models. The results demonstrate that the effects of probiotics depend on the model and inflammatory agent. These results may begin to explain why the effects of one strain or mixture of probiotics may vary between subjects and between disease states (e.g. IBD vs. IBS, active vs. remission).

1.6.2. Therapeutic effects of probiotics in animals

The following section provides details on the therapeutic application of probiotics to BALB/c and IL-10 KO mice. Inflammation was assessed using cytokine production (IL-4 IL-6, IL-12p40, INF-γ and TNF-α) histology scoring for inflammation, MyD88 and MPO activity. In addition to their

prophylactic study, Mane and colleagues[55] fed *L. fermentum CECT 5716* to BALB/c mice after TNBS was administered. Body mass was similar in mice fed *L. fermentum* after TNBS treatment compared with control mice; however, body mass recovered more rapidly in mice treated with *L. fermentum*. In response to TNBS, histology scores were lower in mice fed *L. fermentum* at weeks two and three compared with control mice. IL-6 synthesis from cultured colon explants was also lower in mice fed *L. fermentum* at week two compared with the control group. Immunohistochemistry staining for MyD88 was greater in colon explants cultures from mice fed *L. fermentum* compared with control animals at week 2, but the difference was much less apparent by week three. Together with the prophylactic data, these results demonstrate that *L. fermentum CECT 5716* is effective as both a prophylactic or therapeutic intervention. Their results also indicate that probiotics may exert their effects at different points along the TLR cell signalling pathways. Prophylactically, probiotics activated pro- and anti-inflammatory mediators which initiate TLR-2, TLR-4 and appropriate immune responses, whereas therapeutically, probiotics increased MyD88 concentration to achieve a similar (but potentially weaker) response. TLR's (in particular TLR-2 and TLR-4) play a central role in the immune response by detecting foreign substances and sending appropriate signals to the immune system. These signals can trigger the activation of NF-κB and the production of pro- or anti-inflammatory cytokines.

Using a colitis model involving BALB/c mice with DSS induced colitis, Ukena et al.[70] and Chen and colleagues[71] both found probiotics of various strains [EcN[70], *E. faecalis, L. acidophilus, Clostridium butyricum* or *B. adolescentis*[71]] were able to reduce, at least in part, the effects of colitis. Effects of DSS-induced colitis included: body mass loss,[70, 71] higher disease activity index scores,[71] shortening of the colon,[70] reduced ZO-1 mRNA expression in IECs,[70] increased leukocyte infiltration in the colon,[70] increased Na^+ absorption in the colonic mucosa,[70] increased MPO activity[71] and colonic IL-1β production, coupled with lower IL-4 production.[71] All strains were effective in reducing the effects of colitis, EcN and *E. faecalis* proved most effective.

The results of Chen and colleagues[71] show that in the same model of colitis, different strains of probiotics are capable of achieving similar effects. When the model of colitis is changed however, so does the aetiology of the disease and the effects of probiotics. This is evident by Pena et al[72] who found no change to IL-4 production when IL-10-deficient C57BL/6 mice were exposed to *H. hepaticus*. Pena et al[72] isolated RNA from cecal tissue samples to asses mRNA expression of IL-4, IL-12p40, IFN-γ and TNF-α. IL-10 deficient mice with *H. hepaticus* stimulation had increased mRNA expression of TNF-α, IL-12p40, and IBD-like lesions, but no effect on IL-4. Administering *L. paracasei* 1602 and *L. reuteri* 6798 attenuated TNF-α and IL-12p40 mRNA expression, but did

not prevent formation of the IBD-like lesions. The probiotics also reduced cecocolic junction lesion scores in female mice, but not in male mice. In contrast, probiotics administered to mice without *H. hepaticus* showed no effect on cytokine production.

Overall, the therapeutic effect of probiotics in animals appears to improve histology scores, increase the recovery of body mass and reduce pro-inflammatory cytokine production. The results support the notion that the same probiotic strain may not induce the same response from model to model, and that different probiotic strains may induce a different response or magnitude of response when applied within the same setting.

1.6.3. Therapeutic effects of probiotics in patients with IBD

The therapeutic effect of probiotics in patients with IBD is largely unknown because the majority of research has focused on IBS. Garcia Vilela and colleagues[73] provided *Saccharomyces boulardii* to patients with Crohn's disease and healthy volunteers. Patients with Crohn's disease presented with increased intestinal permeability compared with healthy volunteers. Supplementation with *Saccharomyces boulardii* improved intestinal permeability in patients with Crohn's disease compared with the placebo group. Probiotic treatment did not restore intestinal permeability completely, however.

1.6.4. Therapeutic effects of probiotics in patients with IBS

The following section evaluates the efficacy of probiotics in treating patients with existing IBS. The efficacy was assessed by comparing abdominal pain and discomfort, symptom scores for bowel habit satisfaction, flatulence and production of C-reactive protein (CRP) and cytokines (IL-2, IL-4, IL-6,IL-10, IL-12, IFN-γ and TNF-α,) before and after probiotics treatment.

One of the problems with probiotic therapy is administering the 'ideal' dose. As each probiotic strain may require a different dose and different disease etiologies may require a different dose, finding the 'ideal' dose is complex. Whorwell and colleagues[74] investigated the effects of three different doses of *B. infantis* 35624 (1×10^6, 1×10^8 & 1×10^{10} cfu/mL) in treating primary-care IBS patients. The dose of 1×10^8 cfu/mL proved superior in relieving abdominal pain compared with the placebo and other doses. Further investigation of the highest dosage demonstrated that the probiotics "coagulated" into a firm glue-like mass making them resistant to acid and agitation. The lowest dose of probiotics may not have been effective because of the duration of the study, or

insufficient biological activity. These findings highlight the potential importance of how probiotics are administered in order to maximise bioavailability within the GIT.

To assess the efficacy of probiotics, improvements in disease symptoms is typically used due to its invasive nature. Probiotics have been shown to reduce abdominal pain and discomfort and symptom scores when patients with IBS when administered *L. acidophilus*[75], *L. plantarum* 299V[76] or ProSymbioflor (a combination of *Escherichia coli* DSM 17252 and *E. faecalis* DSM 16640)[77] compared to a placebo. In contrast, Drouault-Holowacz and colleagues[78] found that *B. longum* LA 101, *L. acidophilus* LA 102, *L. lactis* LA 103 and *S. Thermophilus* LA 104 were not superior to the placebo treatment in relieving disease symptoms due to a strong placebo effect. Probiotics supplementation did, however, significantly relieve abdominal pain to a greater extent compared with placebo treatment. Further analysis of the IBS sub-groups revealed that patients with alternating bowel habits reported significantly less abdominal pain, and patients with constipation-predominant IBS reported less constipation. These results indicate that different disease aetiologies may exist between IBS sub groups and that some probiotics may be more efficient than others in treating symptoms within these sub groups. These results also point to the need to classify patients into relevant sub-groups when assessing the efficacy of probiotic therapy.

A number of studies investigating the effects of probiotics within specific sub-groups of IBS have found beneficial effects of probiotics. Zeng and colleagues[79] separated patients with IBS into sub-groups, treating diarrhoea-predominant IBS (D-IBS) patients with *S. thermophilus, L. bulgaricus, L. acidophilus* and *B. longum.* The proportion of patients with increased small bowel permeability decreased significantly after treatment, and these patients also showed an improvement in their IBS score, abdominal pain and flatulence. Similarly, VSL#3 provided to subjects with either diarrhea-predominant IBS (D-IBS)[80] or IBS with bloating (B-IBS)[81] was shown to reduce IBS symptoms. In subjects with B-IBS, VSL#3 reduced flatulence scores and retarded colonic transit time, without altering bowel function. In patients with D-IBS, VSL#3 only relieved abdominal bloating, with no effect on mean transit measures, bowel function scores or satisfactory relief of symptoms. VSL#3 has also been shown to be superior to a placebo in children with IBS. VSL#3 supplementation improved overall IBS symptoms, abdominal pain/discomfort, abdominal bloating/gassiness and assessment of family life disruption.[82]

To date, monitoring disease symptoms has been the most prevalent way to test the efficacy of probiotics in IBS and IBD patients. This subjective measure, usually assessed by the patient themselves, does not help to understand the underlying mechanisms of probiotics or the disease

aetiology. In an attempt to understand the physiological mechanisms of probiotics, Kajander and colleagues supplemented patients with IBS with LGG, *L. rhamnosus* LC705, *B. breve* Bb99 and *P. freudenreichii* ssp. *shermanii* JS in combination[83] or *L. rhamnosus* GG, LGG, *L. rhamnosus* Lc705 (DSM 7061), *P. freudenreichii* ssp. *Shermanii JS* (DSM 7067) and *B. animalis* ssp. *lactis* Bb12.[84] Serum C-reactive protein (CRP) and cytokine (IFN-γ, TNF-α, IL-2, IL-4, IL-6 and IL-10) concentrations were generally below the limit of detection, and therefore did not indicate any differences between the treatment groups.[84] Both studies did, however, report an improvement in the IBS scores from baseline, particularly for distention and abdominal pain.

In contrast with the benefits of probiotics in relieving IBS symptoms described above, some research has found few or no beneficial effects of probiotics. O'Mahony and colleagues[85] showed disparity in the effects when providing *L. salivarius* UCC4331, *B. infantis* 35624 or a placebo to subjects with IBS and healthy volunteers. Following supplementation, the composite score (weeks 1–8), pain/discomfort (weeks 1, 2, 4, 5, and 7), bloating/distention (weeks 2, 5, and 6) and bowel movement difficulty (weeks 2, 3, 5, and 6) was lower in the *B. infantis* group than in the placebo group. Composite score was only lower in the *L. salivarius* group than the control group in the second week of supplementation, indicating that the effects of *L. salivarius* are short-lived and intermittent. In vitro production of IL-10 and IL-12 by isolated mononuclear cells (peripheral blood mononuclear cells [PBMC]) from whole blood was dysregulated at baseline in patients with IBS. Patients with IBS had low levels of IL-10 and high levels of IL-12 synthesis compared with healthy volunteers. Supplementation with *B. infantis* restored IL-10 and IL-12 synthesis to levels similar to those observed in healthy volunteers.

Adding to the lack of effect of probiotics, the placebo effect or natural healing cycle needs to be considered. Niv and colleagues[86] provided *L. reuteri* ATCC 55730 or a placebo to subjects with IBS. Following supplementation, an improvement in IBS symptoms was reported, however, a similar response occurred in the placebo group. This may demonstrate a strong placebo effect or the natural healing cycle of the disease with IBD and IBS patients frequently entering periods of remission.

Taken together, the therapeutic effect of probiotics in humans supports other benefits of probiotics. While some effects are inconsistent, probiotics treatment appears to reduce symptoms of IBS, particularly abdominal pain, and restore the balance of pro and anti-inflammatory cytokines. Separation of IBS into sub-classifications of disease confirmed that some probiotic strains may have effects that are symptom-specific. Combining selected stains of probiotics into a single multi-strain

probiotic blend appears to provide greater efficacy compared to the single strains alone. Based on these findings, further research is required looking more specifically at the various effects of each probiotic when applied to different disease classifications and in combination. To overcome potential placebo effects or inconsistent results, emphasis should be placed on sample size. Future research is vital to help find how and why probiotics are effective in each specific disease state. This in turn may help map the aetiology of particular inflammatory conditions, thereby helping to develop a prophylactic or therapeutic intervention.

1.7. CONCLUSION

Inflammation is a necessary physiological response by body tissues to injury, chemical irritation or an assault by pathogenic bacteria. Once the insult is neutralised, normal physiological function needs to be restored. For example, in the gastrointestinal tract an inflammatory response is elicited to clear pathogenic bacteria. Probiotic bacteria can then subsequently reduce the inflammatory response, thereby promoting a 'regulated' pro–anti inflammatory state, and assist in reducing the symptoms of conditions such as irritable bowel syndrome. The clinical evidence for these benefits is equivocal, however. This review indicates that probiotics provide both a prophylactic and therapeutic benefit in improving IBD and IBS by regulating cytokine and cell signalling pathways. Cell culture studies investigating probiotics used a range of cell lines, probiotic doses and inflammatory stimulants. The majority of studies employed similar cell culture conditions relating to incubation periods and dosage of probiotics and inflammatory stimulants. The concentrations of probiotics applied to cell cultures are similar to those concentrations typically recommended for human consumption. The amount of probiotics applied was, however, greater than the amount of viable probiotics that reach the intestines.[87-89] A number of methodological issues are worth considering for the design of future studies, and I briefly discuss these issues below.

1.7.1. Cell culture studies

The predominant cell lines reviewed were Caco-2 and T84 cells. With the exception of one study reporting that Caco-2 cells are a less stable model of inflammation[36], similar results were seen between all the different cell lines used. Applying probiotic strains to the different cell lines gave varying results. The weight of the evidence indicates that probiotics were beneficial in preventing or reducing inflammation, however. A reduction in inflammation was achieved through a number of mechanisms, including maintaining a strong transepithelial resistance and cell membrane integrity. Maintaining the cell membrane integrity increased the transepithelial resistance by restoring ZO-1 and ZO-2 to the cell membrane and PKC-ζ to the cytosol. Another inflammatory mediator strongly

affected by probiotic stimulation was the production of cytokines. Regulation of cytokine production strongly influences the development and progression of inflammation. Regulation of pro- and anti-inflammatory cytokine production varied greatly between studies. Overall, probiotics reduced or prevented the production of pro-inflammatory cytokines and increased or maintained the production of anti-inflammatory cytokines.

The specific effects of a probiotic strain varied depending on the model and mode of inflammation, however. *L. acidophilus* applied to HT29,[45, 46] Caco-2, [45, 46] Colo329,[47] SW480,[47] Raw264.7,[52] YAMC cells[52] and splenocytes[69] resulted in no effects[52, 69] increased TER,[45, 46] modulated protein expression,[45] increased COX-2 activity and PGE2[47] secretion. These differences highlight the variable and specific effects of probiotics in different models of inflammation. One explanation for the differences may come from the different signalling pathways responsible for inflammation. Each strain of probiotic appears to have a specific ability to up- or down-regulate specific inflammatory pathways. For example, inflammation resulting from a dysregulated Nedd8 pathway may require different strains of probiotics to re-regulate inflammation compared with inflammation resulting from dysregulated cytokine production. Inflammation stemming from a cytokine imbalance (pro- or anti-inflammatory) may require different strains of probiotic again, depending on the specific cytokine profile.

In summary, all the cell lines presented are suitable models for inducing inflammation of the GIT. More important is the cause of inflammation and identifying the dysregulated pathways. Identifying the cause of inflammation assists in selecting appropriate probiotics to counteract inflammation. More work is warranted to examine the efficacy of specific probiotics in different cell lines and models of inflammation.

1.7.2. Animal studies

Probiotic studies involving animals have largely been conducted in mice. The use of multiple strains of mice and variations in probiotic strains, combinations, concentrations and supplementation periods make comparisons difficult. Induction of experimental colitis increased the expression of pro-inflammatory mediators (e.g., IL-1β, IL-12, IFN-γ and TNF-α). Data from several studies indicates that when strains of *Lactobacillus, Bifidobacterium and/or Streptococcus* bacteria are administered, anti-inflammatory mediators (e.g., IL-2, IL-4, IL-10 and MyD88) are up regulated[39, 51, 55, 66, 69], whereas pro-inflammatory mediators (e.g., IL-1β, IL-6, IL-12, TNF-α and IFN-γ) are down regulated.[10, 22, 39, 51, 55, 72]

The most common animal models used were BALB/c, IL-10 KO and C57 background mice. These strains of mice appear to differ with respect to the progression of inflammation. Melgar and colleagues reported that BALB/c mice recovered from DSS-induced colitis within four weeks upon cessation of DSS, while C57 mice continued to developed chronic colitis following cessation of DSS.[90] C57 background mice may be more appropriate to use when inducing inflammation with DSS. Using a model that maintains a degree of inflammation is critical to help eliminate natural recovery from the results.

Similar to cell culture experiments, the effects of probiotics vary between different models of inflammation in animals. Inflammation in animals is initiated using DSS, TNBS, *Bacteroides stercoris*, and deleting the IL-10 gene. TNBS is applied rectally which requires that the animal is anesthetised. Anesthetizing an animal adds additional stress and the enema procedure itself has the inherent risk of adding 'artificial' inflammation by causing damage and even perforating the intestinal lining. This undue stress to the animal may alter cell signalling, giving false results. The invasive nature of TNBS also means that it is difficult to apply multiple low doses of TNBS over a short period. Accordingly, typically one large dose is applied. DSS is given to the animal in their drinking water or by gavage. Using DSS removes the need for anesthesia, and allows treatment with low doses over a set time (in the water). IL-10 KO mice spontaneously develop colitis from 20 weeks of age. These mice continue to develop chronic colitis, but are expensive and eliminate one of the key targets for probiotic activity.

1.7.3. Human studies

Transferring results from an animal model to a human model of inflammation is difficult. Due to the invasive nature of procedures required to collect tissue for analysis of gastrointestinal effects, most human studies have assessed the efficacy of probiotics in humans by evaluating changes in the quality of life, disease symptoms and pain scores. Crohn's disease and IBS patients have been treated with probiotics prophylactically (in remission) or therapeutically (active disease). The majority of studies involving humans have been conducted as randomised controlled trials in a double-blind fashion. A small number of the studies failed to show any benefit of probiotics consumption; one of these reported the lack of effect due to a strong placebo effect.[78] Overall, results indicate that probiotics relieve IBS symptoms (abdominal pain, flatulence, bloating and bowel habits) and reduce the number of relapses that occur. Clinical trials on the efficacy of probiotics are limited by subject availability. The invasive nature and duration of some studies, combined with strict inclusion criteria, make patient recruitment difficult. The restrictions imposed in some studies resulted in only 11 subjects participating, with only five completing the trial.[65]

Other studies have recruited sufficient numbers of subjects, but achieving a homogenous group has been difficult. Some studies have adjusted for the different disease classifications within the groups,[78, 79, 81] whereas others have not. Several disease classifications and levels of severity exist for both IBD and IBS. The different disease classifications and severities may represent different aetiologies, which may respond differently to probiotics.

1.8. Future directions

Future directions for research may involve exploring the optimal doses of probiotics, duration of treatment, the effects in different models and suitability as a prophylactic or therapeutic treatment. Probiotics reach the intestinal tract, but the percentage of ingested viable bacteria that reach the intestinal tract is not well known. Studies aimed at calculating the amount of viable bacteria that reaches the intestines may be useful. There is a clear lack of evidence on the effect probiotics have on patients with IBD. While alleviating the symptoms of IBD and IBS is clinically relevant, future research may also benefit from collecting colon tissue from Crohn's disease and ulcerative colitis subjects for analysis of gastrointestinal inflammation and bacterial adhesion. This would provide a better understanding of the underlying mechanisms of each condition and possibly lead to better treatment strategies. Ultimately, probiotic research may also need to examine the synergistic benefits associated with individual strains that are currently used in commercially available probiotic mixtures.

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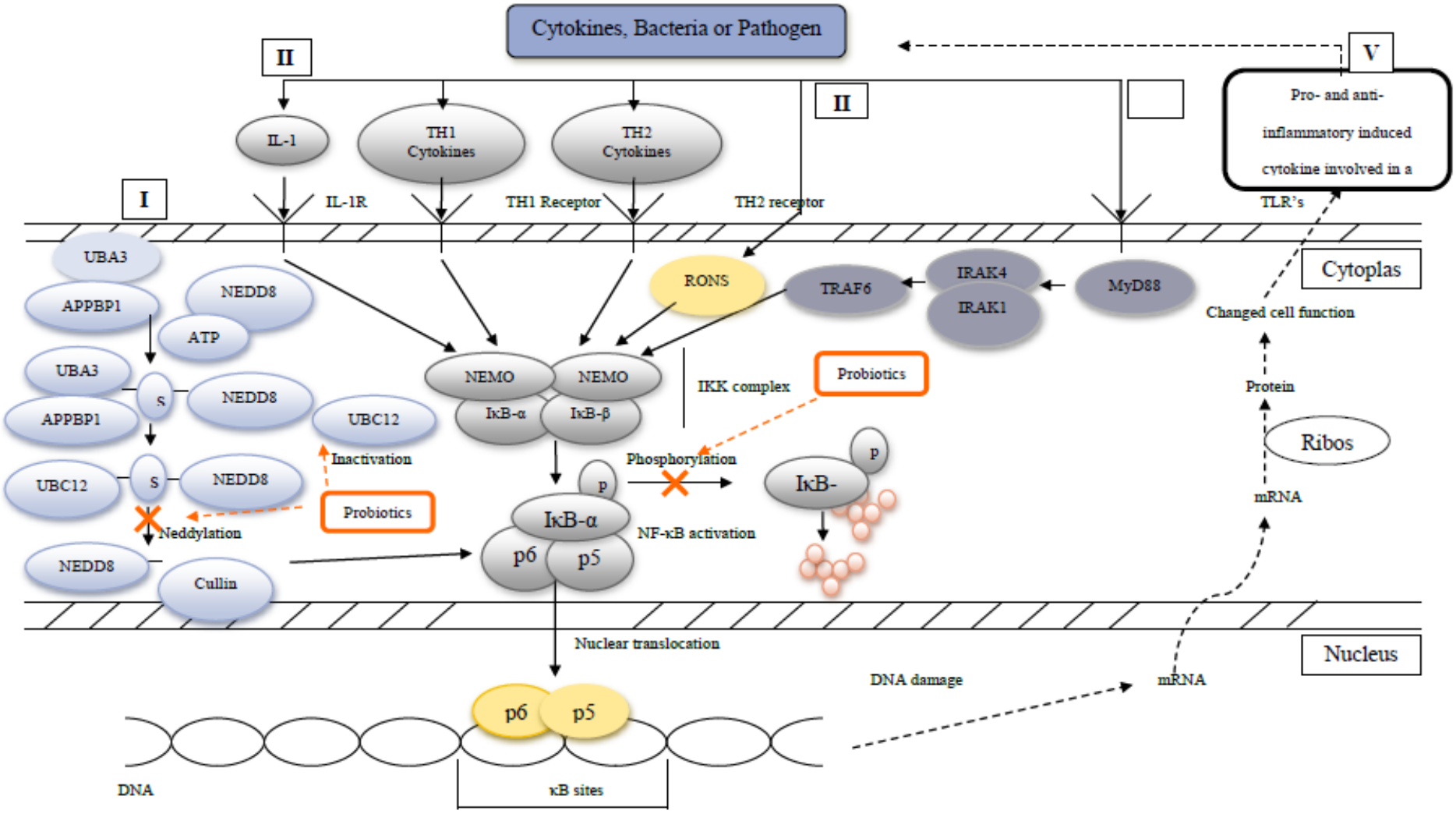
Cytokines, Bacteria or Pathogen
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II
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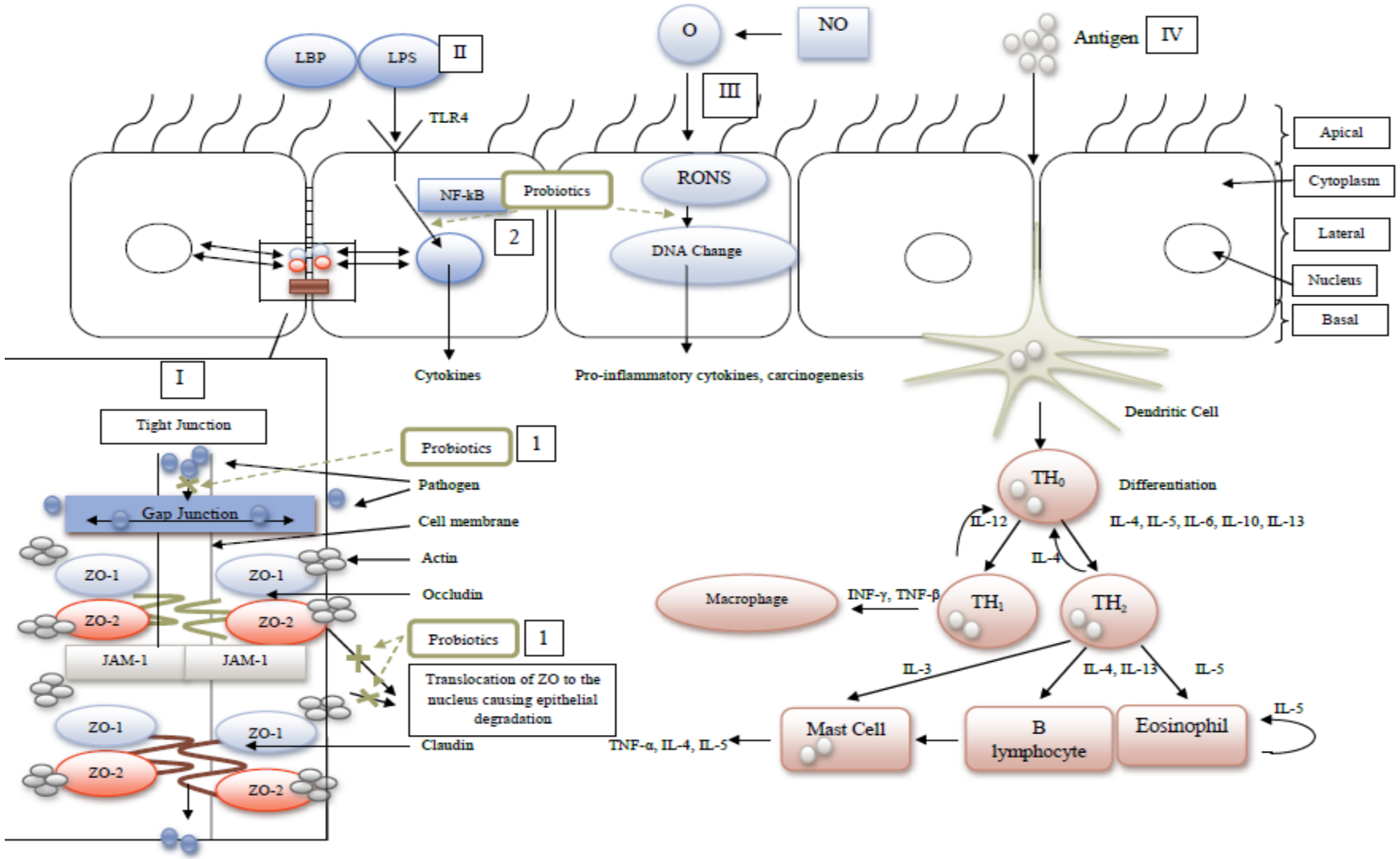
IL-1
TH1 Cytokines
TH2 Cytokines
Pro- and anti-inflammatory induced cytokine involved in a
I
IL-1R
TH1 Receptor
TH2 receptor
TLR's
Cytoplas
UBA3
APPBP1
NEDD8
ATP
RONS
TRAF6
IRAK4
IRAK1
MyD88
Changed cell function
UBA3
APPBP1
S
NEDD8
UBC12
NEMO
NEMO
IκB-α
IκB-β
IKK complex
Probiotics
Protein
Ribos
Inactivation
Phosphorylation
UBC12
S
NEDD8
Probiotics
p
IκB-α
IκB-
p
mRNA
Neddylation
NF-κB activation
NEDD8
Cullin
p6
p5
Nuclear translocation
Nucleus
DNA damage
mRNA
p6
p5
DNA
κB sites

Figure 1


LBP
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II
TLR4
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III
Antigen
IV
Apical
Cytoplasm
Lateral
Nucleus
Basal
NF-kB
Probiotics
2
RONS
DNA Change
Cytokines
Pro-inflammatory cytokines, carcinogenesis
Dendritic Cell
I
Tight Junction
Probiotics
1
Pathogen
Gap Junction
Cell membrane
Actin
ZO-1
ZO-2
Occludin
JAM-1
Probiotics
1
Translocation of ZO to the nucleus causing epithelial degradation
Claudin
TH0
Differentiation
IL-4, IL-5, IL-6, IL-10, IL-13
IL-12
IL-4
Macrophage
INF-γ, TNF-β
TH1
TH2
IL-3
IL-4, IL-13
IL-5
IL-5
TNF-α, IL-4, IL-5
Mast Cell
B lymphocyte
Eosinophil

Figure 2

1.9. Figures

Figure 1.1: A single epithelial cell demonstrating inflammatory signalling pathways that involves: I) The NEDD8 pathway leading to binding of NEDD8 and cullin and subsequent activation of the NF-κB pathway. II) Example of one of the cytokine pathways and its receptor. III) Activation of NF-κB via an imbalance in RONS. IV) MyD88 pathway activated by pathogens binding to TLR leading to the IKK complex and NF-κB pathway. V) Translocation of the p50 and p65 subunits results in changes to the cells function, which may provide positive or negative feedback to the extracellular space. NF-κB activation may result in the feedback changing the cytokine response to regulate the inflammatory response in a positive or negative way. In cases of IBD, one or more of these pathways can be dys–regulated leading to excessive inflammation due in part to an over production or lack of termination of pro-inflammatory cytokine signals. Probiotics may reduce or prevent the resulting inflammation by: 1) blocking or reducing the binding of NEDD8 to cullin via regulated production of ROS inactivating UBC12 and 2) Prevent or reduce the phosphorylation of IκB-α, preventing the translocation of the p50 and p65 subunits to the nucleus.
[This figure was constructed from the published data of: Collier-Hyams et al 2005; Wu et al 2005; Kabelitz et al 2006; Kumar et al 2007; Ivison et al 2010; Hooper and Macpherson 2010.
[IL: interleukin; RONS: reactive oxygen and nitrogen species; TH: T-helper; TLR: toll like receptor; NEMO: NF-κB essential modulator]

Figure 1.2: A diagrammatic representation of part of the epithelial barrier of the gastrointestinal tract. I) Tight junction and gap junction between two epithelial cells. ZO translocates away from the cell boundary towards the nucleus reducing the transepithelial resistance allowing pathogens to move between the cells and through the cell wall into the *lamina propria*. Once the pathogen enters the *lamina propria* it is able to move throughout the gastrointestinal system and systemic circulation causing sever inflammation. II) LPS mediated pathway of inflammation. III) RONS mediated induction of cytokine production and inflammation. IV) Antigens presenting on the epithelial surface may be detected and consumed by dendritic cells. The antigen is presented to TH0 cells which enter the TH1 or TH2 pathway depending on the antigen. Probiotics help regulate and reduce inflammation by 1) preventing the translocation of ZO to the nucleus helping maintain the TER and the integrity of gap junctions preventing the migration of pathogens past the epithelial barrier and 2) by preventing the activation of NF-κB caused by pathogens and compounds like LPS and RONS.

[This figure was constructed from the published data of: Nunbhakdi-Craig et al 2002; Schneeberger Lynch 2004; Parasol et al 2005; Zyrek et al 2007.

[JAM–1: Junctional Adhesion Molecule LPS: Lipopolisaccharides]

Table 1. Probiotic strains and their effects when administered with no inflammatory stimulants

Strain/s	Model	Dose	Supplementation Duration	Findings	Reference
L. rhamnosus	Caco-2, HeLA, T84 (human) and IEC-6 (rat) cells	MOI 1:1	1 hour	Oxidation of thioredoxin-1 and glutathione, ↑ in the chemiluminescence of luminal, ↓ neddylation of Cul-1, loss of IκB-α ubiquination	Kumar *et al.*, 2007
L. rhamnosus	Female BABL/c mice	1×10^8 cfu by gavage	Sacrificed after 30 minutes	Oxidative stress in the mucosa of the small bowel and loss of Cul-1 neddylation in mucosal lysates.	Kumar *et al.*, 2007
L. reuteri	Female BALB/c mice	1×10^{10} cfu/mL daily	On days 1-4 and again on days 21-24	Enhanced mucosal TNF-α, IL-2 and IL-1β producing cells	Maassen, van Holten-Neelen et al. 2000
L. brevis	Female BALB/c mice	1×10^{10} cfu/mL daily	On days 1-4 and again on days 21-24	Enhanced mucosal TNF-α and IL-2 producing cells	Maassen, van Holten-Neelen et al. 2000
B. infantis	Germ free WT & IL-10 KO mice	Monoassociated infection	24 weeks	No effects	Moran, Walter et al. 2009
B. bifidum	Germ free WT & IL-10 KO mice	Monoassociated infection	24 weeks	No effects	Moran, Walter et al. 2009
B. animalis	Germ free WT and IL-	Monoassociated infection	24 weeks	IL-10 KO mice had thickening of the duodena with cellular infiltration of mononuclear cells into the lamina	Moran, Walter et al. 2009

	10 KO mice	propria. Mucosal ulceration localised to the duodenum and ↑ spontaneous release of IL-12/IL-23 p40 from colonic explants

Abbreviations: *L.* = *lactobacillus; B.* = *Bifidobacterium*; TNF = tumour necrosis factor; IL = interleukin; cfu = colony forming units; WT = wild type; KO = knockout; ↑ = increase/improvement; ↓ = decrease/reduction; MOI = multiplicity of infection.

Table 2. Probiotic strains and their effects when applied simultaneously with inflammatory stimulants

Strain/s	Model	Dose	Supplementation Duration	Findings	Reference
L. casei	T84 cells	1×10^6 cfu/mL	3 hours	No effect on EPEC- induced ZO-1 alteration	Parassol, Freitas et al. 2005
L. casei	T84 cells	1×10^7 & 1×10^8 cfu/mL	3 hours	Maintained ZO-1 distribution	Parassol, Freitas et al. 2005
Escherichia coli strain Nissle 1917	T84 and Caco-2 cells	MOI 100:1	1 hour	Maintained TER	Zyrek, Cichon et al. 2007
B. breve	RAW264.7	3×10^8 bacteria cells/mL	16 hours	Attenuated increase in mRNA IL-1β & IL-12p40. Inhibited IκB-α phosphorylation and ↑ mRNA levels of IL-10, SOCS1 and SOCS3	Okada, Tsuzuki et al. 2009
B. adolescentis	RAW264.7	3×10^8 bacteria cells/mL	16 hours	Attenuated increase in IL-1β and TNF-α. Inhibited IκB-α phosphorylation and ↑ mRNA levels of SOCS1 and SOCS3	Okada, Tsuzuki et al. 2009
B. Longum	RAW264.7	3×10^8 bacteria cells/mL	16 hours	Attenuated the IL-12p40 levels. Inhibited IκB-α phosphorylation and ↑ mRNA levels of SOCS1 and SOCS3	Okada, Tsuzuki et al. 2009
B. Longum	LPMCs derived from UC patients mucosal biopsies	106 cfu/mL of medium	24 hours	Reduced tissue supernatant production of TNF-α, IL-8, and NF-κB p65	Bai, Ouyang et al. 2006
L. Paracasei	Caco-2 cells	10^8 bacteria/mL	20 hours	Substantial IL-6, HSP70 and HSP 27 production, no effect on IL-8	Reilly, Poylin et al. 2007

L. Plantarum	Caco-2 cells	10^8 bacteria/mL	20 hours	Substantial IL-6, HSP70 and HSP 27 production, no effect on IL-8	Reilly, Poylin et al. 2007
L. rhamnosus GG	$CD4^+$ T cells from CD and HV	1×10^6 cfu/mL	4 days	↓ IFN-γ production in HV cells	Hvas, Kelsen et al. 2007
L. rhamnosus GG and L. acidophilus	$CD4^+$ T cells from CD and HV	1×10^6 cfu/mL	4 days	↓ IFN-γ production in HV cells	Hvas, Kelsen et al. 2007
S. Boulardii	male Sprague-Dawley rats	5×10^9 viable cells	7 days	restored the histological appearance and weight/length of the colon, abolished the TNBS down-regulation of mRNA PPAR-γ expression and up-regulation of IL-8, IL-1β, IL-6, IL-8R, TNF-α and iNOS mRNA in the colon tissue	Lee *et al.*, 2009b
Escherichia coli strain Nissle 1917	Female BALB/c mice	$1.5\text{-}2.0 \times 10^8$ twice a day	8 days	Protected against intestinal barrier dysfunction, maintained a greater body mass and colon length. Up-regulation of ZO-1	Ukena, Singh et al. 2007
L. plantarum	IL-10 gene KO mice	$1 \times 10^{9\text{-}10}$ cfu/mL in drinking water (average 5mL/day)	4 weeks	Reduced diarrhoea and spontaneous colonic mucosal IL-12 and IFN-γ production, improved colonic, rectal and cecal histology scores	Schultz, Veltkamp et al. 2002
L. GG	male Wistar rats	1×10^8 cfu/mL	3 days	Improved colonic tissue architecture and small ↑ in the relative amounts of *L.*	*Amit-Romach, Uni et al. 2008*
S. thermophilus, L. acidophilus and *B. lactis* (YO-MIX™	male Wistar rats	1×10^8 cfu /mL	3 days	elevated the *Bifidobacteria* population	Amit-Romach, Uni et al. 2008

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Abbreviations: *L.*, *B.*, TNF, IL, cfu, WT, KO, ↑, ↓, MOI - see table 1; *S.* = streptococcus; EPEC = enteropathogenic Escherichia coli; TER = transepithelial resistance; IκB = Ikappa B; NF-κB = nuclear factor kappa B; HSP = heat shock protein; LPMCs = lamina propria mononuclear cells; mRNA = messenger ribonucleic acid; CD = Crohn's disease; UC = ulcerative colitis; HV = healthy volunteers; IFN = interferon; SOCS = suppressor of cytokine signalling.

Table 3. Probiotic strains and their prophylactic effects

Strain/s	Model	Dose	Supplementation Duration	Findings	Reference
S. thermophilus	HT29/cl.19A and Caco-2 cells	MOI 10:1	Up to 48 hours	Only Prophylactic able to alter INF-γ or TNF-α effects	Resta-Lenert and Barrett 2006
L. acidophilus	HT29/cl.19A and Caco-2 cells	MOI 10:1	Up to 48 hours	Only Prophylactic able to alter INF-γ or TNF-α effects	Resta-Lenert and Barrett 2006
L. reuteri	T84, HT-29 and Caco-2 cells	1×10^9 cells	2 hours	Inhibited TNF-α; ↑ IL-8 production	Ma, Forsythe et al. 2004
S. thermophilus	HT29/cl-19A and Caco-2 cells	MOI 50:1	1 hour	Increased TER	Resta-Lenert and Barrett 2003
L. acidophilus	HT29/cl-19A and Caco-2 cells	MOI 50:1	1 hour	Increased TER	Resta-Lenert and Barrett 2003
S. thermophilus and *L. acidophilus*	HT29/cl-19A and Caco-2 cells	MOI 50:1	1 hour	Increased TER above single strains	Resta-Lenert and Barrett 2003
S. Boulardii	HT-29 cells	5-20 yeasts per cell	24 hours	↑ mRNA expression of PPAR-γ and decreased IL-8 expression	Lee *et al.*, 2009b
L. acidophilus	Colo329 and SW480 cells	1×10^6 cfu/mL	6 hours	↑ COX-2 promoter activity, COX-2 protein expression and PGE_2 secretion	Otte, Mahjurian-Namari et al. 2009
VSL#3	Colo329 and SW480 cells	1×10^6 cfu/mL	6 hours	↓ the gastrin and TNF-α induced COX-2 expression and PGE_2 secretion	Otte, Mahjurian-Namari et al. 2009

Escherichia coli strain Nissle 1917	Colo329 and SW480 cells	1×10^6 cfu/mL	6 hours	↓ the gastrin and TNF-α induced COX-2 expression and PGE_2 secretion	Otte, Mahjurian-Namari et al. 2009
L. acidophilus	murine RAW 264.7 macrophage and YAMC cells	2×10^9 cfu/mL	4 hours	No effect	Petrof, Claud et al. 2009
L. Paracasei	murine RAW 264.7 macrophage and YAMC cells	2×10^9 cfu/mL	4 hours	No effect	Petrof, Claud et al. 2009
L. Plantarum	murine RAW 264.7 macrophage and YAMC cells	2×10^9 cfu/mL	4 hours	Inhibited TNF-α stimulated NF-κB activation and binding of the p50/p65 isoform. Attenuated the release of MCP-1	Petrof, Claud et al. 2009
L. fermentum	Mice	1×10^9 cfu daily for two weeks prior to TNBS	2 weeks	Maintained a greater body weight and developed a milder form of colitis. ↑ PGE_2, IL-2 and IL-4 production in colon explant supernatants	Mane, Loren et al. 2009
L. fermentum	Female Wistar rats	5×10^8 cfu	3 weeks	Reduced diarrhoea, colonic damage scores, adhesions, colonic MPO activity and colonic secreted TNF-α levels	Peran, Sierra et al. 2007
B. infantis	female Wistar rats	5×10^8 cfu	3 weeks	Reduced levels of colonic secreted TNF-α	Peran, Sierra et al. 2007

L. casei	female Wistar rats	5×10^8 cfu	3 weeks	Little or no effects	Peran, Camuesco et al. 2007
L. acidophilus	female Wistar rats	5×10^8 cfu	3 weeks	Lower colonic damage scores, adhesions, colonic MPO activity, colonic LTB_4 production and expression of iNOS	Peran, Camuesco et al. 2007
B. longum	male ICR mice	2×10^{10} cfu/kg body weight	6 days	Inhibited GAG degradation, prevented colon shortening and inflammation (protein expression of IL-1β and TNF-α, MPO activity and activation of NF-κB)	Lee, Lee et al. 2009a
L. plantarum	male ICR mice	2×10^{10} cfu/kg body weight	6 days	Inhibit GAG degradation, prevented colon shortening, inflammation (protein expression of IL-1β and TNF-α, MPO activity and activation of NF-κB)	Lee, Lee et al. 2009a
L. salivarius	IL-10 KO mice (develop colitis ≥ week 20)	1×10^9 cfu/mL in milk – 4-7 mL drank/day	19 weeks	Attenuated spontaneously developed colitis	McCarthy, O'Mahony et al. 2003
B. infantis	IL-10 KO mice (develop colitis ≥ week 20)	1×10^8 cfu/mL in milk – 4-7 mL drank/day	19 weeks	↓ *in vitro* production of IFN-γ, TNF-α and IL-12 in isolated lymphocytes	McCarthy, O'Mahony et al. 2003
Escherichia coli Nissle 1917	pathogen-free C57BL/6	5×10^8 cfu/mL	10 days	Prevented weight loss, shortening and thickening of the colon. ↓ DAI score, ulceration and inflammatory cell infiltration	Kamada, Inoue et al. 2005
Escherichia coli	IL-10 KO mice	5×10^8 cfu/mL	10 days	↓ colon weights, spontaneous production of IFN-	Kamada, Inoue

Nissle 1917				γ and MIP-2 from total RNA extracted from LPMCs	et al. 2005
VSL#3	mononuclear cells from mice intestinal tissue	11.25 × 10^9 bacteria/day	7 days	Altered distribution of DC within the intestinal mucosa	Wang, O'Gorman et al. 2009
L. acidophilus Bar 13 and *B. Longum* Bar 33	BALB/c mice	1 × 10^9 cfu of each strain	3 weeks	Prevented loss of body mass, ↓ $CD4^+$subpopulation, ↓ serum concentrations of IL-12, IFN-γ, TNF-α, MCP-1 and IL-10 and ↓ inflammation	Roselli, Finamore et al. 2009
L. plantarum Bar 10, *Streptococcus thermophilus* Bar 20 and *B. animalis* subspecies *lactis* Bar 30	BALB/c mice	1 × 10^9 cfu of each strain	3 weeks	Prevented loss of body mass, serum TNF-α and MCP-1 concentrations and inflammation. ↑ serum IL-10	Roselli, Finamore et al. 2009
Saccharomyces boulardii	CD (n = 32)	1 g	6 months – open RCT	Fewer patients clinically relapsed	Guslandi, Mezzi et al. 2000

Abbreviations: VSL#3 = A multi-strain probiotic blend consisting of: *B. breve, B. longum, B. infantis, L. acidophilus, L. plantarum, L. paracasei, L. bulgaricus and Streptococcus thermophilus. L.*, *B.*, TNF, IL, cfu, ↑, ↓, MOI – see table 1; S., TER, NF-κB, LPMCs, CD, IFN – see table 2; YAMC = young adult mouse colon; MCP-1 = monocyte chemotactic protein-1; COX = cyclooxygenase; PGE_2 = prostaglandin E_2; TNBS = trinitobenzene sulfonic acid; MPO = myeloperoxidase; LTB_4 = leukotriene B4; iNOS = inducible nitric oxide synthase; g = gram; kg = kilogram; GAG = glycosaminoglycan; DAI = disease activity score; MIP = macrophage inflammatory protein; RNA = ribonucleic acid; DC = dendritic cell; RCT = randomised controlled trial.

Table 4. Probiotic strains and their therapeutic effects

Strain/s	Model	Dose	Supplementation Duration	Findings	Reference
B. breve	monocytes purified from healthy blood donors	Bacteria:host ratio 2:1, 10:1 and 40:1	24 hours	↑ TNF-α, IL-1β, IL-6, IL-10, IL-12 and IFN-γ	Latvala, Pietila et al. 2008
L. rhamnosus	monocytes purified from healthy blood donors	Bacteria:host ratio 2:1, 10:1 and 40:1	24 hours	Weak inducer of cytokine expression	Latvala, Pietila et al. 2008
B. animalis	monocytes purified from healthy blood donors	Bacteria:host ratio 2:1, 10:1 and 40:1	24 hours	↑ TNF-α, IL-1β, IL-6, IL-10, IL-12 and IFN-γ	Latvala, Pietila et al. 2008
L. mesenteroides	monocytes purified from healthy blood donors	Bacteria:host ratio 2:1, 10:1 and 40:1	24 hours	Weak inducer of cytokine expression	Latvala, Pietila et al. 2008
B. bifidum	Splenocytes	1×10^7 cells	72 hours	Failed to alter IL-17 production	Tanabe, Kinuta et al. 2008
	colon tissue	1×10^5 cells	24 hours	Suppressed IL-17 and eotaxin production	Tanabe, Kinuta et al. 2008
B. catenulatum	Splenocytes	1×10^7 cells	72 hours	Failed to alter IL-17 production	Tanabe, Kinuta

					et al. 2008
	colon tissue	1×10^5 cells	24 hours	Suppressed IL-17 and eotaxin production	Tanabe, Kinuta et al. 2008
B. infantis	Splenocytes	1×10^7 cells	72 hours	Suppressed IL-17 and promoted IL-27, IL-5 and IL-12 production and ↑ IL-10	Tanabe, Kinuta et al. 2008
	colon tissue	1×10^5 cells	24 hours	Suppressed IL-17 and eotaxin production and ↑ IL-10	Tanabe, Kinuta et al. 2008
L. bulgaricus	Splenocytes	1×10^7 cells	72 hours	Failed to significantly exert an effect on IL-17 production	Tanabe, Kinuta et al. 2008
	colon tissue	1×10^5 cells	24 hours	Suppressed IL-17 and eotaxin production	Tanabe, Kinuta et al. 2008
L. acidophilus	Splenocytes	1×10^7 cells	72 hours	Failed to significantly exert an effect on IL-17 production	Tanabe, Kinuta et al. 2008
	colon tissue	1×10^5 cells	24 hours	Suppressed IL-17 and eotaxin production	Tanabe, Kinuta et al. 2008
L. fermentum	Mice	1×10^9 cfu daily	2 weeks	↓ colon histology scores and IL-6 production in colon explant supernatants, ↑ colonic MyD88 staining	Mane, Loren et al. 2009
L. paracasei and *L. reuteri*	L-10 KO mice	~1×10^9 cfu	2 days before and a third after 3 *H. Hepaticus* doses	Attenuated TNF-α and IL-12p40 mRNA but not the IBD-like lesions	Pena, Rogers et al. 2005

L. acidophilus	BALB/c mice	2×10^8 cfu daily	12 days	Prevented weight loss, ↓ DAI score, MPO activity and IL-1β mRNA levels and ↑ IL-4 mRNA levels in the colon	Chen, Wang et al. 2009
B. adolescentis	BALB/c mice	2×10^8 cfu daily	12 days	Prevented weight loss, ↓ DAI score, MPO activity and IL-1β mRNA levels and ↑ IL-4 mRNA levels in the colon	Chen, Wang et al. 2009
E. faecalis	BALB/c mice	2×10^8 cfu daily	12 days	Prevented weight loss, ↓ DAI score, MPO activity and IL-1β mRNA levels and ↑ IL-4 mRNA levels in the colon	Chen, Wang et al. 2009
Clostridium butyricum	BALB/c mice	2×10^8 cfu daily	12 days	Prevented weight loss, ↓ DAI score, MPO activity and IL-1β mRNA levels and ↑ IL-4 mRNA levels in the colon	Chen, Wang et al. 2009
B. infantis	IBS (n= 362)	1×10^6 cfu/mL	4 weeks – multicenter, double-blind RCT	No change	Whorwell, Altringer et al. 2006
	IBS (n= 362)	1×10^8 cfu/mL	4 weeks – multicenter, double-blind RCT	Relieved abdominal pain	Whorwell, Altringer et al. 2006
	IBS (n= 362)	1×10^{10} cfu/mL	4 weeks – multicenter, double-blind RCT	Coagulated becoming resistant to acid and agitation	Whorwell, Altringer et al. 2006
L. acidophilus	IBS (n = 40)	2×10^9 cfu/mL	4 weeks – double-blind RCT	Relieved abdominal pain and associated symptoms	Sinn, Song et al. 2008

L. plantarum	IBS (n = 40)	1×10^{10} cfu/day	4 weeks – double-blind RCT	Relieved pain, constipation and flatulence	Niedzielin, Kordecki et al. 2001
Saccharomyces boulardii	CD (n = 34)	1.2×10^{9} cells/day	3 months – double-blind RCT	↑ intestinal permeability	Garcia Vilela, De Lourdes De Abreu Ferrari et al. 2008
B. infantis 35624	IBS (n= 77)	1×10^{10} live bacteria cells	8 weeks – single blind RCT	Lower composite score and restored *in vitro* production of IL-10 and IL-12 by PBMCs	O'Mahony, McCarthy et al. 2005
L. salivarius UCC4331	IBS (n= 77)	1×10^{10} live bacteria cells	8 weeks – single blind RCT	Lower composite score only at week 2	O'Mahony, McCarthy et al. 2005
Escherichia coli and E. faecalis	IBS (n = 297)	3.0 to 9.0 $\times 10^{7}$ cfu/day	8 weeks – double-blind RCT	Improved global symptom score and abdominal pain	Enck, Zimmermann et al. 2008
B. longum, L. acidophilus and *S. thermophilus*	IBS (n = 116)	1×10^{10} cfu/day	4 weeks - double-blind RCT	Strong placebo effect but relieved abdominal pain	Drouault-Holowacz, Bieuvelet et al. 2008
S. thermophilus, L. bulgaricus, L.	D-IBS (n = 42)	2.6×10^{10} cfu/day	4 weeks – single blind RCT	Small bowel permeability ↓, improved IBS scores, abdominal pain and flatulence	Zeng, Li et al.

acidophilus and *B. longum*					2008
VSL#3	D-IBS (n = 25)	4.5 × 10^{11} bacteria/day	10 weeks - double-blind RCT	Relieved abdominal bloating	Kim, Camilleri et al. 2003
VSL#3	B-IBS (n = 48)	4.5 × 10^{11} bacteria/day	4 and 8 weeks – double-blind RCT	Reduced flatulence scores and slowed colonic transit. Cytokines below detection level	Kim, Vazquez Roque et al. 2005
VSL#3	IBS	4.5 or 9.0 × 10^{11} bacteria/day	6 weeks – double-blind RCT cross over design	↑ IBS symptoms, ↓ abdominal pain/discomfort, ↓ abdominal bloating/gassiness and ↓ disruption of family life	Guandalini et al.
L. rhamnosus GG, *L. Rhamnosus* Lc705, *Propionibacterium freudenreichii* ssp. *Shermanii* JS & *B. Animalis* ssp. *Lactis* Bb12	IBS (n = 86)	4.8 × 10^{9} cfu/day	5 months – double-blind RCT	Improved IBS scores. Cytokines largely below level of detection	Kajander, Myllyluoma et al. 2008
L. reuteri	IBS (n = 54)	2 × 10^{8} cfu/day	6 months – double-blind RCT	Improvement in IBS symptoms but also placebo effect	Niv, Naftali et al. 2005
L. GG	CD (n = 45)	1.2 × 10^{10} cfu/day	1 year – double-blind RCT	Unsuccessful in reducing the severity of CD	Prantera, Scribano et al. 2002

Abbreviations: *L.*, *B.*, cfu, ↑, ↓, TNF, IL, KO – see table 1; IFN, mRNA – see table 2; MPO, DAI, RCT – see table 3; *H.* = helicobacter; E. = enterococcus CD = Crohn's disease; IBD = inflammatory bowel disease; IBS = irritable bowel syndrome; D-IBS = diarrhea predominant irritable bowel syndrome; PBMC = peripheral blood mononuclear cell.

Chapter 2

Publication 1

A review of the pharmacobiotic regulation of gastrointestinal inflammation by probiotics, commensal bacteria and prebiotics

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Inflammopharmacology

REVIEW

A review of the pharmacobiotic regulation of gastrointestinal inflammation by probiotics, commensal bacteria and prebiotics

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Abstract The idea that microbes induce disease has steered medical research toward the discovery of antibacterial products for the prevention and treatment of microbial infections. The twentieth century saw increasing dependency on antimicrobials as mainline therapy accentuating the notion that bacterial interactions with humans were to be avoided or desirably controlled. The last two decades, though, have seen a refocusing of thinking and research effort directed towards elucidating the critical inter-relationships between the gut microbiome and its host that control health/wellness or disease. This research has redefined the interactions between gut microbes and vertebrates, now recognizing that the microbial active cohort and its mammalian host have shared co-evolutionary metabolic interactions that span millennia. Microbial interactions in the gastrointestinal tract provide the necessary cues for the development of regulated pro- and anti-inflammatory signals that promotes immunological tolerance, metabolic regulation and other factors which may then control local and extra-intestinal inflammation. Pharmacobiotics, using nutritional and functional food additives to regulate the gut microbiome, will be an exciting growth area of therapeutics, developing alongside an increased scientific understanding of gut-microbiome symbiosis in health and disease.

Keywords Pharmacobiotics · Gastrointestinal · Inflammation · Probiotics · Commensal · Bacteria · Prebiotics · Symbiotics

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Introduction

All body mucosal and extra-mucosal tissue sites that include the GIT, mouth, hair, nose, ears, vagina, lungs, and skin have their own unique microbiomes (Dominguez-Bello et al. 2010). There are purported to be thousands of bacterial species in the GIT and the number residing within the body of the average healthy adult human is estimated to out number human cells by a factor of 10 to 1 (O'Hara and Shanahan 2006). The microbiota within the human distal GIT are the largest body community and it provides an excellent millieu to investigate inflammatory processes. Recent evidence suggests that the bacterial load and the products of the intestinal microbiota might positively influence inflammatory disease pathogenesis (Wen et al. 2008; Mazmanian et al. 2008).

The bacteria that colonize the GIT perform a number of functions that include (1) regulating the normal development and function of the mucosal barriers (Xu and Gordon 2003); (2) assisting the maturation of immunological tissues, which in turn promotes immunological tolerance to antigens from foods/environment or potentially pathogenic organisms (Berg and Savage 1975); (3) controlling nutrient uptake and metabolism (Mazmanian et al. 2005;

Rakoff-Nahoum et al. 2004), and (4) preventing the propagation of pathogenic micro-organisms (Tappenden and Deutsch 2007). Changes in the profile of resident GIT bacteria may reduce their beneficial functions and affect the regulation of GI immune and inflammatory responses. Hence, in addition to its inherited constitution of genes, the GIT microbiota and the effects of the environment may constitute prime factors in the causation of disease, as alluded to more than half a century ago (Pickering 1950).

The GIT, immunological tolerance and the control of inflammation

Pre-birth mammalian young are sterile in utero. Therefore, could a germ-free GIT be desirable and could it be conducive to better health outcomes, significantly reducing the risk of disease such as inflammatory bowel diseases or large bowel cancer? Although this notion may provide a useful hypothesis for further thought, this premise is beyond the scope of this review. Given that mammals on this planet engage with bacterial species throughout a lifetime in vivo studies with germ-free animals tend to suggest otherwise (Cert-Bensussan and Gaboriau-Routhiau 2010). Comparative studies between mice that were raised in conventional versus germ-free environments highlight the importance of the intestinal microbiota for the development of the peripheral immune system in immunocompetent hosts. Most notably, the spleens of germ-free mice contain fewer and smaller germinal centers (Bauer et al. 1963) and decreased numbers of memory CD4$^{?}$ T cells, and cytokine production by these T cells shows a TH2-type profile (Mazmanian et al. 2005). Moreover, a balanced microbiome prevents the growth of disease-causing bacteria within the intestine. The GIT microbiota produce vitamins (e.g. vitamin K) and are also important for maintaining the muscular activity of the small intestine. The bacteria that colonize the adult human GIT hence functions collectively as a metabolic organ (Backhead et al. 2007) and within this evolutionary paradigm, the development of an immune-metabolic-competent host may be a necessary response for survival.

The environmental/microbiological picture, hence, is that from the time of birth, there is an assailment and colonization of all mucosal surfaces and the skin with bacteria that triggers the natural development and maturation of the immune system. Specifically studies reveal that the functions of the human GIT immune system are only partially encoded in the host's genes and that cues are required from the symbiotic microbial cohort for its full development (Hooper 2004). The microbiota that colonize the human GIT exhibit a high phylogenetic diversity reflecting their immense metabolic potential. How bacteria colonize the GIT provides initial clues as to the cues the GIT needs to develop a regulated immuno-metabolic-competent profile.

Up-regulated immune responses in an individual are necessary to clear the GIT of pathogenic cells. The immune system achieves this by initiating a pro-inflammatory response. The microbiota act partly in an immune-surveillance role by detecting pathogenic bacteria, stimulating the immune system and subsequently initiating an appropriate eradicative inflammatory response (Eckmann 2006). Once the pathogenic cells are cleared, anti-inflammatory signals are switched on to restore the pro-inflammatory response back to a normal level. Accordingly, the healthy gut is in a constant state of regulated inflammation. The role that microbiota play in triggering the anti-inflammatory response is still unclear. Failure to re-regulate inflammatory responses can increase the risk of developing inflammatory conditions of the host's gut architecture such as inflammatory bowel diseases (IBD) or irritable bowel syndrome (IBS).

Accumulating evidence indicates that the balance of commensal[1] bacteria within the GIT may be associated with the development of some GI disorders (Swidsinski et al. 2002). Patients IBD or IBS have been reported to present with increased pro-inflammatory or potentially pathogenic bacterial species such as Bacteroides (Swidsinski et al. 2002), Escherichia coli (Mylonaki et al. 2005; Martin et al. 2004), Enterococci and decreased bifidobacteria and lactobacilli species (Giaffer et al. 1991; Van de Merwe et al. 1988). The etiology of IBD is not fully understood, but is considered to be a T-cell-driven inflammation resulting from a persistent preponderance of pro- over anti-inflammatory cytokine production (Hvas et al. 2007).

Crohn's disease (CD) and ulcerative colitis (UC) are the two main types of IBD. CD is driven by T-helper 1 (TH1) immune responses (Matsuoka et al. 2004; Fuss et al. 1996), and can affect any part of the GIT, i.e. from the mouth to the anus. By contrast, UC is T-helper 2 (TH2) driven, and is restricted to the mucosa of the colon and rectum (Heller et al. 2005; Fuss et al. 1996) (Fig. 1). IBS is a functional bowel disorder affecting mostly the large intestine with the prime symptoms including abdominal pain, diarrhoea and constipation.

The hygiene hypothesis and GIT inflammation

The past six decades have seen a significant increase in the prevalence of autoimmune diseases (Mackay et al. 2001; Sironi and Clerici 2010). This was the catalyst that led to the formulation of the hygiene hypothesis. Over the passt two decades, the hygiene theory has been tested and

[1] Literally meaning eating at the same table.

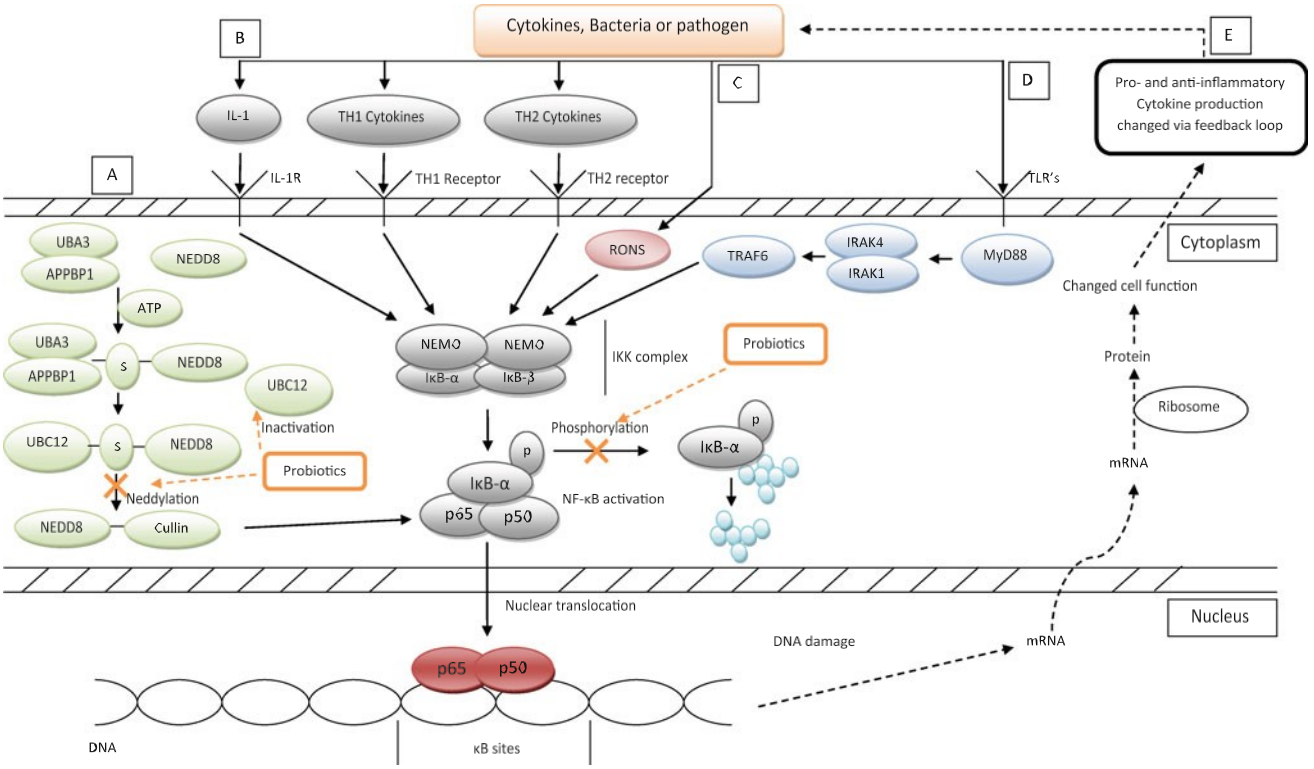

Fig. 1 Inflammatory signalling pathways of a single epithelial cell. a The NEDD8 pathway leading to binding of NEDD8 and cullin and subsequent activation of the NF-**j**B pathway. b Example of one of the cytokine pathways and its receptor. c Activation of NF-**j**B via an imbalance in RONS. d MyD88 pathway activated by pathogens binding to TLR leading to the IKK complex and NF-**j**B pathway. e Translocation of the p50 and p65 subunits results in changes to the cells function, which may provide positive or negative feedback to the extracellular space. NF-**j**B activation may result in the feedback changing the cytokine response to regulate the inflammatory response in a positive or negative way. In cases of IBD, one or more of these pathways can be dys-regulated leading to excessive inflammation due to signals. Probiotics may reduce or prevent the resulting inflammation by: (1) blocking or reducing the binding of NEDD8 to cullin via regulated production of ROS inactivating UBC12 and (2) Prevent or reduce the phosphorylation of I**j**B-a, preventing the translocation of the p50 and p65 subunits to the nucleus. This figure was constructed from the published data of Collier-Hyams et al. 2005, Wu et al. 2005, Kabelitz et al. 2006, Kumar et al. 2007, Ivison et al. 2010, Hooper and Macpherson 2010). IL interleukin, RONS reactive oxygen and nitrogen species, TH T-helper, TLR toll-like receptor, NEMO NF-**j**B essential modulator

tweaked, expanded and extended (Sironi and Clerici 2010). This hypothesis provides a biologically plausible explanation for the trend that implicates diminished exposure in early childhood to those commensal infections that boost immune defenses. This deficit subsequently enhances the risk, for later life, of GIT inflammatory problems that disrupt normal/regulated GIT inflammatory responses and increases the susceptibility to developing autoimmune diseases (Bach 2002). The hypothesis proposed that there was reduced exposure to infections in early childhood owing to a combination of diminishing family size and better personal hygiene, which might then increase the risk of developing allergic diseases (Bach 2002). The interface of the microbial environment with the innate immune system could be significantly modulated so that its ability to impart instructions to adaptive/regulatory immune/ inflammatory responses would be adversely affected, par- ticularly when such interactions occurred in utero and/or were presaged in early life. Bach (2002) documented this in part to an over production or lack of termination of pro- inflammatory cytokine trend highlighting that an epidemic of both GI autoimmune diseases in which the immune response was dominated by Th1 cells (such as type 1 diabetes mellitus, CD, multiple sclerosis) and allergic diseases in which the immune response was dominated by Th2 cells (such as asthma, allergic rhinitis, and atopic dermatitis) was becoming increasingly prevalent in Western communities.

Evolution has naturally endowed the human species with immune/inflammatory regulatory mechanisms activated by the interactions with both the external and internal microbial environments (Ley et al. 2008). These then serve to fine-tune both Th1 and Th2 antigen-driven effector responses (Wills-Karp et al. 2001). The innate immune system senses the environment and accordingly modulates the T regulatory arm, the ultimate keeper of the balance between antigen tolerance and responsiveness. The efficiency of the regulatory interface in its current state would paradoxically be jeopardized by a decrease in the microbial burden that the immune system has co-evolved with (Wills-Karp et al. 2001).

Studies exploring the molecular mechanisms that might underpin the hygiene hypothesis have focused mostly on the interactions between bacterial products and Toll-like receptors (TLRs)—the main transducers of microbial signals to the innate immune system and critical regulators of CD4 T-cell activation and regulation (O'Neill 2006; Pasare and Medzhitov 2004). Therapeutically, a recent review has highlighted how in those individuals with chronic helminth infections there is often an association with a reduced prevalence of inflammatory disorders, including allergic diseases (Hussaarts et al. 2011). Mechanistically, it was reported that by inducing or expanding regulatory B cells with helminths may open novel avenues for the treatment of inflammatory diseases, such as allergic asthma (Hussaarts et al. 2011).

Probiotics and IBD

Probiotics are living organisms in food and dietary supplements that upon ingestion can improve the health of the host beyond their inherent basic nutritional content (Fuller 1989). CD and UC, collectively referred to as IBD, are chronic aggressive disorders with a prevalence of approximately 0.1–0.5% in Western countries. Probiotics may have a significant benefit in preventing and treating IBD.

Environmental factors such as the composition and metabolic activity of the gut flora, immune system reactivity and genetic factors are all believed to play a role in the progression of IBD states (Lakatos et al. 2006). Clinical observations suggest that certain intestinal and extra-intestinal bacterial infections may perhaps precede or reactivate chronic intestinal inflammation. A number of microbial agents have been implicated as initiating factors in the pathogenesis of IBD, including Mycobacterium paratuberculosis, measles virus, Listeria monocytogenes, and adherent E. coli (Sartor 2005). However, results implicating any single micro-organism in the etiology of IBD are equivocal. Moreover, recently it was reported that subjects diagnosed with IBD were more likely to have been prescribed antibiotics 2–5 years before their diagnosis (Shaw et al. 2011). This then suggests that antibiotic administration may be implicated as a predisposing factor in IBD etiology. The plausible mechanism for causality is the disruption of the GIT microbiome.

One mechanism by which pathogenic micro-organisms may drive intestinal inflammation in susceptible individuals is via disruption of the mucosal barrier. This could then lead to an increased uptake of luminal antigens or mimics of self-antigens and activate the mucosal immune system via modulation of transcription factors such as NFkB (Sartor 2006) (Fig. 2) by sustaining an up-regulating activity.

Data from in vitro studies, from experiments using animal models of intestinal inflammation, and clinical trials have all suggested a critical role for normal luminal bacteria in the pathogenesis of IBD (Sartor 2006).

The most compelling evidence for the interactive role of bacteria, immune system and genes has been derived from experimental animal models of both Crohn's-like and colitis-like disease (Duchmann et al. 1999; Mow et al. 2004; Sartor 2006). There are more than 20 different spontaneously occurring or genetically engineered (either the knockout type or transgenic) animal models of IBD (Sartor 2006). Colonization with an enteric flora is required for full expression of disease. Thus, the normal flora is a common factor driving the inflammatory process irrespective of the underlying genetic predisposition and immunological effector mechanism. A recent phenotype/genotype investigation demonstrated that in a subset of patients with CD and UC, there was an altered intestinal-associated microbial compositions (Frank et al. 2011). Hence, it is possible that changes in the GIT microbiota/microbiome profile in individuals with specific susceptible genotypes may lead to adverse inflammatory profiles. A recent review has documented the results of clinical trials that investigated the efficacy of probiotics (single, multiple strains and probiotic blends with prebiotics) (Table 1) in CD, UC and pouchitis (Mack 2011). The review concluded that there was little evidence for the benefit of currently used probiotic microbes in CD or associated conditions affecting extra-intestinal organs. However, the review also reported that clinical practice guidelines in Canada were now including a probiotic as an option for recurrent and relapsing antibiotic sensitive pouchitis. Further, the use of probiotics in mild UC was provocative and suggested a potential for benefit in selected patients (Mack 2011).

Rescue of an disrupted GIT microbiome may depend on the introduction of multi-strain probiotics rather than single strains. Given the extensive array of micro-organisms that inhabit the GIT, probiotic mixtures may be a more biologically plausible therapeutic option in rescuing GIT microbiome functionality. Recently, it was reported that multi-strain probiotics are more effective against a wide range of end points (Chapman et al. 2011). Chapman et al. (2011) also reported that based on a limited number of studies, multi-strain probiotics may show greater efficacy than single strains, including strains that are components of the mixtures themselves. Altering the GIT microbiome/microbiota through pharmacological or nutritional means has the potential to influence the onset, progression and recovery from inflammatory disorders of the GIT.

Influence of probiotic therapies

A number of reports describe the influence of probiotic supplements on colitis in animal trials. In particular, the IL-

Table 1 Clinical studies reported for probiotics [adapted and updated from Vitetta and Sali (2008)]

Probiotics			
Conditions and symptoms	Probiotic supplement source and associated bacterial strains L = Lactobacillus strains B = Bifidobacterium strains	Level of evidence[a]	References
Diarrhoea from antibiotic use for bacterial infections	L. rhamnosus GG	I	Hawrelak et al. (2005)
	L. reuteri MM53	II	de Vrese and Marteau (2007)
	L. reuteri ATCC 55730		Frohmader et al. (2010)
	VSL#3		Guandalini (2011)
			Hickson (2011)
			Cimperman et al. (2011)
			Johnston et al. (2011)
Atopic eczema			Kalliomäki et al. (2001, 2003); Kajander et al. (2008)
Prevention	L. rhamnosus GG	I	Giovannini et al. (2007)
Treatment	L. fermentum PCC	II	Kalliomäki et al. (2001, 2003, 2007)
		II	Betsi et al. (2008)
Allergies			Majamaa and Isolauri (1997)
Food allergy	L. rhamnosus GG	III	Mukerji et al. (2009)
Rhinosinusitis	L. rhamnosus R0011		
Bacterial gastroenteritis			Saavedra et al. (1994)
Vancomycin resistant enterococci	L. rhamnosus GG	II	Shornikova et al. (1997)
	L. rhamnosus GG	III	Lomax and Calder (2009)
C. difficili			Rohde et al. (2009)
Colic	L. reuteri DSM17938	II	Savino et al. (2007)
			Savino et al. (2010)
Chronic fatigue syndrome	L. paracasei ssp.	III	Rao et al. (2009)
	L. paracasei F19	IV	Sullivan et al. (2009)
	L. acidophilus NCFB 1748		Lakhan and Kirchgessner (2010)
	B. lactis Bb12		
	L. casei strain Shirota		
Constipation	In adults: effective strains		Chmielewska and Szajewska (2010)
	B. lactis DN-173 010, L. casei		
	Shirota Escherichia coli Nissle 1917		Tabbers et al. (2009)
			Quigley (2011)
	In children: effective strains	I/II	
	L. casei rhamnosus Lcr35	II	
	B. lactis DN-173 010		
Helicobacter pylori infection	L. reuteri MM53	II	Cremonini et al. (2002)
	L. acidophilus La5	III	Lesbros-Pantoflickova et al. (2007)
	B. lactis Bb12		Francavilla et al. (2008)
			Mourad-Baars et al. (2010) (ineffective in children)

Table 1 continued

Probiotics			
Conditions and symptoms	Probiotic supplement source and associated bacterial strainsL = Lactobacillus strainsB = Bifidobacterium strains	Level of evidence[a]	References
Irritable bowel syndrome	L. acidophilus	II	Niedzielin et al. (2001)
	L. plantarum	II	Madden and Hunter (2002)
			Whorwell et al. (2006)
	L. rhamnosus		Drouault-Holowacz et al. (2008)
	B. breve B. lactis		Ki Cha et al. (2011)
	B. longum		
	S. thermophilus		
Inflammatory bowel syndrome	B. breve	II	Friedman and George (2000)
	B. bifidum	II	Gionchetti et al. (2000a, b) UC
	L. acidophilus	II	Fedorak and Dieleman (2008)
CD	L. acidophilus		Hammer (2011)
Pouchitis	L. paracasei		Mack (2011)
	L. bulgaricus		(refer text forefficacy outcomes)
	B. breve		
	B. longum		
	B. infantis		
	S. thermophilus		
	L. fermentum PCC		
	L. plantarum 299V		
	VSL#3		
Immunity decreased—reducing rates of infections:			Kontiokari et al. (2001)
	L. rhamnosus GG	I	Hatakka et al. (2001)
URTIs	L. rhamnosus GR-1	II	Falagas et al. (2006)
Candidiasis	L. reuteri RC-14	II	Forestier et al. (2008)
HIV	B. lactis HN019	III	Hao et al. (2011)
Day care infections in children	L. acidophilus La5		Hummelen et al. (2011)
	L. plantarum CECT 7315		Mañé et al. (2011)
ICU infections			Davidson et al. (2011)
Adjuvant to influenza			
Vaccine—improve efficacy			
Prevention of preterm necrotizing enterocolitis	L. bifidus,	I	AlFaleh and Bassler (2010)
	S. thermophillus		
	B. infantis		
Prevention of:	Probitics—Yakult	II	Twetman and Stecksen-Blicks (2008)
			Näse et al. (2001)
Dental caries	L. rhamnosus GG	III	Slawik et al. (2011)
Gingival inflammation			Koduganti et al. (2011)
Periodontal disease			
Radiotherapy-induced diarrhoea	VSL#3[b]	II	Delia et al. (2007)
Chemotherapy—	L. casei (Yakult Honsha)	III	Naito et al. (2008)
Prevention of recurrence–	F. prausnitzii	I	Prisciandaro et al. (2011)

Table 1 continued

Probiotics			
Conditions and symptoms	Probiotic supplement source and associated bacterial strainsL = Lactobacillus strainsB = Bifidobacterium strains	Level of evidence[a]	References
Bladder cancer (combined with epirubicin)	L. plantarum		
	B. bifidum Yakult		
	B. breve Yakult		
Chemotherapy-induced intestinal mucositis	S. boulardi		
	B. infantis		
	L. rhamnosus GG		
	L. plantarum LP31		
	L. plantarum 423		
	L. johnsonii NCC533 Ecologic®		
	641		
	L. fermentum CECT571		
	VSL#3		
	L. acidophilus		
	L. plantarum 299V		
Ulcerative colitis			
Inducing remission	VSL#3	III	Friedman and George (2000)
Maintenance/remission	L. rhamnosus GG35/VSL#3	II/III	Gionchetti et al. (2000a)
Prevention	VSL#3	II	Gionchetti et al. (2003)
Pouchitis	L. rhamnosus GG36	III	Mimura et al. (2004)
Viral gastroenteritis	L. casei Shirota	II	Lin et al. (2009)
Prevention	L rhamnosus GG	II	Nagata et al. (2011)
Treatment	B. lactis Bb12	I	
	L. rhamnosus GG	II	
	L. reuteri MM53		

[a] Level I: from a systematic review of all relevant randomised controlled trials—meta-analyses, Level II: from at least one properly designed randomised controlled clinical trial, Level III: from one or more well-designed pseudo-randomised controlled trials (alternate allocation or some other method), Level IV: opinions of respected authorities based on clinical experience, descriptive studies or reports of expert committees

[b] VSL#3, a multi-strain product composed of—B. longum, B. infantis, B. breve, L. acidophilus, L casei, L. plantarum, L. delbruekii ssp. bulgaricus, S. thermophilus

10 knockout mouse has been extensively studied because it develops colitis when colonized with a conventional flora but remains disease-free when maintained under germ-free conditions (Paul et al. 2011).

Although it is unclear whether the abnormal composition of the enteric flora contributes to the pathogenesis of IBD, evidence from clinical observations (e.g., from evidence that antibiotics are effective in certain patients) (Talley et al. 2011) has prompted the examination of a wide variety of probiotic strains in the treatment of IBD (Cain and Karpa 2011).

Probiotics have also been used to treat patients with existing IBS (Cain and Karpa 2011). The efficacy was assessed by comparing abdominal pain and discomfort, symptom scores for bowel habit satisfaction, flatulence and production of C-reactive protein (CRP) and cytokines (IL-2, IL-4, IL-6, IL-10, IL-12, IFN-c and TNF-a,) before and after probiotics treatment (Lee and Bak 2011).

One of the problems with probiotic therapy is finding and administering the optimal dose. As each probiotic strain may require a different dose and the different disease aetiologies may require different doses, finding the 'ideal' dose is complex. Whorwell et al. (2006) investigated the effects of three different doses of B. infantis 35624 ($1 \cdot 10^6$, $1 \cdot 10^8$ and $1 \cdot 10^{10}$ cfu/ml) in treating primary-care IBS patients. The dose of $1 \cdot 10^8$ cfu/ml proved superior in relieving abdominal pain compared with the placebo and other doses. Further investigation of the highest dosage demonstrated that the probiotics "coagulated" into a firm glue-like mass making them resistant to acid and agitation. The lowest dose of probiotics may not have been effective because of the duration of the study, or

insufficient biological activity. These findings highlight the potential importance of how probiotics are administered to maximize bioavailability within the GIT.

To assess the efficacy of probiotics, improvements in disease symptoms are monitored. Probiotics have been shown to reduce abdominal pain and discomfort and symptom scores such as when patients with IBS were administered L. acidophilus (Sinn et al. 2008), L. plantarum 299V (Niedzielin et al. 2001) or ProSymbioflor (a combination of E. coli DSM 17252 and E. faecalis DSM 16640) (Enck et al. 2008) compared with a placebo. In contrast, Drouault-Holowacz et al. (2008) found that B. longum LA 101, L. acidophilus LA 102, L. lactis LA 103 and S. thermophilus LA 104 were not superior to the placebo treatment for relieving disease symptoms, due to a strong placebo[2] effect. Probiotic supplementats did, however, significantly relieve abdominal pain to a greater extent compared with placebo treatment. Further analysis of the IBS sub groups revealed that patients with changing bowel habits (alternations in bowel habits and short durations of symptom exacerbation and remission) reported significantly less abdominal pain, and patients with constipation predominant IBS reported less constipation. These results indicate that different disease etiologies may exist between IBS sub groups and that some probiotics may be more efficient than others for treating symptoms within these sub groups. These findings also point to the need to further classify patients into relevant sub groups whenever possible for assessing the efficacy of a probiotic.

A number of studies investigating the effects of probiotics within specific subgroups of IBS have shown the beneficial effects. Thus, Zeng et al. (2008) first separated patients with IBS into sub groups (those with increased small bowel permeability and those with increased colonic permeability), treating diarrhoea-predominant IBS patients with S. thermophilus, L. bulgaricus, L. acidophilus and B. longum. The proportion of patients with increased small bowel permeability (lactulose/mannitol ratio ▛0.025) decreased significantly (P ⧹ 0.023) after treatment. These patients also showed improvements in their IBS score diminished abdominal pain and flatulence. Similarly, the symptoms were relieved after treatment with the probiotic VSL#3 in subjects with either diarrhoea-predominant IBS (Kim et al. 2005) or IBS with bloating (Kim et al. 2003). In subjects with IBS with bloating, the VSL#3 reduced flatulence scores and retarded colonic transit time, without altering bowel function. In patients with diarrhoea-predominant IBS, the VSL#3 only relieved abdominal bloating, having no effect on mean transit measures, bowel function scores or satisfactory relief of symptoms. VSL#3 has also been shown to be superior to a placebo in children with IBS. VSL#3 supplementation improved overall IBS symptoms as assessed by abdominal pain/discomfort, abdominal bloating/gassiness and on family life disruption (Guandalini et al. 2010).

To date, monitoring disease symptoms has been usually used to assess the efficacy of probiotics in IBS and IBD patients. These subjective measures, usually self-assessed by the patients, have provided slight indications of the underlying mechanisms of probiotics or the disease etiology. In an attempt to understand the physiological actions of probiotics, Kajander et al. (2005) administered patients with IBS the mixture of probiotic supplements containing LGG, L. rhamnosus LC705, B. breve Bb99 and P. freudenreichii ssp. shermanii JS (Kajander et al. 2005) or L. rhamnosus GG, LGG, L. rhamnosus Lc705 (DSM 7061), P. freudenreichii ssp. Shermanii JS (DSM 7067) and B. animalis ssp. lactis Bb12 (Kajander et al. 2005). Serum CRP and pro-inflammatory and anti-inflammatory cytokine (IFN-c, TNF-a, IL-2, IL-4, IL-6 and IL-10) concentrations were generally below the limit of detection and, therefore, did not indicate any differences between the treatment groups (Kajander et al. 2008). Both studies did, however, report an improvement in the IBS scores from baseline, particularly for distention and abdominal pain. The IBS score had at 5 months decreased by 14 points (95% CI −19 to −9) with the multispecies probiotic versus 3 points (95% CI −8 to 1) with placebo (P = 0.0083). Moreover, the study also reported that there was a stabilization of the microbiota. As the microbiota similarity index increased with the probiotic supplementation (1.9 ± 3.1), it decreased with placebo (−2.9 ± 1.7).

In contrast to the benefits of probiotics for relieving IBS, symptoms other studies have found few or no beneficial effects of probiotics. O'Mahony et al. (2005) found disparate effects when providing L. salivarius UCC4331, B. infantis 35624 or a placebo to subjects with IBS and to healthy volunteers. Following supplementation, the composite score (weeks 1–8), pain/discomfort (at weeks 1, 2, 4, 5, and 7), bloating/distention (at weeks 2, 5, and 6) and difficulty with bowel movements (at weeks 2, 3, 5, and 6) were generally lower in the B. infantis group than in the placebo (malted milk drink) group. Composite score was only lower in the L. salivarius group compared with the control group in the second week of supplementation, indicating that the effects of L. salivarius were short-lived and intermittent. In vitro production of IL-10 and IL-12 by isolated mononuclear cells [peripheral blood mononuclear cells (PBMC)] from whole blood was a pro-inflammatory profile at baseline in patients with IBS. Patients with IBS had low levels of IL-10 and high levels of IL-12 synthesis

[2] A placebo is a substance containing no medication benefit and prescribed to reinforce a patient's expectation of possibly attaining a beneficial effect.

compared with healthy volunteers. Notably, however, supplementation with B. infantis restored IL-10 and IL-12 synthesis to levels similar to those observed in healthy volunteers.

To understand the apparent lack of effect of probiotics in some clinical studies, it is essential that the placebo effect or natural healing cycle needs to be objectively investigated. Niv et al. (2005) provided L. reuteri ATCC 55730 or a placebo to subjects with IBS. Following supplementation, an improvement in IBS symptoms was reported. However, a similar response occurred in the placebo group (treatment versus placebo $P = 0.0714$ and $P = 0.0971$, respectively). This may demonstrate a strong placebo effect or stimulation of the natural healing cycle of the disease allowing some IBD and IBS patients to more frequently enter periods of remission.

Overall the therapeutic effect of probiotics in human studies supports additional benefits. While some results were inconsistent, probiotics treatment generally reduced symptoms of IBS, particularly abdominal pain, and restored the balance of pro- and anti-inflammatory cytokines. Separation of IBS into sub-classes of disease activity confirmed that some probiotic strains may have effects that are symptom-specific (Fedorak and Dieleman 2008; Hammer 2011). Based on these findings, further research is required to identify more specifically the various effects of each probiotic when applied to different disease classifications. To overcome potential placebo effects and minimize inconsistent results, a greater emphasis should be placed on sample size. Future research is vital to help find how and why probiotics are effective in each specific disease state. This, then may further assist to map the aetiology of particular inflammatory conditions that serve to develop rationally designed prophylactic and therapeutic interventions.

The symbiotic control of GIT inflammation

Inflammation is an essential physiological response by body tissues to injury, chemical irritation or an assault by generally pathogenic bacteria (Mazmanian et al. 2008). Once the insult is neutralized, normal physiological function needs to be restored. In the GIT, an inflammatory response is elicited to clear pathogenic bacteria with adaptive responses by commensal and probiotic bacteria that can then subsequently reduce the inflammatory response. This, thereby promotes a regulated pro- or anti-inflammatory state, and assists in reducing the symptoms of conditions such as IBS. Figures 1 and 2 illustrate diagrammatically the complexity exhibited by the GIT in the regulation of inflammation. Research (Parassol et al. 2005; Zyrek et al. 2007) supports the notion that increased intestinal permeability resulting from the disruption of the epithelial tight junction may initiate or promote dys-regulated inflammation. Maintaining and protecting the tight junctions preserve barrier function (Nunbhakdi-Craig et al. 2002; Schneeberger and Lynch 2004). It has been demonstrated in vitro that treatment of T84 and Caco-2 cells with probiotics restored or maintained tight junction complexes, thereby restoring the epithelial barrier function in enteropathogenic E. coli-stimulated cells. Incubation with Escherichia coli Nissle 1917 or L. casei following or during enteropathogenic E. coli infection restored the integrity of the epithelial cell barrier (Parassol et al. 2005, Zyrek et al. 2007) (Fig. 2).

Commensal bacteria and vertebrate immune systems form a symbiotic relationship and have a co-evolutionary profile (such that proper immune development and function rely on colonisation of the GIT by commensal bacteria and the maturation cues elicited by the bacterial cohort.

Modification of the gut flora has strong therapeutic implications. The demonstration that commensal bacteria are not sequestered by the gut epithelium but are instead recognized by TLRs under normal steady-state conditions attests to this complexity. Indeed, the interaction of commensal bacterial products with host microbial pattern recognition receptors plays a crucial role in resistance to epithelial injury and promoting intestinal homeostasis (Rossi et al. 2011). Because mammalian TLRs recognize products of both pathogenic and commensal bacteria, they might have at least two distinct functions, namely: (1) protection from infection and (2) control of mucosal homeostasis, both of which are dependent on the recognition of microorganisms—pathogens and commensals, respectively. This dual function might explain why some of the TLR-induced gene products, such as inflammatory cytokines and chemokines, are intricately involved in both host defense and tissue repair.

Although the clinical evidence for the benefits of probiotics is equivocal, the data presented in this review indicate that probiotics provide both a prophylactic and therapeutic benefit by regulating cytokine and cell signaling pathways (Mencarelli et al. 2011). We have previously reviewed the published human studies of probiotics and of prebiotics (a nutritional supplement favoring the growth and increasing the lifespan of probiotic bacteria) and their effects on several clinical scenarios (Vitetta and Sali 2008). The beneficial effects of probiotics and prebiotics can occur when the internal human environment meets the enhanced commensal/probiotic environment throughout the digestive tract. Understanding both the bacteria–bacteria interactions and the bacteria–host interactions, especially in the distal GIT, will provide further opportunities for modulating the bacterial flora for therapeutic gain. Although the trials summarized in Tables 1 and 2

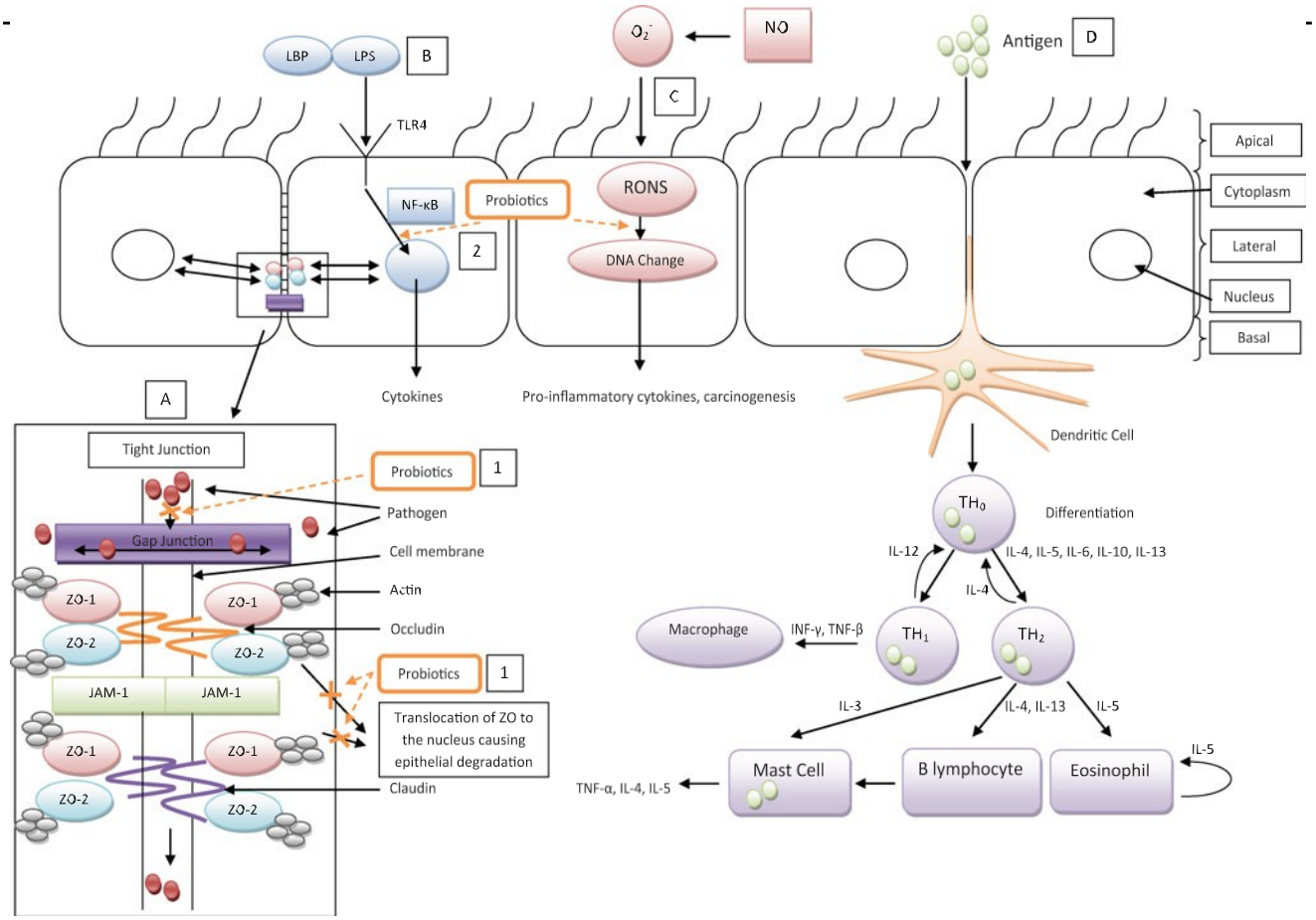

Fig. 2 Epithelial cell barrier of the gastrointestinal tract. a Tight junction and gap junction between two epithelial cells. ZO translocates away from the cell boundary towards the nucleus reducing the transepithelial resistance allowing pathogens to move between the cells and through the cell wall into the lamina propria. Once the pathogen enters the lamina propria it is able to move throughout the gastrointestinal system and systemic circulation causing sever inflammation. b LPS-mediated pathway of inflammation. c RONS-mediated induction of cytokine production and inflammation. d Antigens presenting on the epithelial surface may be detected and consumed by dendritic cells. The antigen is presented to TH0 cells which enter the TH1 or TH2 pathway depending on the antigen. Probiotics help regulate and reduce inflammation by (1) preventing the translocation of ZO to the nucleus helping maintain the TER and the integrity of gap junctions preventing the migration of pathogens past the epithelial barrier and (2) by preventing the activation of NF-**j**B caused by pathogens and compounds like LPS and RONS. This figure was constructed from the published data of Nunbhakdi-Craig et al. (2002), Schneeberger and Lynch (2004), Parassol et al. (2005), Zyrek et al. (2007). ZO-1 and -2 zolulin occludin, JAM-1 junctional adhesion molecule, LPS Lipopolisaccharides

indicate promising trends, the present consensus is that a number of larger controlled trials will be necessary before warranting the use of probiotic supplements as a routine medical treatment for numerous gastrointestinal conditions.

There is considerable public, media and scientific interest in various natural products that include probiotics and prebiotics in modulating intestinal activities. Probiotic bacteria are becoming more important in the context of human nutrition, as scientific evidence continues to accumulate on the properties, functionality, and benefits of probiotics for promoting human health. Manipulating this most complex ecosystem is challenging. This is especially evident when therapeutic interventions aim to regulate the GIT flora for the effective treatment of diseases such as irritability and inflammation of the GIT and possibly cancer of the large bowel. However, the promise is often admixed with the hype. We do not believe one probiotic will cure all diseases, but rather that probiotics are certainly an integral part of the integrative approach to health.

Prebiotics and synbiotics

The introduction of prebiotics (Table 2) in Japan and Europe as food additives justifies the need for controlled clinical studies, before prebiotics can be unequivocally recommended as a food additive for infant formulas and yogurts or as dietary supplements that should be consumed on a daily basis. No human studies have been conducted to confirm the suggested in vitro and animal study effects of

Table 2 Clinical studies reported for prebiotics and symbiotics [adapted and updated from Vitetta and Sali (2008)]

Prebiotics and symbiotics			
Conditions and symptoms	Type of prebiotic and (daily dose)	Level of evidence[a]	References
Constipation in elderly adults	Galacto-oligosaccharides (12 g/day)	III	Scheppach et al. (2001)
	Fructo-oligosaccharides (10 g)	III	Welters et al. (2002)
			Sairanen et al. (2007)
			Yen et al. (2011)
Infant weight gain	Symbiotic treatment	II	Chouraqui et al. (2008)
	B. longum BL999 (BL999) ? L. rhamnosus LPR, BL999 ? LPR ? 4 g/L of 90% galacto-oligosaccharide/10% short-chain fructo-oligosaccharide (GOS/SCFOS), or BL999 ? L. paracasei ST11 (ST11) ? 4 g/L GOS/SCFOS		
IBD	Dietary inulin 24 g/day	III	Welters et al. (2002)
Active CD	Symbiotic treatment	II	Furrie et al. (2005)
Chronic pouchitis	B. longum ? 6 g of synergy (inulin plus oligofructose mixture b.i.d. synergy 15 g/day)		Lindsay et al. (2006)
Active UC			
Prevention of atopic eczema	Galacto-oligosaccharides and Fructo-oligosaccharides	II	Kukkonen et al. (2007)
(infants on formula feeding regimens)	(0.8 g/100 ml of formula)		
Immunity decreased—reducing rates of infection	Fructo-oligosaccharides (2 g for infants)	II	Shadid et al. (2007)
Enhanced calcium absorption	Fructo-oligosaccharides (8 g)	II	Scholz-Ahrens et al. (2007)

Synbiotics are products that contain both a probiotic and prebiotic component (Bengmark and Martindale 2005; Scholz-Ahrens et al. 2007)

[a] Level I: from a systematic review of all relevant randomised controlled trials—meta-analyses, Level II: from at least one properly designed randomised controlled clinical trial, Level III: from one or more well-designed pseudo-randomised controlled trials (alternate allocation or some other method)

prebiotics on carcinogenesis. Long-term trials with prebiotics, perhaps among patients with chronic digestive diseases such as colon cancer-prone patients, would certainly be useful (de Vrese and Schrezenmeir 2008).

The efficacy of a synbiotic combination of combining prebiotics with probiotics needs to be further evaluated and quantified. Such studies could include investigations as to whether there is altered bacterial colonization in the gut following the ingestion of both prebiotics and probiotics. For example, by determining the natural adaptation of the gut to re-colonization with commensal bacteria and their growth-promoting nutrients (after pre/probiotic treatment) may establish how dietary factors could influence the pathogenesis of inflammatory diseases of the digestive system.

The FAO/WHO Expert Consultation and Working Group on probiotics presented their recommendations to Codex (Pineiro and Stanton 2007) with the hope that these will be used for a science-based risk assessment process for managerial decisions concerning probiotics. These recommendations will have major implications by limiting future health claims that can be attributed to probiotics as therapeutic preparations.

The resolution of some human diseases does not reside solely within the host but rather could involve the host's interface with the microbial environment. Manipulating the gut flora is a realistic therapeutic and prophylactic strategy for many infectious, inflammatory and neoplastic diseases within the gut. But the promise of pharmacobiotics (therapeutic exploitation of the commensal flora) is only likely to be fulfilled following greater understanding of the endogenous enteric microflora. The GIT flora is certainly a rich repository of metabolites that can be exploited for therapeutic benefit. Elucidating the molecular details of host–gut flora interactions is, therefore, a prerequisite for a bacteria derived metabolomic program of discovery that may provide novel metabolites for the control of GIT inflammation. Reports that have demonstrated that dietary fructo-oligosaccharide (neosugar) can significantly influence the feacal flora and activities of reductive enzymes (Buddington et al. 1996) certainly warrant further study.

Synbiotics are products that contain both probiotic and prebiotic components (Bengmark and Martindale 2005; Scholz-Ahrens et al. 2007). The rationale for such combination products is that together the formulation enhances the survival of probiotic bacteria in transit through the proximal GIT, improves colonization of the probiotic in the large bowel and stimulates the growth of the endogenous flora as well (Bengmark and Martindale 2005). This effect may rescue the GIT from a dys-regulated inflammatory response that may increase risk of disease.

Future research

Future directions for research may involve exploring the optimal doses of probiotics, duration of treatment, their effects in different models (in vitro and animal) of inflammatory disease and suitability as a prophylactic or therapeutic treatment. Probiotics and prebiotics can be delivered to the GIT but the proportion of ingested viable bacteria that reach the intestinal tract is not well characterized. Studies aimed at calculating the quantity of viable probiotic bacteria that reaches the upper and lower GIT may be useful. There is still a clear lack of evidence about the effect probiotics has on patients with IBD in terms of restoring the GIT microbiome profile. While alleviating the symptoms of IBD and IBS is clinically relevant, future research may also benefit from collecting colon tissue from CD and UC subjects for analysis of gastrointestinal inflammation, bacterial adhesion to normal epithelial cells and colonic crypts histology. This would certainly provide a better understanding of the underlying mechanisms of each condition and possibly lead to better strategies for treatment. Ultimately, probiotic research may also need to examine the synergistic benefits associated with individual bacterial strains that are currently used to formulate commercially available probiotic mixtures.

Mechanistically, the causal relationship between reactive oxygen species and the unbridled damage proposed to macromolecules has led to an over simplification of complex biological processes. We have previously reported that the formation of superoxide anion/hydrogen peroxide and nitric oxide does not conditionally lead to random macromolecular damage; under normal physiological conditions, their production is actually regulated consistent with their second messenger roles (Linnane et al. 2007). As for the GIT, we would expect that it too would behave in a manner that sustains a redox regulated state. Intestinal cells that maintain a redox balance preserve the environment that supports physiological processes and orchestrates networks of enzymatic reactions whereby inflammation remains regulated. Furthermore, the innate immune system presents a wide array of different receptors that can recognize specific bacterial molecular patterns. Hence an enhanced understanding of the role played by individual probiotic molecular patterns becomes crucial to evolve the current complex area of live probiotic bacteria toward improved efficacious pharmacobiotic strategies (Caselli et al. 2011).

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Chapter 2

Publication 2

Probiotics, prebiotics and the gastrointestinal tract in health and disease

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REVIEW

Probiotics, prebiotics and the gastrointestinal tract in health and disease

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Abstract The microbiome located in the human gastrointestinal tract (GIT) comprises the largest community (diverse and dense) of bacteria, and in conjunction with a conducive internal milieu, promotes the development of regulated pro- and anti-inflammatory signals within the GIT that promotes immunological and metabolic tolerance. In addition, host-microbial interactions govern GIT inflammation and provide cues for upholding metabolic regulation in both the host and microbes. Failure to regulate inflammatory responses can increase the risk of developing inflammatory conditions in the GIT. Here, we review clinical studies regarding the efficacy of probiotics/prebiotics and the role they may have in restoring host metabolic homeostasis by rescuing the inflammatory response. The clinical studies reviewed included functional constipation, antibiotic-associated diarrhoea, Clostridium difficile diarrhoea, infectious diarrhoea/gastroenteritis, irritable bowel syndrome, inflammatory bowel diseases and necrotizing enterocolitis. We have demonstrated that there was an overall reduction in risk when probiotics were administered over placebo in the majority of GIT inflammatory conditions. The effect size of a cumulative reduction in relative risk for the GIT conditions/diseases investigated was 0.65 (0.61–0.70) ($z = 13.3$); $p < 0.0001$ that is an average reduction in risk of 35 % in favour of probiotics. We also progress a hypothesis that the GIT comprises numerous micro-axes (e.g. mucus secretion, Th1/Th2 balance) that are in operational homeostasis; hence probiotics and prebiotics may have a significant pharmacobiotic regulatory role in maintaining host GIT homeostasis in disease states partially through reactive oxygen species signalling.

Keywords Microbiome · Clinical trials · Reactive oxygen species · Probiotics · Lactobacillus · Bifidobacteria · Prebiotics · Gastrointestinal tract · Inflammation · Internal environment · Nutrition

Introduction

Inflammatory reactions are defence mechanisms triggered by injury to tissues that can be prompted by either internal or external insults (Koch and Nusrat 2012). The functional interactions between the anatomical sub-structures of the gastrointestinal tract (GIT) (e.g. epithelial cell lining, mucosal tissues), the microbiota that inhabits this site and the milieu that ensues, lead to functional connections with complex metabolic outcomes.

It is reported that the products of bacterial metabolism in the GIT act as signalling molecules that impact the host's metabolic responses (Tremaroli and Bäckhed 2012). Although contentious, the idea that humans in utero are germ-free may no longer be accurate, (Jiménez et al. 2008) instead bacteria or bacterial antigen exposure at this developmental stage may occur indicating that signalling of mucosal development may actually commence in utero rather than in the neonatal stage. Furthermore, diet and medications (i.e. antibiotics) that a neonate is exposed to, in combination with the GIT microbiome in early life, may hold the key to aberrant molecular signals that predispose

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to chronic inflammatory disease development in adulthood (Neuman and Nanau 2012).

Recent analytical studies report that it is possible to classify humans into just three broad bacterial enterotypes (irrespective of whether chronic intestinal diseases are present or absent), hence these being dominated by three different genera, namely the Bacteroides and Prevotella (both belonging to the phylum Bacteroidetes) and Ruminococcus (Arumugam et al. 2011). Furthermore, it has been reported that this overall enterotype profile was conserved independently of gender, body mass index and geographical region/nationality, while notwithstanding the significant differences that exist in the long-term dietary habits between people from Western countries and those from Asian countries. In another study that investigated what linkages may exist between long-term dietary patterns with GIT microbial enterotypes, it was reported that higher fat and lower fiber intakes were associated with specific enterotypes (Wu et al. 2011). That is that the enterotypes seemed to be determined by the type of long-term diet exposure. Hence, the Bacteroides enterotype was positively related with animal protein and saturated fats, whereas the Prevotella enterotype was associated with a mostly plant-based dietary profile that consisted of high carbohydrates and low meat and dairy consumption. Further, a recent investigation demonstrated that short-term macronutrient changes in the diet, being either composed of entirely animal or plant products, substantially alter the microbial profile and microbial gene expression in humans (David et al. 2013). The authors reported that the animal-based diet increased the abundance of bile-tolerant microbes (Alistipes, Bilophila and Bacteroides) and decreased the levels of Firmicutes that metabolize dietary plant polysaccharides (Roseburia, Eubacterium rectale and Ruminococcus bromii). The reverse was true for the plant-based diet. Whereas the animal-based diet increased the abundance and activity of Bilophila wadsworthia and altered faecal bile acid profiles that are associated with IBD.

The increased consumption of fat-to-fiber ratio that occurs in Western diets has been reported to be among the major triggering factors of metabolic impairments and gut dysbiosis that can lead to obesity and type II diabetes mellitus (T2DM) (Roberfroid 2007). A recent study in mice showed that the gut microbiota could be regarded as a stamp of the metabolic phenotypes that inhabit the GIT and that this was independent of differences in host genetic make-up and dietary profile (Serino et al. 2012). This then proposes the notion that there may be a co-operative microbial–host induction of metabolic adaptation. Consequently, modifying the gut microbiota by administering appropriate dietary changes together with probiotic species and prebiotic fibers may represent a promising strategy to control or prevent inflammatory metabolic diseases of the GIT.

Methodology

A systematic search of the literature was conducted using PubMed, the Cochrane Library, Science Direct, Scopus, EMBASE, MEDLINE and CINAHL.

Search terms

Articles were identified using the search terms, “Diet” OR “Overweight” OR “Obesity” AND “Probiotics” OR “Prebiotics” OR “Commensal Bacteria” AND “Gastrointestinal Tract” and “Inflammation” AND “Crohn’s Disease” AND ‘Ulcerative colitis” AND “Irritable Bowel Syndrome” AND” Constipation” AND “Diarrhoea” AND “Gastrointestinal Infections” AND “Necrotizing Enterocolitis.” The inclusion criteria for this review were: (1) an RCT and/or cross-over clinical trial that used either a placebo comparator or other as a control published on or after the year 2000, (2) human participants diagnosed with or without GIT inflammatory conditions, (3) other epidemiological observational and mechanistic studies, (4) the clinical study was published in English; and (5) the clinical study presented data in the form of a relative risk (RR) (95 % CI) reduction (test: probiotic with or without prebiotics versus a placebo or appropriate comparator) or that

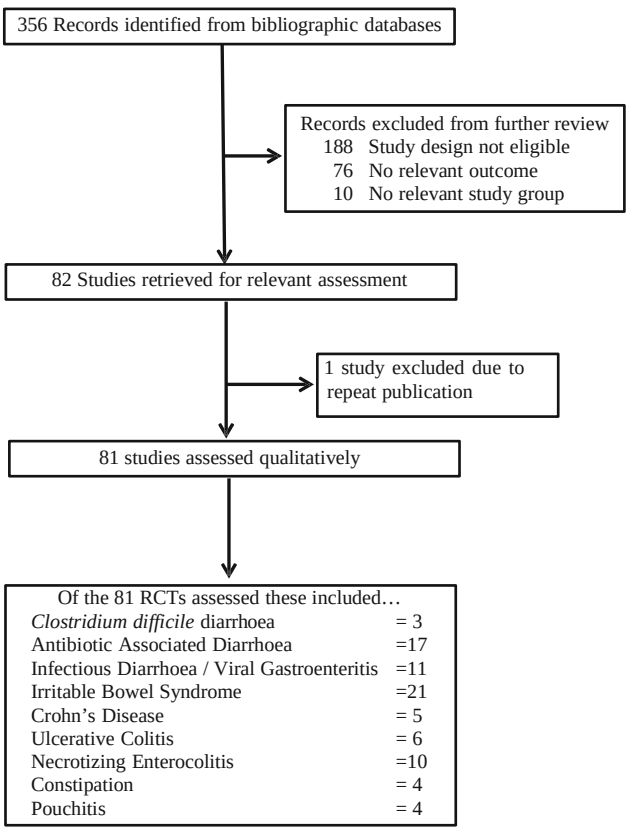

Fig. 1 Flow diagram of literature search for systematic review

Table 1 RCTs that administered probiotics with/without prebiotics reporting a benefit or otherwise for inflammatory conditions/diseases of the gastrointestinal tract as an effect size (risk ratio reduction and 95 % CI)

Conditions/diseases references	Strain(s), Dose regimen L. = Lactobacillus B. = Bifidobacterium S. = Streptococcus E. = Escherichia	Effect size RR (95 % CI)*	Outcome
Functional constipation			
Koebnick et al. 2003	L. casei (Shirota) Dose: 6.5 × 10^9 CFU/65 mL/day/ 5 weeks	0.27 (0.1–0.72)	Significant improvement in chronic constipation
Yang et al. 2008	B. lactis DN-173010 Dose: 1.25 × 10^{10} CFU/100 g of fermented milk/day/2 weeks	0.32 (0.23–0.43)	Significant improvement in stool frequency by probiotic (71 %) over control (8.3 %) in 2-week study period
Banaszkiewicz and Szajewska 2005	L. rhamnosus GG Dose: 1 mL/kg/day of 70 % lactulose plus 10^9 CFU/day/24 weeks	1.1 (0.58–1.9)*	No significant difference between treatments
Bu et al. 2007	Lcr35 8 × 108 CFU/day (250 mg/two capsules/b.i.d./4 weeks)	0.25 (0.1–0.61)	Significant improvement with probiotic over placebo
Tabbers et al. 2011[17]	B. lactis DN-173 010 Dose: 4.25 × 10^9 CFU/125 g/pot/b.i.d./ 3 weeks	0.86 (0.70–1.10)*	Non-significant ↓stool frequency over control
Mazlyn et al. 2013[14]	L. casei strain Shirota Dose: 3 × 10^{10} CFU/80 mL/day/4 weeks	0.71 (0.55–0.92)	↓severity in constipation/significant only after 4 weeks (authors noted that a longer intervention was required to properly assess this study outcome)
Antibiotic-associated diarrhoea			
Surawicz et al. 2000	S. boulardii Dose: 1 g/day (administered as 2x250 mg capsules b.i.d./4 weeks	0.33 (0.11–1.06)*	S. boulardii ? high-dose vancomycin non-significant ↓67 % efficacy prevention of CDD recurrences over high-dose vancomycin alone
Szajewska et al. 2001	L. rhamnosus GG Dose: 6 × 10^9 CFU/day/until discharged	0.2 (0.1–0.66)	Significant↓nosocomial diarrhoea
Thomas et al. 2001	L. rhamnosus GG Dose: 20 × 10^9 CFU/day/2 weeks	0.98 (0.68–1.4)*	No statistically significant difference between probiotic and placebo in reducing antibiotic-associated diarrhoea
Armuzzi et al. 2001	L. rhamnosus GG Dose: 6 × 10^9 CFU/b.i.d./2 weeks	0.01 (0.03–0.43)	↓bloating ↓diarrhoea ↓taste disturbances
Cremonini et al. 2002	L. casei subsp. rhamnosus (GG) 6 × 10^9/ sachet Saccharomyces boulardii 5 × 10^9/sachet L. acidophilus and B. lactis 5 × 10^9/ sachet Dose: administered b.i.d./2 weeks	0.17 (0.02–1.27)*	↓diarrhoea—all probiotic combinations not significantly better than placebo
Jirapinyo et al. 2002	L. acidophilus and B. infantis Dose: 10^8 CFU/b.i.d./2 weeks	0.47 (0.18–1.21)*	Non-significant ↓diarrhoea
La Rosa et al. 2003	L. sporogens ? prebiotic of Fructo-oligosaccharides Dose : 10^7 CFU/t.i.d./2 weeks	0.47 (0.29–0.77	Non-significant↓number of days and duration of events with antibiotic-induced diarrhoea

Table 1 continued

Conditions/diseases references	Strain(s), Dose regimen L. = Lactobacillus B. = Bifidobacterium S. = Streptococcus E. = Escherichia	Effect size RR (95 % CI)*	Outcome
Beniwal et al. 2003	Vanilla-flavored yogurt containing 10^6 CFU/g of L. acidophilus, L. bulgaricus, and S. thermophilus combined Dose: 227 g/b.i.d./8 days	0.52 (0.28–0.97)	Significantly reduced the incidence and duration of antibiotic-associated diarrhoea
Seki et al. 2003	Clostridium butyricum Dose: 10^7 CFU/g viable spores administered at 1–4 g/day/1 weeks	0.12 (0.05–0.28)	Effective treatment and prophylaxis for antibiotic-associated diarrhoea
Nista et al. 2004	Bacillus clausii Dose: 2 × 10^9 spores/t.d.s./2 weeks	0.88 (0.50–1.56)*	A non-significant ↓prevalence of diarrhoea
Pereg et al. 2005	L casei DN-114 001 Dose: 100 mL of yogurt with 10^8 CFU/mL/day/8 weeks (6 days/week)	0.76 (0.49–1.17)*	A non-significant trend for reduction of the incidence of diarrhoea was reported
Corrêa et al. 2005	B. lactis S. thermophilus Dose: 10^7 and 10^6 CFU, respectively, of each/day/2 weeks	0.52 (0.21–1.23)*	Non-significant ↕stool frequency and ↓stool consistency
Kotowska et al. 2005	S. boulardii Dose: 250 mg/b.i.d./duration of antibiotic treatment	0.19 (0.07–0.55)	Significant↓prevalence of diarrhoea
Wenus et al. 2008	L. rhamnosus GG L. acidophilus La-5 B. lactis Bb-12 Dose: 10^8 CFU/mL of LGG/Bb-12 and 10^7 CFU/mL La-5 administered as 250 mL/day/2 weeks	0.21 (0.05–0.93)	Significant ↓risk of antibiotic-associated diarrhoea
Frohmader et al. 2010[19]	S. thermophilus B. breve B. longum, B. infantis L. acidophilus, L. plantarum, L. paracasei, L. delbrueckii subsp. Bulgaricus Dose: 45 × 10^{10} multi–strain CFU/day/2 weeks	0.50 (0.27–0.93)	Significant↓frequency of liquid stool
Song et al. 2010	L. rhamnosus R0011 L. acidophilus R0052 Dose: 2 × 10^9 CFU/capsule/b.i.d./2 weeks	0.54 (0.17–1.74)*	Non-significant ↓antibiotic-associated diarrhoea
Cimperman et al. 2011[20]	L.reuteri ATCC 55730 Dose: 1 × 10^8 CFU/day/4 weeks	0.15 (0.02–1.11)*	Non-significant↓frequency of diarrhoea

Table 1 continued

Conditions/diseases references	Strain(s), Dose regimen L. = Lactobacillus B. = Bifidobacterium S. = Streptococcus E. = Escherichia	Effect size RR (95 % CI)*	Outcome
Clostridium Difficile diarrhoea			
Wullt et al. 2003	L. plantarum 299v Dose: metronidazole (400 mg t.i.d.) orally for 10 days in combination with either a fruit drink containing oats fermented with L. plantarum 299v (5 × 10^{10} CFU/d) or placebo (fruit drink with chemically acidified oats) once a day for 38 days.	0.55 (0.22–1.35)*	Although efficacy was not significant probiotic group had less symptom recurrence.
Plummer et al. 2004	L. acidophilus B. bifidum Dose: one capsule 2 × 10^{10} CFU/capsule/ day/3 weeks	0.33 (0.07–1.59)*	Non-significant;diarrhoea events for test over control (NS)
Lawrence et al. 2005	L. rhamnosus GG Dose: LGG 2.8 × 10^{11} CFU/capsule (40 mg lyophilized LGG and 320 mg inulin) or one placebo capsule (360 mg inulin)/t.i.d. adjunctively with anti-C. difficile antibiotics	1.53(0.54–4.35)*	No significant difference between treatments
Infectious/traveller's diarrhoea/gastroenteritis			
Guandalini et al. 2000	L. rhamnosus GG Dose: rehydration solution containing 10^{10} CFU/250 mL/until diarrhoea resolved	0.57 (0.34–0.81) 0.35 (0.12–0.59)	Significantly shorter duration of diarrhoea in retrovirus positive children Significantly reduced duration of diarrhoea
Szajewska et al. 2001	L. rhamnosus GG Dose: 2.46 g powder at 6 × 10^{9} CFU/ sachet b.i.d./for duration of hospital stay	0.13 (0.02–0.79)	Significant improvement of probiotic over control.
Chouraqui et al. 2004	B. lactis Bb 12 Dose: 10^{6} CFU/g powder resulting in 1.5 × 10^{8} CFU/L/day/on average approx. 20–21 weeks	0.94 (0.53–1.66)*	No significant difference between groups
Salazar-Lindo et al. 2004	L. casei strain GG Dose: 10^{9} CFU/mL in a milk formula of 150 mL/kg/day—maximum administered 1,000 mL	1.01 (0.46–2.21)*	No improvement in management of diarrhoea
Weizman et al. 2005	B. lactis Bb-12 or L. reuteri SD 2112 Dose: all at 1 × 10^{7} CFU/g powder/day/ 12 weeks	0.39 (0.19–0.79) 0.05 (0.01–0.34)	Both strains effective in ;diarrhoea
Margreiter et al. 2006	L. gasseri and B. longum versus Enterococcus faecium Dose: 25 mg of 2 × 10^{7}–2 × 10^{8} CFU/ capsule/t.i.d. versus 75 × 10^{6} CFU/ capsule/t.i.d.	1.9 (0.74–4.96)**	This study reported equivalent therapeutic efficacy for the 2 treatment regimens.
Grossi et al. 2010	L. paracasei B 21060 ? prebiotic Dose: 7 g sachet dissolved in water/juice of symbiotic preparation administered at 10^{11} CFU/day/10 days	0.43 (0.12–1.62)	No difference in acute diarrhoea incidence at the end of the study However, significant difference in duration of diarrhoea

Table 1 continued

Conditions/diseases references	Strain(s), Dose regimen L. = Lactobacillus B. = Bifidobacterium S. = Streptococcus E. = Escherichia	Effect size RR (95 % CI)*	Outcome
Nagata et al. 2011[41]	L. casei Shirota Dose: 4 × 10^{10} CFU/80 mL bottle/day/ 12 weeks	1.25 (0.88–1.79)*	No difference in number of cases with viral gastroenteritis between groups ; mean duration days of fever after onset significantly decreased by test over placebo
Virk et al. 2013	Synbiotic containing: 4.5 × 10^{9} CFU Enterococcus faecium, a probiotic yeast of 5 × 10^{8} CFU S cerevisiae strain CNCM I 4444 and a prebiotic fructo–oligosaccharide Dose: 2 capsules/day.	1.13 (0.87–1.5)*	No significant difference of test synbiotic over placebo
Pouchitis			
Gionchetti et al. 2000[22]	S. Thermophiles B. breve B. longum, B. infantis L. acidophilus L. plantarum L. paracasei L. delbrueckii subsp. Bulgaricus Dose: 5 × 10^{11} multi-strain CFU/g/6 g/ day/36 weeks	0.85 (0.71–1.02)	; frequency of flare-ups of chronic pouchitis effective in maintaining remission
Gionchetti et al. 2003[23]	S. Thermophiles B. breve B. longum B. infantis L. acidophilus L. plantarum L. paracasei L. delbrueckii subsp. Bulgaricus Dose: 9 × 10^{11} multi-strain CFU/day/ 52 weeks	0.25 (0.06–1.03)	; frequency of flare-ups of chronic pouchitis effective in maintaining remission
Mimura et al. 2004[24]	S. Thermophiles B. breve B. longum B. infantis L. acidophilus L. plantarum L. paracasei L. delbrueckii subsp. Bulgaricus Dose: 30 × 10^{11} CFU/g × 3/day/ 52 weeks	0.16 (0.06–0.46)	; frequency of flare–ups of chronic pouchitis—effective in maintaining remission at 1 year
Crohn's disease			
Guslandi et al. 2000	S. boulardii Dose: mesalamine 1 g t.i.d. or mesalamine 1 g b.i.d. plus a preparation of S. boulardii 1 g/day/ 24 weeks	0.17 (0.02–1.8)*	Relapse in the probiotic treatment group was 6.25 % as compared to the control 37.5 %. The low participant numbers precluded significance

Table 1 continued

Conditions/diseases references	Strain(s), Dose regimen L. = Lactobacillus B. = Bifidobacterium S. = Streptococcus E. = Escherichia	Effect size RR (95 % CI)*	Outcome
Prantera et al. 2002	L. rhamnosus GG Dose: 2.46 g satchet of 6 × 10^9/b.i.d./ 52 weeks	1.50 (0.90–2.4)*	No significant difference from placebo in disease remission or disease improvement
Schultz et al. 2004	L. rhamnosus GG Dose: 2 × 10^9 CFU/day/24 weeks	1.2 (0.1–14.7)*	No significant difference in remission between test and placebo groups. Groups with low numbers
Marteau et al. 2006	L. johnsonii, LA1, Nestle® Dose: 2 × 10^9 CFU/day/24 weeks	0.77 (0.53–1.11)*	No difference in recurrence between test and placebo groups
Van Gossum et al. 2007	L. johnsonii, LA1, Nestle® Dose: 10^{10} CFU/day/12 weeks	1.10 (0.27–4.4)*	Post elective ileo-caecal resection recurrence
Ulcerative Colitis			
Ishikawa et al. 2003	B. breve B. bifidum L. acidophilus Dose: fermented milk 100 mL of 10^9CFU/ day/52 weeks	0.30 (0.11–0.81)	Probiotic supplementation maintained remission
Kato et al. 2004	B. breve strain Yakult B. bifidum strain Yakult L. acidophilus Dose: 10^9 CFU/100 mL bottle/day/ 12 weeks	0.43 (0.15–1.2)*	While there was a greater response to the probiotic treatment the result was not significant highlighting the low participant numbers.
Kruis et al. 2004	E. coli of strain Nissle 1917 (serotype O6:K5:H1) Dose: 2.5–25 × 10^9 CFU/day/52 weeks	1.06 (0.86–1.19)*	Probiotic equal efficacy to pharmaceutical in maintaining remission
Sood et al. 2009	S. Thermophiles B. breve B. longum B. infantis L. acidophilus L. plantarum L. paracasei L. delbrueckii subsp. Bulgaricus Dose: 3.6 × 10^{12} multi-strain CFU/b.i.d./ 12 wks	0.68 (0.55–0.84)	Significant; UC disease activity index and remission in the active treatment over placebo at 6 and 12 weeks
Matthes et al. 2010	Escherichia coli strain Nissle 1917 Dose: regimes tested were 20 mL of 4 × 10^9 versus 20 mL of 2 × 10^9 versus 10 mL of 10^9 CFU versus placebo/day/8 weeks	0.87 (0.54–1.4)*	Re: in the intention to treat analysis…time to remission was shorter in the 40 mL administered group. The result was not significant highlighting the low participant numbers

Table 1 continued

Conditions/diseases references	Strain(s), Dose regimen L. = Lactobacillus B. = Bifidobacterium S. = Streptococcus E. = Escherichia	Effect size RR (95 % CI)*	Outcome
Tursi et al. 2010	S. thermophiles B. breve B. longum B. infantis L. acidophilus L. plantarum L. paracasei L. delbrueckii subsp. Bulgaricus Dose: 3.6 × 10^{12} multi-strain CFU/b.i.d./ 8 weeks	0.66 (0.45–0.97)	;UC disease activity index
Irritable bowel syndrome			
Nobaek et al. 2000	L. plantarum (DSM9843) Dose: 400 mL of 5 × 10^{7} CFU/mL/day/ 4 weeks	0.59 (0.38–0.92)	A non-significant;abdominal pain and flatulence
Niedzielin et al. 2001[29]	L. plantarum 299 V Dose: 400 mL of 5 × 10^{7} CFU/mL/day/ 4 weeks	0.10 (0.01–0.40)	Significant;abdominal pain and overall IBS symptomatology normalisation of stools frequency test versus placebo
Kim et al. 2003	S. thermophiles B. breve B. longum B. infantis L. acidophilus L. plantarum L. paracasei L. delbrueckii subsp. Bulgaricus Dose: 45 × 10^{11} multi-strain CFU/b.i.d./ 8 weeks	1.1 (0.6–1.95)*	A non-significant;bloating stool-related symptoms in diarrhoea-associated IBS
Kim et al. 2005	S. thermophiles B. breve B. longum B. infantis L. acidophilus L. plantarum L. paracasei L. delbrueckii subsp. Bulgaricus Dose: 45 × 10^{11} multi-strain CFU/b.i.d./ 8 weeks	0.77 (0.42–1.4)*	A non-significant improvement in overall symptomatology of IBS
Kajander et al. 2005	L. rhamnosus GG (ATCC 53103, LGG) L. rhamnosus Lc705 (DSM 7061), P. freudenreichii ssp. Shermanii JS (DSM 7067) B. animalis ssp. Lactis Bb12 (DSM15954) Dose: ONE cap all at 8–9 × 10^{9} multi-strain CFU/day/24 weeks	0.42 (0.23–0.77)	Significant change in total IBS symptom score including ;abdominal pain ? ;bloating/distension ? ;flatulence ? ;borborygmi

Table 1 continued

Conditions/diseases references	Strain(s), Dose regimen L. = Lactobacillus B. = Bifidobacterium S. = Streptococcus E. = Escherichia	Effect size RR (95 % CI)*	Outcome
Whorwell et al. 2006[33]	B. infantis 35624 Note 3 dose regimens were investigated and reported Dose 1: 1 × 10^6 CFU/day/4 weeks Dose 2: 1 × 10^8 CFU/day/4 weeks Dose 3: 1 × 10^{10} CFU/day/4 weeks Overall RR reduction:	0.96 (0.72–1.30)* 0.88 (0.69–1.11)* 1.16 (0.90–1.50)* 0.90 (0.76–1.10)*	Combined variable of scores for abdominal pain/discomfort, bloating, and bowel habit satisfaction Non-significant improvements ;abdominal pain ;bloating ;bowel dysfunction
Guyonnet et al. 2007	B. animalis DN-173 010 Dose: 1.25 × 10^{10} CFU/day/6 weeks	0.88 (0.66–1.75)*	Non-significant improvement in constipation—predominant IBS and health-related quality of life
Gawronska et al. 2007	L. rhamnosus GG Dose: 3 × 10^9 CFU/day/4 weeks	0.70 (0.50–0.99)	Significant improvements in pain frequency and severity
Drouault-Holowacz et al. 2008[35]	L. rhamnosus GG Dose: 1 × 10^{10} CFU/day/4 weeks	0.98 (0.69–1.37)*	;abdominal pain borderline significance (p ↘ 0.048) trend toward lower abdominal pain score (p ◧ 0.05)
Enck et al. 2008[37]	E. coli (DSM 17252) E. faecalis (DSM 16440) Dose: 3–9 × 10^7 CFU/day/8 weeks	0.51 (0.39–0.66)	Significant;abdominal pain ;global symptom score
Andriulli et al. 2008	Symbiotic formulation each 7 g sachet contains L. paracasei B21060 [5 × 10^9 CFU xylo-oligosaccharides (700 mg) glutamine (500 mg) arabinogalactone (1,243 mg) Dose: 7 g in 100 mL of water/b.i.d./ 12 weeks	0.93 (0.68–1.3)* 0.46 (0.24–0.89)	Overall IBS symptomatology not significantly different between groups Significant ; in diarrhoea between groups in favour of the test
Hong et al. 2009	B. bifidum BGN4 B. lactis AD011 L. acidophilus AD031; L. casei IBS041 Dose: 20 × 10^9 CFU/sachet/b.i.d./ 8 weeks	0.89 (0.54–1.46)*	No significant change between groups in overall IBS symptomatology
Simrén et al. 2010	L. paracasei, ssp L. paracasei F19, L. acidophilus La5, B. lactis B1. Dose: 400 mL of fermented milk of 5 × 10^7 CFU/day/8 weeks	0.85 (0.62–1.7)*	No significant treatment effect between active and control.
Cui and Hu 2012	No clear information given re species or dose. Test: 2 Bifid triple viable capsules administered t.i.d./4 weeks Placebo: 200 mg of placebo administered t.i.d./4 weeks	0.51 (0.30–0.85)	Significant difference trend of overall management of IBS symptomatology of test probiotic over placebo

Table 1 continued

Conditions/diseases references	Strain(s), Dose regimen L. = Lactobacillus B. = Bifidobacterium S. = Streptococcus E. = Escherichia	Effect size RR (95 % CI)*	Outcome
Ki Cha et al. 2012[39]	S. thermophiles B. breve B. longum B. infantis L. acidophilus L. plantarum L. paracasei L. delbrueckii subsp. Bulgaricus Dose: 1 × 10^{10} multi-strain CFU/day/ 8 weeks	0.59 (0.4–0.89)	↓abdominal pain ↓bloating
Kruis et al. 2012	E. coli Nissle 1917 Dose: 2.5–25 × 10^{9} CFU/day/12 weeks	0.81 (0.57–1.17)*	No difference between groups response to treatment was significant in the probiotic group versus placebo in a subgroup prior to IBS development
Ducrotté et al. 2012	L. plantarum 299v (DSM 9843) Dose: 1 × 10^{10} CFU/capsule/day/ 4 weeks	0.24 (0.17–0.35)	Significant effective symptom relief, particularly of abdominal pain and bloating
Dapoigny et al. 2012	L. casei rhamnosus Lcr35 Dose: 6 × 10^{8} CFU/day/4 weeks		1.20 (0.8–1.80) 0.64 (0.44–0.93) 0.92 (0.6–1.54)* 5.4 (2.1–13.8)
Roberts et al. 2013	B. lactis (strain I-2494 DN-173 010) S. thermophiles (CNCM strain I-1630) L. bulgaricus (CNCM strain I-1632 and I-1519) Dose: 1.25 × 10^{10} CFU B. lactis and 1.2 × 10^{9} CFU/cup of S. thermophiles and L. bulgaricus/day/4 weeks and 8 weeks		0.71 (0.46–1.16)* 0.80 (0.52–1.24)* 0.84 (0.6–1.19)*Overall IBS symptomatology improved in placebo over test with significant ↓in diarrhoea sub analysis in test over placebo
Capello et al. 2013	Symbiotic preparation contains thermophile bacteria: 5 × 10^{9} L. plantarum 2 × 10^{9} L. casei subp. Rhamnosus 2 × 10^{9} L. gasseri 1 × 10^{9} B. infantis 1 × 10^{9} B. longum 1 × 10^{9} L. acidophilus 1 × 10^{9} L. salivarus 1 × 10^{9} L. sporogenes 5 × 10^{9} S. thermophiles Prebiotic inulin 2.2 g 1.3 g of tapioca-resistant starch Dose: 5 g sachet/b.i.d./4 weeks		4 weeks no difference between groups 8 weeks placebo more significantly effective (p = 0.001) Symbiotic preparation demonstrated a non-significant beneficial effect in ↓ flatulence and bloating severity in IBS. Overall RR reduction not significant

Table 1 continued

Conditions/diseases references	Strain(s), Dose regimen L. = Lactobacillus B. = Bifidobacterium S. = Streptococcus E. = Escherichia	Effect size RR (95 % CI)*	Outcome
Yoon et al. 2014	B. longum B. bifidum B. lactis L. acidophilus L. rhamnosus S. thermophiles Dose: all at 5 ⨀ 10 CFU/capsule (500 mg)/b.i.d./4 weeks	0.51 (0.27–0.98)	Significant difference between test probiotic multi-strain and placebo groups re overall improvement in IBS symptomrtology
Necrotizing Enterocolitis			
Dani et al. 2002	L. rhamnosus GG Dose: 6 ⨀ 10^9 CFU/day/1 week	0.49 (0.15–1.61)*	No effective reduction in incidence
Costalos et al. 2003	Saccharomyces boulardii Dose: 50 mg/kg every 12 h	0.59 (0.19–1.78)*	No improvement
Bin-Nun et al. 2005	B. infantis S. thermophilus B. bifidus Dose: 10^9 CFU/day/until discharged	0.1 (0.01–0.77)	Significant reduction in incidence and severity and with no deaths from necrotizing enterocolitis
Lin et al. 2005	L. acidophilus B. infantis 125 mg/kg/dose/b.i.d. with breast milk until discharged	0.21 (0.05–0.94)	Significant reduction in incidence and severity
Mohan et al. 2006	B. lactis Bb12 Dose: 1.6 ⨀ 10^9 CFU/day 1–3 and 4.8 ⨀ 10^9 CFU/4–21 days	1.62 (0.16–16.4)*	No improvement in reduction of antibiotic resistant organisms
Manzoni et al. 2006	L. casei subspecies rhamnosus Dose 6 ⨀ 10^9 CFU/day/6 weeks maximum	1.0 (0.71–1.40)	Significant reduction in gut fungal (candida) colonization
Lin et al. 2008	L. acidophilus NCDO 1748 B. bifidum NCDO 1453 Dose: added to breast milk or mixed feeding all at 1 ⨀ 10^9 CFU/125 mg/kg/b.i.d./6 weeks	0.35 (0.4–3.23)	Significant reduction in incidence and severity and death
Rougé et al. 2009	L. rhamnosus GG B. longum BB536 Dose:10^8 CFU/day/until discharged	2.18 (0.2–23.2)*	No improvement in gastrointestinal tolerance to enteral feeding
Samanta et al. 2009	B. infantis B. bifidum B. longum L. acidophilus Dose: each at 2.5 ⨀ 10^{10} CFU (125 g/kg) with expressed breast milk/b.i.d./till discharged	0.35 (0.13–0.92)	Enteral administration significantly reduced morbidity due to necrotizing enterocolitis

Table 1 continued

Conditions/diseases references	Strain(s), Dose regimen L. = Lactobacillus B. = Bifidobacterium S. = Streptococcus E. = Escherichia	Effect size RR (95 % CI)*	Outcome
Fernández-Carrocera et al. 2013	L. acidophilus 1.0 × 10^9 CFU/g L. rhamnosus 4.4 × 10^8 CFU/g L. casei 1.0 × 10^9 CFU/g L. plantarum 1.76 × 10^8 CFU/g B. infantis 2.76 × 10^7 CFU/g S. thermophilus 6.6 × 10^5 CFU/g Dose: 1 g pack/day/until discharged	0.50 (0.20–1.26)	Post-hoc analysis showed significant risk reduction for necrotizing enterocolitis or death

* p < 0.05 b.i.d. = twice per day, t.i.d. = three times per day

** This study not included in the final overall analysis due to equivalent efficacy between the two treatments

the data allowed for the calculation of a RR (95 % CI). A flow diagram of the literature search for articles included in this systematic review is presented in Fig. 1.

Probiotics, prebiotics and inflammatory git conditions

Numerous clinical studies suggest probiotics can improve health outcomes in various end-organs (Vitetta and Sali 2008; Vitetta et al. 2012). Hence, probiotics have been profiled accordingly, and that is that upon administration can improve the health of the host beyond their intrinsic and basic nutritional content (Fuller 1989). Hence it was noted that probiotic bacteria employed in clinical trials investigated in this review have included organisms from different genera (i.e. Bifidobacteria, Lactobacilli); different species from a specified genera (i.e. Lactobacillus acidophilus; Lactobacillus bulgaricus, Lactobacillus rhamnosus); as well as those organisms from different strains within a species (i.e. Lactobacillus acidophilus La–1, Lactobacillus acidophilus NCFM) whilst administered as single- or multi-strain preparations as well as symbiotics (preparations of probiotics and prebiotic mixtures). This hierarchical profiling serving to highlight that different strains from the same species vary and hence may have the capacity to elaborate different physiological functions within the GIT as demonstrated by the different effects on different inflammatory GIT conditions/diseases.

Bifidobacteria and Lactobacilli have been the predominant genera studied and have demonstrated significant clinical efficacy in a number of health GIT conditions (Table 1). High level evidence-based studies have reported significant efficacy with specific probiotic strains in GIT inflammatory conditions such as constipation (in adults and children), diarrhoea (in adults and children), Crohn's disease (CD), ulcerative colitis (UC), irritable bowel syndrome (IBS) viral gastroenteritis, pouchitis and necrotizing enterocolitis.

The demonstrated efficacy in this critical review has been presented as an effect size of relative risk reduction with the administration of a probiotic versus a comparator. The effect sizes as a relative risk reduction (95 % CI) for probiotics over a placebo/comparator for the GIT conditions reviewed (Fig. 2) were significant for antibiotic-associated diarrhoea [0.72 (0.62–0.84); p < 0.0001]; infectious diarrhoea/gastroenteritis [0.68 (0.55–0.84); p < 0.0001]; IBS [0.71 (0.65–0.77); p < 0.0001]; ulcerative colitis [0.63 (0.53–0.76); p < 0.0001]; necrotizing enterocolitis [0.4 (0.27–0.59); p < 0.0001]; constipation [0.59 (0.5–0.68); p < 0001] and pouchitis [0.19 (0.11–0.32); p < 0.0001]. Whereas, and although the trend was in favour of probiotics the effect size relative risk reduction was not significant for clostridium difficile diarrhoea [0.70 (0.32–1.55); p = 0.38; and for Crohn's disease [0.86 (0.63–1.16); p = 0.30. This latter result, possibly a reflection of the few studies included in this review. Others have reported significant risk reductions in these GIT conditions (Ritchie and Romanuk 2012). The overall effect size relative risk reduction for the administration of probiotics versus a placebo/comparator was statistically significant for probiotics 0.65 (0.61–0.70) (z = 13.3); p < 0.0001, reflecting a clinical risk reduction of 35 %.

Manipulating metabolic changes in the GIT—a mechanistic overview

The commensal microbiome contribution

Microbiota that colonizes the human GIT exhibits a high phylogenetic diversity, reflecting their vast metabolic

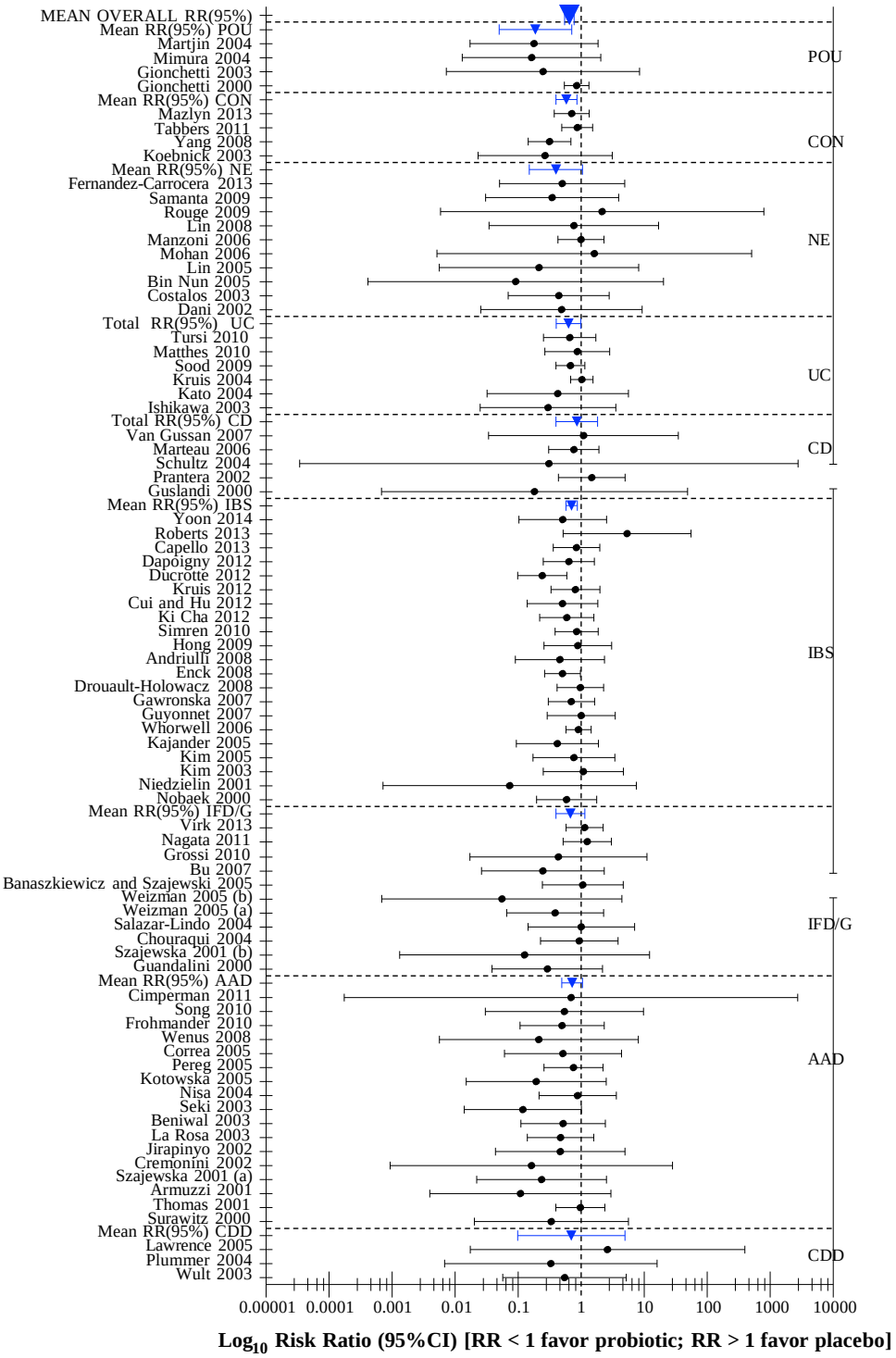

Fig. 2 Effect size [risk ratio (95 % CI)] for the effect of probiotics over placebo/ comparator in the prevention and treatment of gastrointestinal tract inflammatory diseases/ conditions

potential. The environmental/microbiological picture is one that continues throughout life to functionally co-operate with the host which first initiated from the interactions first disseminated from the time of birth and possibly even in utero. Germ-free mice studies have shown that triggering natural development and maturation of the immune system are only partially encoded in the host's genes, demonstrating that fundamental cues are required from the symbiotic microbial cohort for homeostatic development (Hooper 2004). How bacteria colonize the GIT provides initial clues as to the signals required by the GIT to develop a regulated immune–metabolic–inflammatory competent

profile. Up-regulated immune responses in an individual are necessary to protect the GIT from pathogenic cells. The immune system achieves this by initiating a pro-inflammatory response. The microbiota, act partly in an immune-surveillance role by detecting pathogenic bacteria and stimulating the immune system, subsequently initiating an appropriate eradicative inflammatory response (Eckmann 2006). Once the pathogenic cells have been cleared, the requisite is for an anti-inflammatory signal response that restores the balance between pro-and anti-inflammatory reactions. Accordingly, the healthy gut may be seen as one that is in a constant state of regulated inflammation. The role that commensal bacteria play in promoting an anti-inflammatory response is not well understood but is reported to be in part accomplished by the interaction of the bacteria with the intestinal epithelial cells that not only provide a physical barrier but also facilitate the interactions between GIT bacteria and host immune cells to achieve mucosal immunological equilibrium (Goto and Ivanov 2013). Failure to re-regulate inflammatory responses can increase the risk of developing inflammatory conditions of the host's gut architecture such as IBD or IBS. Accumulating evidence indicates that the balance of commensal bacteria within the GIT may be associated with the development of some GIT disorders (Swidsinski et al. 2002). Patients with IBD or IBS are reported to present with increased pro-inflammatory or potentially pathogenic bacterial species with the Bacteroides, (Swidsinski et al. 2002) Escherichia coli (Mylonaki et al. 2005; Martin et al. 2004) and Enterococci genera together with decreased Bifidobacteria and Lactobacilli species (Van de Merwe et al. 1988). The etiology of IBD is not fully understood, but is considered to be T cell-driven inflammation resulting from a persistent preponderance of pro- over anti-inflammatory cytokine production (Hvas et al. 2007).

Nutrition/supplementation contribution

GIT commensal bacteria metabolize food components that typically serve as energy sources. Additional factors such as sanitary conditions, birth delivery mode or antibiotic use drive the fluctuations of the microbial community during the first year or two of life (Adlerberth 2008). Furthermore, select studies clearly show that the specific consumption of foods that contain bioactive compounds may enhance health or increase the risk of disease. Such as is the case with human milk oligosaccharides that constitute the third most abundant class of molecules in breast milk optimizing the GIT microbial composition (Li et al. 2009). Other studies show that at least part of the protective effect of cruciferous vegetables is due to their relatively high content of fiber and phytochemicals such as glucosinolates (Marcobal and Sonnenburg 2012). Dietary fiber can be fermented by gut bacteria, to yield short chain fatty acids and other metabolites that may go on to suppress adverse inflammatory conditions. Additional studies that report the specific consumption of dietary compounds such as phytoestrogens show that metabolites elaborated by the GIT microbiome can then provide specific health benefits such as enhanced bone health (Chiang and Pan 2013).

Recent findings suggest that a high-fat diet interacts with GIT bacteria to promote early inflammatory changes in the gut that contribute to the development of obesity and insulin resistance (Ding and Lund 2011). The innate immune system recognizes and responds to the structural components of gram-negative bacteria (e.g. lipopolysaccharide), resulting in inflammation. Toll-like receptors (TLRs) are pattern recognition receptors that have a central role in innate immunity (O'Neill et al. 2013). Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria. LPS binding to the host receptor, TLR4, triggers an inflammatory reaction characterised by the release of large number of inflammatory mediators that allow the host to respond to the invading pathogen (Montero Vega and de Andrés Martín 2008). The mechanism that drives this response is partly through the activation of transcriptional factors such as NF-jB via the balanced action of constitutively expressed nitric oxide/inducible nitric oxide synthase to maintain homeostasis.

Mice fed diets high in saturated fats (72 % energy as fat) for 4 weeks reported an endotoxemia characterised by significant increases in plasma endotoxin levels i.e. LPS (Burcelin et al. 2008). This then shown to be a risk for inducing innate immune responses through the activation of TLR4 leading to inflammation by secreting pro-inflammatory cytokines and chemokines. Moreover, dietary fats and carbohydrates appear to be involved in inflammation through TLR4 activation. Free saturated fatty acids aggravate the expression and activity of TLR4 that is induced by high glucose in human monocytes along with increased signalling molecules such as superoxide generation that increase NF-jB activity leading to increased pro-inflammatory signals such as IL-6. But are these signals fundamentally adverse? It is further postulated that as a consequence, chronic activation of the immune system is associated with the development of obesity, insulin-resistance and T2DM through LPS, free fatty acid and products from dying cells that can bind TLR4 at the surface of innate immune cells and activate inflammatory pathways implicated in the pathogenesis of chronic diseases (Nakamura and Omaye 2012).

Tien et al. (2006) have reported that the anti-inflammatory activity demonstrated by probiotics within the GIT, in particular Lactobacillus casei, is modulated by their targeting the stability of I-jba, the specific NF-jB inhibitor, resulting in the mitigation of this major pro-inflammatory pathway. Therefore, they have hypothesized

that certain commensal microbiota has the ability to actively influence the homeostatic control of intestinal inflammation, inhibiting NF-**j**B activation, even in the presence of pro-inflammatory pathogenic and commensal microorganisms. This data would tend to suggest that increased calorie consumption increases certain bacterial species that promote pro-inflammatory GIT profiles by influencing NF-**j**B activation that then increases the risk of metabolic diseases. Alternatively, a diet that promotes a healthy GIT milieu such as vegetarian, Palaeolithic or Mediterranean diets encourages optimum ratios of bacterial species by re-regulating pre- and pro-inflammatory signals that reduce the risk of metabolic disease development (Kim et al. 2013; Scoditti et al. 2012). The overall requisite is the regulated control of these intracellular molecular responses. It seems plausible to posit that reactive oxygen species (ROS) may be the upstream early signal that provides an overall message to regulate the response that controls GIT inflammation.

Probiotics as signal transducers in the GIT

Over the last few decades, the role of oxidative stress has been proposed to play a major role in the development of diseases such as inflammatory bowel disease (Abdullah et al. 2013). This inference further nurtures support for the administration of antioxidant therapies. We assert that this is incorrect and further that there are no reported clinical trials that support this conclusion.

ROS are known to play a major role in maintaining normal physiological function (Linnane et al. 2007). The investigations on protein albumin thiol oxidations and serum protein carbonyl formations overemphasize the molecular damage that is attributed to ROS activity. These assertions have been previously considered and have challenged the commonly held view that proteins are randomly oxidized in an uncontrolled process by superoxide anion, hydrogen peroxide, nitric oxide and peroxynitrite, thereby contributing directly to the development of inflammatory conditions. This concept is untenable, misrepresenting stringently regulated cellular redox metabolic processes. Elsewhere we have discussed the oxidation of protein amino acid residues (Linnane et al. 2007) and scientifically contended that oxidatively modified proteins do not simply arise as the result of random oxidative damage (e.g. hydroxylation of various amino acid residues, sulphoxidation of methionines and nitrosylation of sulphydryl groups).

Probiotic bacteria have been reported to promote a range of GIT physiological functions that include a regulated control over immune responses, epithelial barrier function and cellular proliferation (Bermudez-Brito et al. 2012). The mechanism proposed for the GIT control of pathogens involves (a) direct anti-microbial activity through the production of bacteriocins or other inhibitors of pathogenic bacteria gene expression, (b) competitive exclusion of pathogenic bacteria by competing for binding sites or stimulation of epithelial barrier function, (c) stimulation of immune responses via increases of sIgA and anti-inflammatory cytokine factors and the rescue and regulation of pro-inflammatory cytokines, and (d) inhibition of virulence gene(s) or protein expression in gastrointestinal pathogenic bacteria (Amalaradjou and Bhunia 2012). The active mechanism that induces this complex control of pathogenic activity implicates ROS.

Recent important advances in cellular signalling have demonstrated that some genera of human commensal GIT bacteria can induce a rapid increase of ROS that then elicit a strong physiological response through the activation of epithelial NADPH oxidase-1 (Nox1) (Neish 2013; Lin et al. 2009). In addition, reports site in vitro experiments with epithelial cells that, when co-cultured with specific probiotic bacteria, show an increased and rapid oxidation reaction of soluble redox sinks, namely glutathione and thioredoxin (Neish 2013; Lin et al. 2009). This very much indicates the presence of a regulated process. This effect was demonstrated as an increase in the oxido-reductase reaction of transcriptional factor activations such as NF-**j**B, NrF2 and the antioxidant response element, reflecting a cellular response to increased ROS production that is regulated (Neish 2013; Lin et al. 2009). This effect must be decisive to elicit a restrained anti-infective response with a minimal chance of pro-inflammatory damage to the tissue. These reactions define potent regulatory effects on host physiological functions that include immune function and intracellular signalling.

The reported mechanisms of action for probiotics are similarly aligned acting to enhance the epithelial barrier, increase bacterial adhesion to the intestinal mucosa with an attendant inhibition of pathogen adhesion to the competitive exclusion of pathogenic microorganisms (Neish 2013; Lin et al. 2009; Lee 2008). Furthermore, probiotic strains have also been reported to generate a range of anti-microbial substances and to positively affect and modulate immune system function. Lee (2008) has reported that the enteric commensal bacteria, by rapidly generating ROS, negotiate an acceptance by the GIT epithelia. Different strains of commensal bacteria can elicit markedly different levels of ROS from contacted cells. Lactobacilli are especially potent inducers of ROS generation in cultured cells and in vivo, though all bacteria tested have some ability to alter the intracellular oxido-reductase environment. Yan et al. (2007) has reported that there are soluble factors that are produced by strains of lactobacilli that are

capable of mediating beneficial effects in in vivo inflammatory models. This result expands our understanding that there are ROS-stimulating bacteria that possess effective specific membrane components and/or secreted factors that activate cellular ROS production to maintain homeostasis.

It has been reported that redox signalling by microbial ROS formation is in response to microbial signals via formyl peptide receptors and the gut epithelial Nox1 (Lin et al. 2009). As we have previously documented (Linnane et al. 2007) ROS generated by Nox enzymes have been shown to function as essential second messengers in multiple signal transduction metabolic pathways through the rapid and transient oxidative inactivation of a distinct class of sensor proteins bearing oxidant-sensitive thiol groups. These redox sensitive proteins include tyrosine phosphatases that attend as regulators of the MAP kinase pathways (Linnane et al. 2007; Lin et al. 2009). These reports focus our understanding on the importance of second messenger functionality for the maintenance of homeostasis and bring into serious question the elimination of ROS by antioxidant supplements for the amelioration of GIT inflammatory diseases such as IBD. The established importance of recent investigations regarding probiotic/microbial-elicited ROS clarifies that stimulated cellular proliferation and motility is strictly controlled and is a regulated signalling process for proper innate immunity and gut barrier function (Lin et al. 2009; Collier-Hyams et al. 2006; Neish et al. 2000). The observations that the vertebrate epithelia of the intestinal tract, supports a tolerable low-level inflammatory response toward the GIT microflora, can be viewed as an adaptive activity that maintains homeostasis.

Discussion

It has become clear from numerous studies derived from different experimental model systems that enteric bacteria are a critical component in the maintenance of health as well as in the initiation and dys-regulation of gastrointestinal inflammation that may lead to dysbiosis and ultimately GIT disease (Howarth and Wang 2013). Also an enhanced understanding of the molecular mechanisms underlying bacterial signalling and tolerance in the small and large bowel may provide clues to the localized microbiotic-controlled axis that operates within the GIT to maintain homeostasis, such as in the secretion of mucus. Mucus production has been reported to be stimulated by high-fiber diets, confirming that under in vivo physiological conditions, an adaptive GIT-derived feedback micro-axis mechanism is in place for sensing and responding to normally induced mechanical stress with an increase in lubrication of the GIT lumen (Enss et al. 1994; Schmidt-Wittig et al. 1996). A more recent study (Miyake et al. 2006) has demonstrated that lubrication in the GIT can be rapidly and precisely fine-tuned to widely fluctuating dietary-dependent levels of mechanical stress. This further supports our contention that feedback GIT micro-axes that are associated with mucus secretion, Th1/Th2 modulation or the secretion of proteins (e.g. secretin) that regulate GIT homeostasis which are fundamentally influenced by the GIT microbiome. The value of ROS signalling, rather than leading to macromolecular damage, has been relatively undervalued. We suggest that proper commensal bacterial signalling is of utmost importance in maintaining GIT homeostasis.

Furthermore, this critical review has highlighted the significant pharmacobiotic importance of certain probiotic genera/strains that can exert a significant health benefit by rescuing a dysbiotic GIT microbiota profile. The microbial community composition is governed by the host's age, diet and internal environment and bacterial phylogeny influencing immunological tolerance and inflammatory responses within individuals (Vitetta et al. 2013). Prudent dietary practices with perhaps regular probiotic/prebiotic supplementation that achieve and preserve the internal milieu may be the rationale for health maintenance and GIT inflammatory disease prevention. Clearly, the number of human intervention studies assessing the effect of probiotics in inflammatory diseases of the GIT supports this contention.

Although overall, the connection between the gut microbiota, energy homeostasis, inflammation and its role in the pathogenesis of GIT inflammatory conditions are increasingly recognized, further studies are required to confirm probiotic, prebiotic and symbiotic relevance, biological acceptability, mode of action and the long-term effects of these supplements. The challenge is for clinical trials with robust designs and sharp end-points.

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Chapter 3
Probiotics Attenuate Non–Alcoholic Fatty Liver Disease in Wild Type Mice Fed a High Fat Diet.

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3.1. Abstract

Objective Obesity is a predisposing factor for chronic diseases. Current research efforts with probiotic bacteria have reported improvement in gastrointestinal tract low–level inflammation and metabolic syndrome associated markers that positively affect energy balance.

Aim We have investigated a multi–strain probiotic preparation containing a combination of probiotic strains including *Bifidobacteria* and *Lactobacilli species and a Streptococcus* species in a mouse model of high fat diet/obesity induced liver steatosis.

Methods Three groups of C57B1/6J mice fed either a standard chow or high fat diet (HFD) for 20 weeks and a third group fed a HFD for 10 weeks and then concomitantly administered probiotics for a further 10 weeks. At sacrifice all animals were weighed and serum, liver and large bowel samples collected for analysis.

Results A multi-strain probiotic administered with a HFD significantly maintained tight junction protein ZO-1 (0.24±0.04 vs. 0.01±0.01) and ZO-2 (0.44±0.12 vs. 0.17±0.08) expression compared to the HFD group only. However probiotics were unable to restore ZO-1 or ZO-2 expression to levels seen in chow fed mice (0.38±0.08 and 0.81±0.19 respectively). Final body mass (33.6g ± 1.91 [chow] vs. 46.5g ± 4.07 [HFD–probiotics] and 45.5g ± 4.75 [HFD+probiotics]; $p<0.001$) was significantly increased with the high fat diet ± probiotics compared to chow controls as were liver (1.72g ± 0.33, 2.40g ± 0.74, 2.73g ± 1.2; $p<0.05$) and fat pad mass (0.90 ± 0.33, 2.60 ± 0.47 and 2.14 ± 0.56; $p<0.01$) respectively. Compared to chow fed mice, HFD ± probiotics elevated hepatic triglycerides ($p<0.001$), serum glucose ($p<0.05$), cholesterol ($p<0.01$), Alanine transaminase (ALT) ($p<0.001$) and Aspartate aminotransferase (AST) ($p<005$) while reducing serum triglycerides ($p<0.05$).

Conclusions The reduced progression of HFD induced steatosis by the administration of a multi–strain probiotic formulation further supports the posit that probiotics may assist with

lipid disposal from a HFD by reducing the accumulation of fat deposits in the liver consistent with reduced steatosis.

Key words: Steatosis, Non –Alcoholic Fatty Liver Disease, High Fat Diet, Probiotics.

3.2. Introduction

Non-alcoholic fatty liver disease (NAFLD) is more commonly diagnosed than alcoholic liver disease, owing to the rapid and increased prevalence and burden of obesity.[91-93] The prevalence of NAFLD is almost proportional to the global increase in obesity and T2DM. Furthermore, NAFLD is the most commonly diagnosed cause of abnormal liver function tests.[94] NAFLD is caused by fat deposited (steatosis) in the liver that is not due to excess alcohol consumption. Disruption of the normal mechanisms for synthesis, transport and removal of fatty acids (LCFA)[95] and triglycerides (TG)[96] underpins the basic mechanism for the development of NAFLD.[97, 98]

Furthermore, the progressive form of NAFLD is designated as non–alcoholic steatohepatitis (NASH). NASH is the progression of steatosis to chronic inflammation of the liver with fat accumulation (steatohepatitis), previously thought to be only associated with alcoholic liver disease (ALD). The progression from NAFLD to NASH may lead to cirrhosis and end stage liver disease that is adverse to health and associated with morbidity. Not all those that develop NAFLD progress to NASH leading to *the second hit* theory.[98] Day and James[98] have reported that different pathogenic factors can lead firstly to NAFLD and secondly to NASH. NASH is increasingly recognized as the leading form of liver dysfunction and cirrhosis in non-alcoholic liver disease, viral hepatitis negative population currently accounting for 5% of all liver transplants.[99]

The major risk factor for NAFLD is over consumption of calorie dense foods and the resulting consequences, namely obesity (abdominal visceral obesity), dyslipidemia, insulin resistance and T2DM.[100, 101] Diets comprising high amounts of saturated fats or

carbohydrates have been associated with increased liver inflammation.[102, 103] Currently, NAFLD has been estimated to affect as much as 34%[104, 105] and NASH between 2–5% of the population. [106] Dividing the population into lean and obese groups further highlights the correlation with obesity. NAFLD has been found in 16% of lean individuals, opposed to 76% of obese individuals.[107] With childhood obesity rates increasing, the prevalence of NAFLD and NASH is expected to rise, increasing the burden of fatty liver disease.

A link between ALD and NAFLD that may help explain the similar pathogenesis between the diseases may be ethanol concentrations. Ethanol production has been linked with bacterial overgrowth and obese mice have been shown to produce greater amounts of ethanol than lean mice.[108] Bacterial overgrowth has been associated with liver disease and found in NASH patients.[109, 110] The usually low levels of bacteria colonizing the small intestine increase several fold small intestinal bacterial overgrowth (SIBO) and naturally produce elevated levels of ethanol via fermentative processes. In order to metabolise ethanol in the liver there is a high energy demand that leads to increased oxygen consumption. There is then a subsequent requisite for an increase in hepatic blood flow. If the supply of oxygen does not match the demand, however, a state of hypoxia can be induced that may contribute to liver damage.[111] Ethanol also increases the production of ROS, mediating lipid peroxidation that has been postulated to promote liver damage.[112] This however can be alternatively interpreted as increased cell signalling by oxidized lipids that interact with the mitochondria.[113]

Excess ethanol can lead to increased intestinal permeability, increased exposure to pathogenic bacteria (and bacterial products such as lipopolysaccharides), to immune surveillance cells and increased passage of undigested macromolecules into the bloodstream. These actions can then activate the immune system resulting in dysregulated inflammation.[114, 115]

The treatment options for NAFLD include weight management, medications, various supplements and recently the administration of probiotics. While probiotics are traditionally

recommended for individuals with gastrointestinal tract (GIT) disorders, it is emerging that probiotics may have an overarching role via the GIT influencing end–organ physiology (e.g., the liver, kidneys).[116] Probiotics may help increase liver function by re–regulating cytokine productions, particularly pro–inflammatory cytokines that affect the liver (i.e., TNF–α, IL–6 and TGF–β).[117, 118] TGF–β in particular, may activate hepatic stellate cells leading to fibrogenesis.[119] Probiotics may also help reduce the inflammatory response via a ROS dependent signalling[120] mechanism reducing lipid peroxidation and preventing the progression of NAFLD and NASH.

The link between liver disease and gastrointestinal bacteria (commensal or pathogenic) may appear abstract, however animal studies correlate pathogenic bacterial overgrowth with the development of liver disease.[121] Subsequent administration of antibiotics reduced the liver damage incurred by gastrointestinal pathogenic bacteria overgrowth by reducing and in some cases eradicating bacteria.[121] Probiotics defined as live bacterial cultures that when consumed in foods (e.g., yoghurts) and dietary supplements can improve the health of the host beyond their inherent basic nutritional content.[122] As such probiotic bacteria have been posited to prevent or treat NAFLD by positively influencing the gastrointestinal microbiome through cytokine productions and regulating immuno-inflammatory responses.

A mechanistic overview postulates that probiotics may reduce inflammation by reducing gastrointestinal epithelial cell permeability, preventing the passage of pathogens (or bacterial by-products such as lipopolysaccharides) from passing across the GIT epithelial barrier. Probiotics have been reported to reduce intestinal permeability by maintaining or increasing the concentration of tight junction proteins (ZO-1, ZO-2, PKC-ζ) at the cell membrane.[35, 36]

This study hypothesized that the administration of a multi–strain probiotic preparation consisting of a mixture of *Bifidobacteria* and *Lactobacilli* strains and a *Streptococcus* strain could mitigate the adverse effects of a HFD on the liver and hence offer new insights into the

cellular mechanisms behind NAFLD. Furthermore the aim of this study was to establish what beneficial effects probiotic bacteria could provide in a mouse model of NAFLD and what GIT cellular mechanism (attenuation of gut dysbiosis) may be contributing to exert their effect on the liver.

3.3. Methods

Study design

Mice were fed either a standard chow diet or a HFD for a total of 20 weeks. At the end of week 10, the HFD group of mice were divided into 2 groups and probiotics were added to the drinking water for the remaining 10 weeks for one group of mice. At the end of week 20 all mice were euthanized, dissected and samples stored for later analysis.

Animals

Young wild type (WT) mice on a C57B1/6J background were randomly divided into one of three groups: 1) a control group, receiving a standard diet (n=10); 2) a HFD fed group (n=10); and 3) HFD fed group supplemented with probiotics (n=10; $1 \times 10^{8\text{-}9}$ Colony Forming Units (CFU)/mL). All animals were housed in a SPF facility maintained at 20°C on a 12 hour light/dark cycle with access to clean water and food.

Ethics Statement

All procedures were carried out in accordance and with approval from The University of Queensland and Queensland Institute of Medical Research (QIMR) ethics committees.

Bacterial Strains

Nine strains of probiotics represented here as percentages of total CFU/mL (*Lactobacillus rhamnosus / Lactobacillus casei* / *Lactobacillus acidophilus / Lactbacillus plantarum / Lactobacillus fermentum* comprising 87% of the total colony forming units; *Bifidobacterium lactis / Bifidobacterium breve / Bifidobacterium bifidum* comprised 13% of the total colony forming units; and *Streptococcus thermophilus* comprising 5% of the total colony forming

units were used as a multi–strain probiotic blend. All strains were lyophilized, water soluble and added to the drinking water as a combination probiotic. The total concentration of probiotic blend added to the drinking water was $1 \times 10^{8\text{-}9}$ CFU/mL. This dosage was chosen as it represents a dose per kilogram of body mass equivalent for a human.

Diet

The standard chow and HFD were purchased from Specialty Feeds (WA, Australia; HFD product no. SF03-020). The chow diet contained 4.8% total fat (mono unsaturated fat 2%, polyunsaturated fat 1.77% and saturated fat 0.74%) providing 14 MJ/Kg of energy while the HFD contained 23% total fat (mono unsaturated fat 7.59%, polyunsaturated fat 2.04% and saturated fat 12.6%) providing 20 MJ/Kg of energy.

Dissection

At the end of week 20, mice were anaesthetized using an intraperitoneal injection of pentobarbital and xylene. Once anaesthetized, blood was collected using a cardiac puncture and allowed to clot at room temperature before serum was removed and stored. Tissue was collected by first removing the pancreas followed by the liver, fat pads, thigh muscle, small intestine, large intestine, spleen and the heart. The mass of the liver and fat pads was recorded. Tissue samples from the different sites were snap frozen in liquid nitrogen, fixed in OCT fluid or placed in formalin for histology. Tissue placed in formalin for fixing was transferred to a 70% ethanol solution after 24 hrs.

Large Intestine / Swiss Roll

The large intestine was cut longitudinally from the caecum to the rectum and opened. The intestinal tract was cleared of faecal matter using a cotton bud and cut longitudinally down the center again providing two separate longitudinal segments. One segment was carefully rolled on a wooden toothpick starting from the colon end with the mucosa on the outside of

the roll. The resulting roll was then carefully placed in formalin for histology. The second segment was cut into two or three pieces and frozen in liquid nitrogen.

Blood Biochemistry

Serum was analysed spectrophotometrically for alanine transaminase (ALT), aspartate transaminase (AST), albumin, glucose, cholesterol and triglycerides. Analysis was performed using a Cobas Integra 400, reagents and calibrators supplied by Roche Diagnostics (NSW, Australia).

Hepatic Triglycerides Quantification

Liver tissue was homogenized in a 1.5% potassium chloride solution (2.3 g KCl in 200 mL water). 500 μl of homogenate was extracted using a 2:1 chloroform/methanol mixture. Extracts were dried and stored at -80°C until analysis. For analysis, samples were reconstituted using 2% triton-x with the aid of sonication. Samples were further diluted with 2% triton-x for a final concentration of 1:6 ready for analysis. Samples were measured spectrophotometrically (Cobas Mira, Roche Diagnostics, Australia) using a kit and calibrators supplied by Novachem (Victoria, Australia).

Protein Quantification

Proteins were measured as per manufacturer's direction using a Pierce BCA protein assay kit supplied by Thermo Scientific (Victoria, Australia)

Tight Junction Proteins – ZO-1, ZO-2

Tight junction protein ZO-1 and ZO-2 rabbit anti-human polyclonal antibodies were purchased from Lifespan Biosciences (WA, USA). A polymer HRP anti-rabbit secondary antibody was purchased from Daco (Vic, Australia). ZO-1 and ZO-2 were analysed on large intestine swiss roll histology slides as per manufacturer's direction. Optimisation of the antibodies gave optimal concentrations of 2.5 μg/ml and 10 μg/ml for ZO-1 and ZO-2 respectively.

Histological Scoring

Histology was blindly scored on large intestinal swiss roll sections with ZO-1 and ZO-2 staining and liver sections with H and E and oil red O staining. Swiss rolls were scored for the expression of ZO-1 and ZO-2. Liver sections were scored for diagnosis, NAFLD activity score, steatosis grade and percentage, portal inflammation, lobular inflammation and ballooning, mallory's hyaline, fibrosis stage, portal score and centrilobular score.

Statistical analysis

All data is presented as mean ± standard deviation unless otherwise stated. Data was tested for normality of the distribution, and analysis was performed with the statistical software GraphPad Prism. Comparison between groups was carried out using a 1-way analysis of variance with a Tukey's multiple comparison post hoc test or a Kruskal-Wallis test for non-parametric data with a Dunn's multiple comparisons post hoc test of significance between individual groups. Differences were considered significant when *P* was less than 0.05 (* = P < 0.05; # = p < 0.01; § = p < 0.001). All significant differences between groups were determined using the HFD group as the reference group.

3.4. Results

Histological examination of the large intestine Swiss rolls for tight junction proteins ZO-1 and ZO-2 showed mice fed a HFD had reduced expression for ZO-1(0.01±0.01 vs. 0.38±0.08) and ZO-2 (0.17±0.08 vs. 0.81±0.19) compared to the chow group. Mice fed a HFD and supplemented with probiotics showed significant recovery in ZO-1 (0.24±0.04 vs. 0.01±0.01) and ZO-2 (0.44±0.12 vs. 0.17±0.08) compared to high fat fed mice (Figure 1). Histological examination of livers from HFD mice demonstrated the development of steatosis with large fat droplets present. Chow fed mice showed no steatosis development (Table 1) or fat droplet accumulation. Compared to mice fed a HFD, mice fed a HFD and supplemented

with probiotics showed a non-significant reduction in steatosis grade (2.44 ± 0.73 and 2.00 ± 1.25 respectively; p = 0.50) and steatosis percentage (72.78% ± 27.17 & 59.2% ± 38.74; p = 0.9) respectively (Table 1) and visible reductions in fat droplets (Figure 2A-C).

Confirming liver histology scores and observation, mice supplemented with probiotics had a two and a half fold reduction in hepatic triglyceride concentrations compared to HFD mice (Figure 3; p<0.001). Hepatic triglyceride concentrations of the HFD fed mice supplemented with probiotics remained elevated by threefold compared to chow fed mice (Figure 3; p<0.001). HFD feeding also decreased serum triglyceride concentrations (Figure 4). Probiotics supplementation was unable to attenuate these effects of the HFD. Chow fed mice showed increased serum triglycerides as hepatic triglycerides increased. HFD fed mice showed a reduction in serum triglycerides as hepatic triglycerides increased (Figure 5).

Serum ALT, AST, glucose and cholesterol were elevated in HFD fed mice compared to chow fed mice (figure 6 & figure 4). Probiotics supplementation was unable to attenuate the effects of the HFD. When outliers were removed (>2 SD) probiotics supplementation significantly reduced ALT and AST concentrations compared to HFD fed mice (Figure 7; p<0.05).

There was no difference in initial body mass of the chow, HFD or HFD plus probiotics groups (23.87g ± 0.95, 24.76g ± 0.72, 24.48g ± 0.84 respectively; p = 0.9). Body mass gain of the experimental groups paralleled the mass gain of the experimental group for the first seven weeks. At week eight, compared to chow fed mice, mice fed a high fat diet or supplemented with probiotics were 4.1% and 7.7% heavier (31.3g ± 1.22 vs. 32.6g ± 2.55 and 33.7g ± 3.49; p<0.05) respectively. By the end of the 20th week, compared to chow fed mice the HFD fed mice and probiotics supplemented mice were 27.7% and 26.2% heavier (33.6g ± 1.91 vs. 46.5g ± 4.07 and 45.5g ± 4.75; p<0.001; Figure 8) respectively. Compared to chow fed mice, mice fed a HFD or supplemented with probiotics also had heavier livers (1.72g ± 0.33 vs. 2.40g ± 0.74 and 2.73g ± 1.2; p<0.05; Figure 9). Plotting liver mass against body

mass showed a steady increase in liver mass in relation to body mass. Once the body mass reached 45 – 47 g, the liver mass increased sharply (Figure 10). Above 45g, 50 – 100% of the body mass increase can be attributed to increased liver mass.

3.5. Discussion

The results from this study showed that mice fed a HFD followed by the administration of a multi-strain probiotic formulation maintained tight junction proteins (i.e., decreased intestinal epithelial cell permeability) and reduced hepatic triglyceride concentrations compared to mice fed a HFD alone. Furthermore, the study demonstrated that supplementation with a multi-strain probiotic preparation non–significantly reduced the effects of a HFD by attenuating the progression of steatosis / NAFLD as evidenced in part from liver tissue histology showing visible reductions in fat droplets.

Increased consumption of calorie dense foods / diets that are high in fat, particularly saturated fat, is a major contributor to obesity and its comorbidities. NAFLD is such an outcome. Despite the increasing prevalence of NAFLD, the pathogenesis remains poorly understood.

NAFLD may have a benign asymptomatic course with a lack of definitive evidence about effective interventions. NAFLD is typically first suspected when the results of liver function tests, measured as part of routine testing are moderately abnormal.[123] The usual observed biochemical pattern in hepatic steatosis due to NAFLD is of increased levels of transaminases, with alanine aminotransferase (ALT) levels exceeding those of aspartate aminotransferase (AST).[123]

While this study did not investigate the profile of the GIT microbiome with and without a HFD, we nevertheless postulated that the development of NAFLD could in part be associated with a dysbiotic GIT (a gut barrier abnormality). In this study we combined a major contributor to the development of NAFLD, that is the over consumption of a high fat diet and

posited that induced changes in TJP expression would follow, that could then be preserved by the administration of probiotic bacteria.

Maintaining the integrity of the GIT and epithelial permeability is one of the most important physiological defenses against pathogenic bacteria and exogenous pathogens. A reduction in TJP, in particular a reduction in TJP at the cell membrane leads to increased intestinal permeability. This in turn can lead to increased inflammatory responses and the translocation of pathobionts across the protective intestinal epithelial barrier. With the liver receiving 70% of its blood supply from the GIT[124], pathobiont interactions across the epithelial barrier can trigger inflammatory responses that can then significantly and adversely influence end–organ physiology.[25] Such inflammatory responses mechanistically can in part lead to the development and progression of NAFLD. Histological analysis of the large intestine showed that the administration of probiotics partly preserved the HFD induced sub-optimal expression of tight junction protein ZO-1 and ZO-2. By maintaining the integrity of the GIT epithelial layer through the administration of probiotics, the liver seems to have been sparred further development / progression of NAFLD. Hence it is possible to hypothesise that this is in part the result of reducing the inflammatory response that a HFD triggered dysbiotic GIT would promote from the effects of exogenous pathogens / pathobiont and by–product translocations across the epithelial gut barrier.

While cellular mechanisms responsible for the observed lipid clearance from the liver were not found, the study showed physiological responses that may lead to the further elucidation of cellular mechanisms involved in the increased deposition of lipids in the liver. Moreover the results also showed that in the chow fed mice as the liver triglyceride concentration increased, the serum triglyceride concentration also was observed increased. In the HFD fed mice however, the observed increase in liver triglycerides was accompanied with decreased serum triglycerides (Figure 5). This result may be due to altered liver lipid mobilisation and

metabolism. Experimental studies have reported that under regulated conditions, as liver triglyceride concentrations increase, there is an adaptation response that may increase or decrease cell signalling (reactive oxygen species dependent) mechanisms in order to increase the mobilisation of fat to clear it from the liver.[125]

Further, an additional mechanism may be the activation and deactivation of hepatic stellate cells (HSC). HSC have been shown to aid in the mobilisation of lipids. Lu[126] showed that HSC activation can be modified by exposure to triglycerides. Triglycerides at a concentration of 400 mg/L had an inhibiting effect on HSC activation, whereas a concentration of 12.5 mg/L of triglycerides promote HSC activation while concentrations in between had no effect on HSC activation. HSC are also involved in liver fibrosis in response to liver damage. Therefore increased HSC activation may remove triglycerides from the liver but also increase fibrosis. Over activation of HSC may therefore lead to the development of cirrhosis. Under normal physiological conditions, HSC activation is regulated to maintain a balance between lipid mobilisation and lipid accumulation. Increased HSC activation from lipid accumulation may potentially result in liver damage and cirrhosis. Therefore it would seem that reducing HSC activity with a subsequent decrease in triglyceride mobilisation, can lead to the prevention of liver cirrhosis.

The results of increased liver mass in the HFD mice provided a physiological response that posits the presence of a body mass *threshold*. Mice fed a HFD consistently gained body mass for the duration of the 20 weeks (Figure 8). A linear relationship was evident between body mass gained and liver mass increase that peaked around 45–47 g of body mass. The mass of the liver was observed to markedly increase, doubling to tripling in mass.

Currently this study suggests that once the body mass of the mice reached 45 g, there was accumulation of most of the additional fat mass in the liver. This could be due to exposing the liver to high levels of triglycerides and to lowered HSC activity, followed by a plausible

reduction in triglyceride mobilisation rather than leading to accumulation of triglycerides in the liver. The reduced liver triglycerides whilst maintaining liver mass following the administration of probiotics, is indicative of a liver–gut axis that in part may be under operational control by the gastrointestinal microbiome.[116] It is possible that the administration of probiotics influenced liver physiology by generating additional liver tissue that replaced that possibly damaged by the excessive accumulation of fat. This result may aid in lipid mobilisation with the increased regenerated liver tissue capable of producing cell signalling molecules and essential fatty acids. Whether activation of HSC is increased with probiotics administration is not known at this stage and is of interest for future research.

Despite the beneficial effects demonstrated by the administration of probiotics, these bacteria cannot be deemed a panacea to a high calorie diet. In this study, 30% of the mice (n=3) showed little effect from the administration of probiotics, consequently additional factors that vary the gastrointestinal microbiome could also be important considerations. Colonization of the GIT is not a uniform event and begins post birth as the newborn is exposed to maternal and environmental microbes.[127] Therefore, it is plausible that animal housing differences, variable bacterial colonisation of the GIT and disease development could have attributed to the observed variation in probiotic effects. Certainly a weakness of this study in this regard was the lack of GIT microbiome studies before and after probiotic administration and in the different groups of animals.

This study clearly proposes that probiotics are not a panacea for an over consumption of calorie dense foods through a high saturated fat diet in the hope of down–regulating GIT inflammation and or liver fatty acid metabolism. Rather, probiotics may help reduce the severity and or rate of progression of NAFLD to cirrhosis or hepatocellular carcinoma, resulting from the consumption and salvage of a high caloric / fat diets, by modulating the

gastrointestinal microbiome, host energy metabolism, gut permeability and inflammatory responses.

Future Research Directions

Further studies should investigate the role of multi-strain probiotics in the modulation of the GIT microbiome, gut dysbiosis and the effect on the gut-liver axis. Moreover investigate the prophylactic effect probiotics would have on NAFLD. Studies have shown that probiotics may have a greater effect when administered prophylactically. Exercise may also have a dramatic effect on NAFLD outcomes. The mice in this study were housed in cages with no exercise wheels and so were considered sedentary. With exercise the development of NAFLD may be further attenuated with the administration of probiotics that we postulate may have an additive positive effect.

3.6. Figures

Figure 3.1: Swiss Roll Staining of the large intestine for ZO-1 A) Chow fed mouse showing normal ZO-1; B) HFD fed mouse with reduced ZO-1; C) Mouse fed HFD and supplemented with probiotics with increased ZO-1 staining compared to HFD alone.

Figure 3.2: Liver histology for lipid deposits A) Mouse fed a standard chow diet with very little fat deposits; B) Mouse fed a HFD with large fat droplets; C) Mouse fed a HFD with probiotics supplementation showing significant reduction in fat droplets.

Figure 3.3: Hepatic triglyceride concentrations

Figure 3.4: Serum biochemical data A: glucose; B: cholesterol; C: triglycerides

Figure 3.5: Liver triglycerides against serum triglycerides. An inverse relationship between serum and liver triglycerides was observed when a HFD was consumed with or without probiotics.

Figure 3.6: Serum biochemical data A: ALT; B: AST; C: Albumin

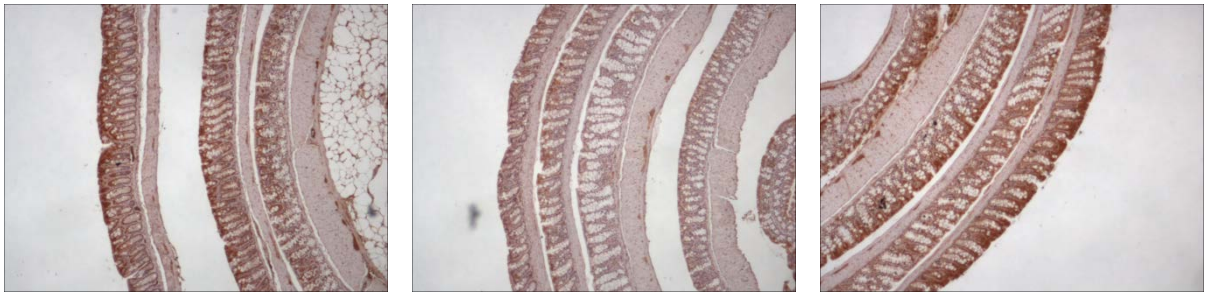
Figure 3.7: Serum biochemical data with three outliers removed A: ALT; B: AST

Figure 3.8: Body mass increase over the 20 weeks

Figure 3.9: A: Fat pad mass; B: Liver mass; C: Liver mass with outliers removed

Figure 3.10: Liver mass plotted against body mass showing at approximately 45 grams in body mass a “liver mass threshold” appeared to occur. At this point the majority of body mass increase is largely due to an increase in liver mass.

Figures



A B C

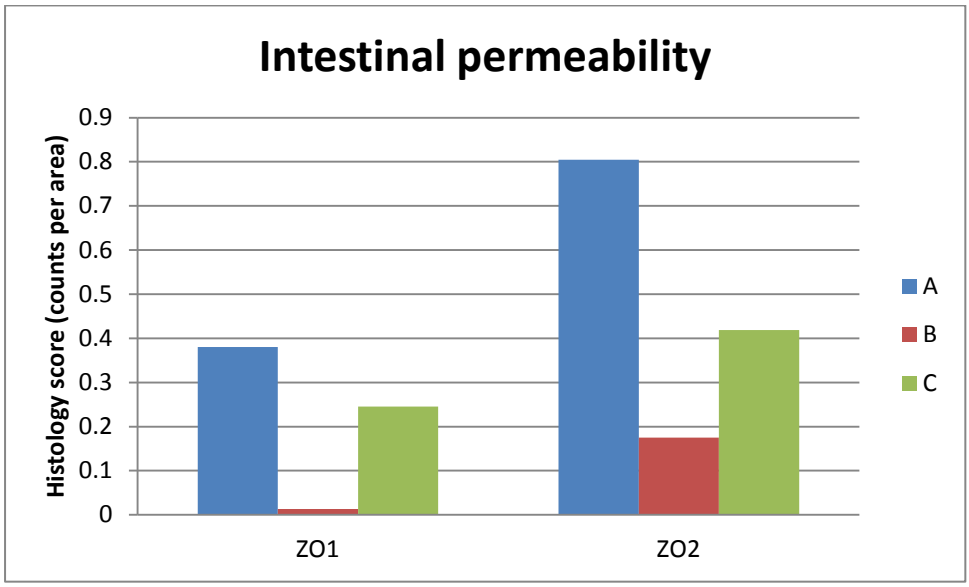
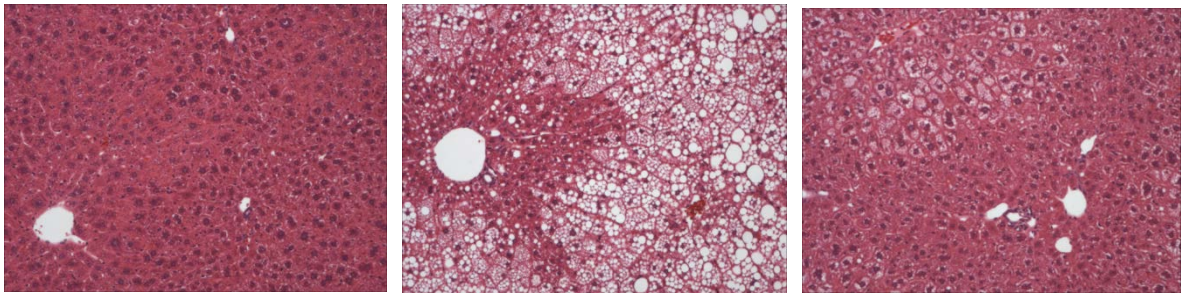


Figure 3.1

Table 3.1: Liver histology grading.

	Chow (n=9)	HFD (n=9)	HFD + probiotics (n=10)
Steatosis Grade	0.0 ± 0.0	2.4 ± 0.7*	2.0 ± 1.3*
Steatosis %	0.1 ± 0.3	72.8 ± 27.2*	59.2 ± 38.7*
Portal Inflammation	0.0 ± 0.0	0.2 ± 0.4	0.0 ± 0.0
Lobular Inflammation	0.6 ± 0.7	1.9 ± 1.0*	1.7 ± 1.2
Ballooning	0.2 ± 0.4	1.3 ± 0.7*	1.1 ± 0.6*

Data are mean ± SD. * = $p<0.05$



A B C

Figure 3.2

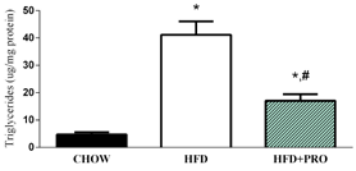


Figure 3.3

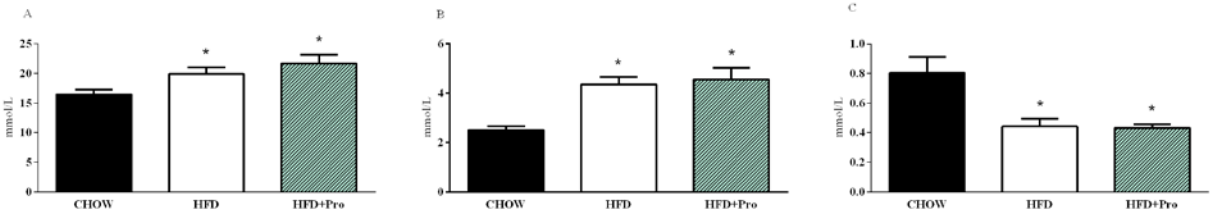


Figure 3.4

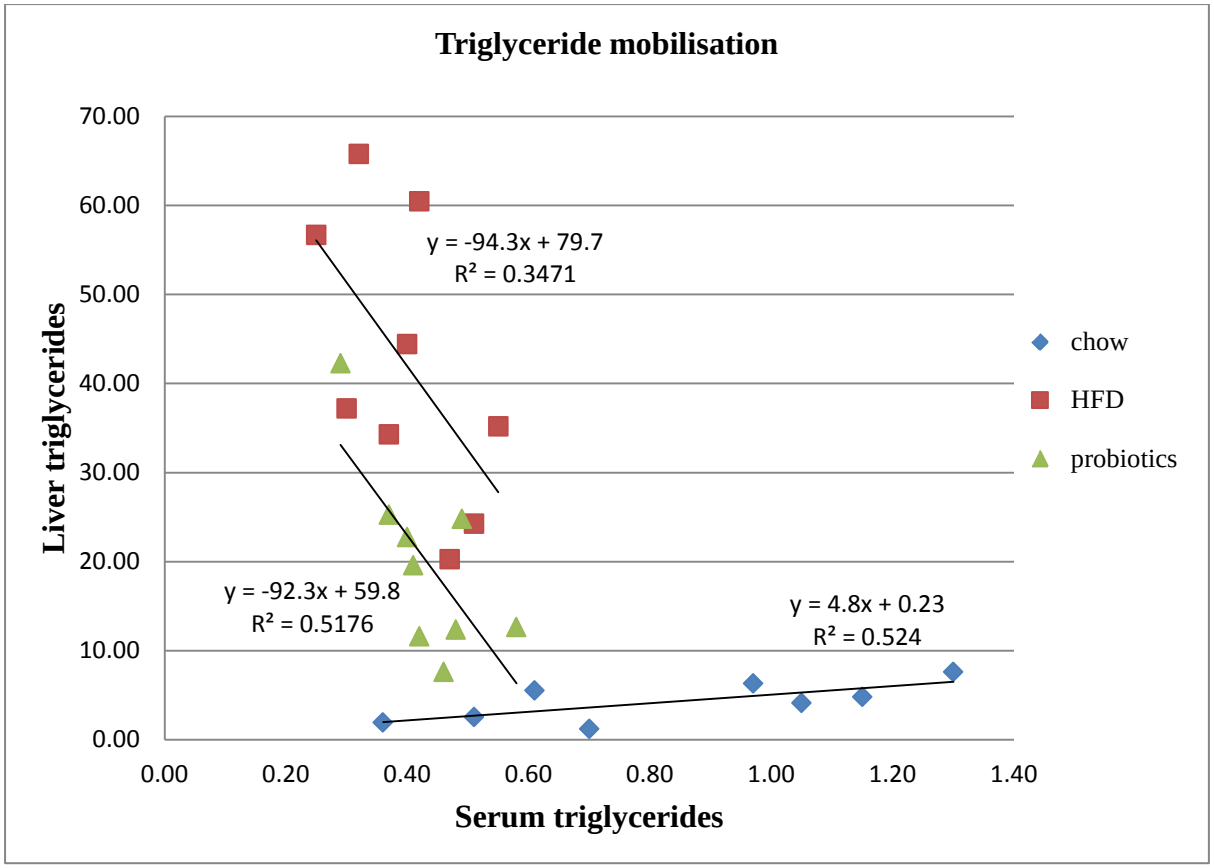


Figure 3.5

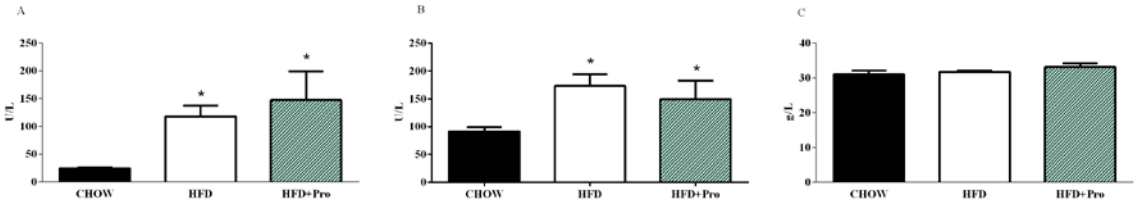


Figure 3.6

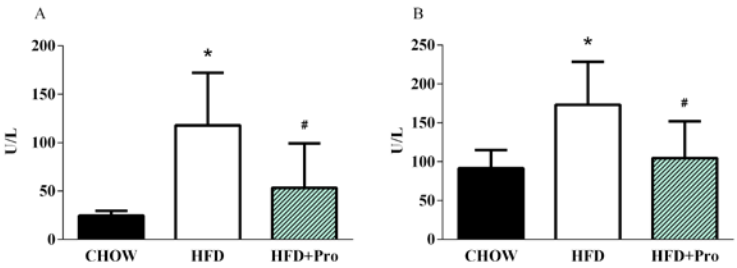


Figure 3.7

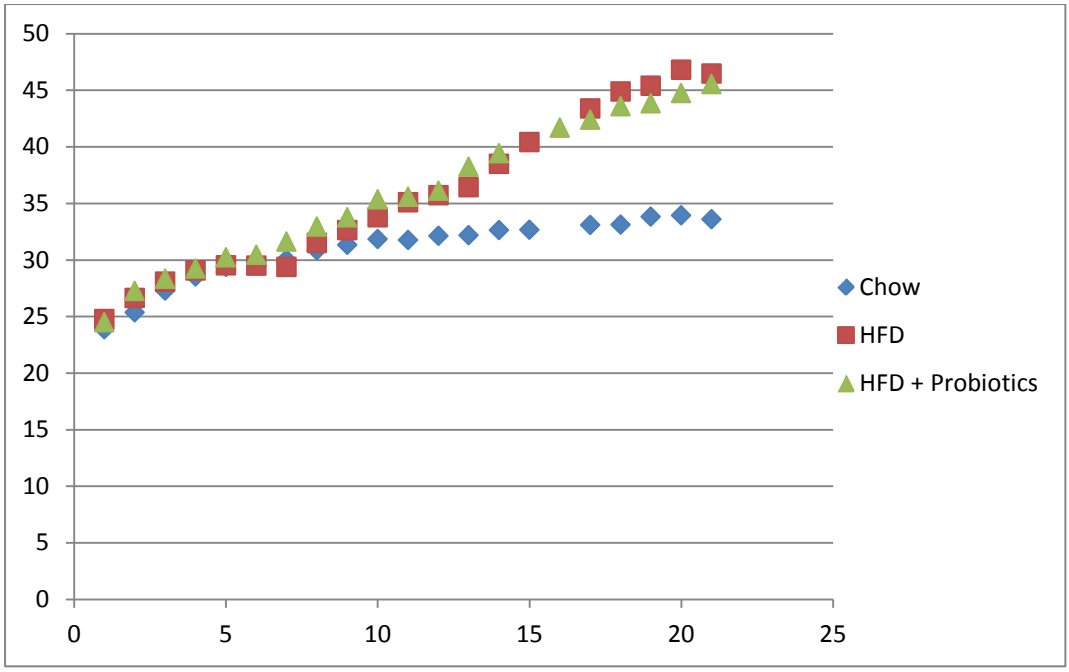


Figure 3.8

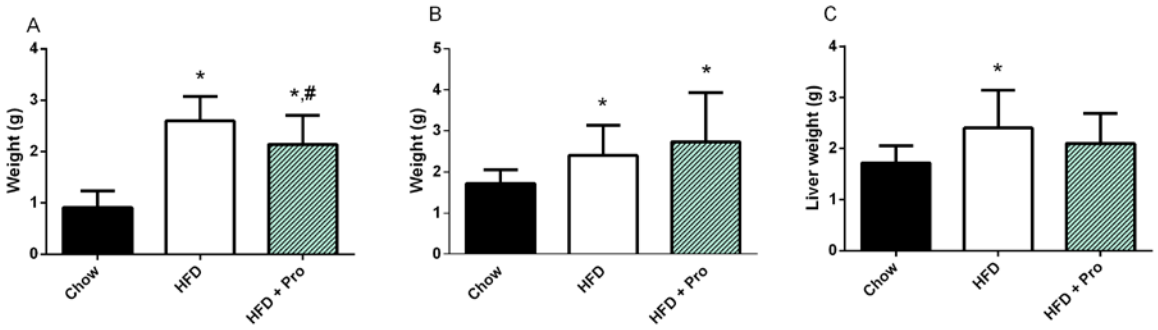


Figure 3.9

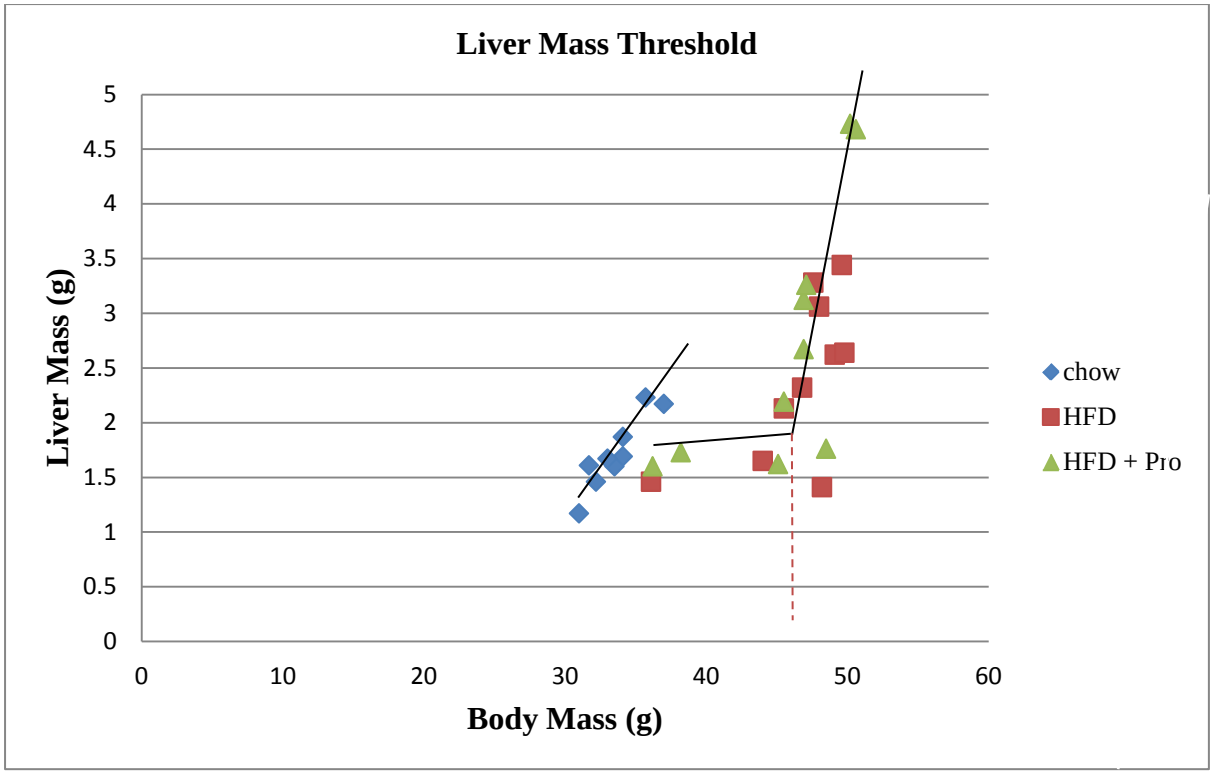

Liver Mass Threshold
Liver Mass (g)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60
Body Mass (g)
chow
HFD
HFD + Pro

Figure 3.10

Chapter 4

The effects of probiotics supplementation on a high fat diet in a high iron environment

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4.1 Abstract

Obesity and non-alcoholic fatty liver disease (NAFLD) are associated with increased extracellular iron concentrations and mutations to the HFE gene, whereas increased extracellular iron alters the microbiome within the gastrointestinal tract. Probiotics have been advanced as a potential therapeutic option that may rescue a dysbiotic gastrointestinal tract by readjusting the local commensal bacterial cohort and its environment. Few studies have investigated the effects of probiotic supplementation on NAFLD in a high iron milieu. **Aim:** This study investigated the effects of a multi-strain probiotic blend on lipid metabolism and liver function in mice with high extracellular iron and fed a high fat diet so as to induce NAFLD. ***Methods*:** Hemochromatosis protein knock out ($hfe^{-/-}$) mice were fed one of three diets: (i) a chow diet, (ii) a high fat diet for 20 weeks, or (iii) a high fat diet (10 weeks) and supplementation with multi-strain probiotics (predominantly *Lactobacillus* and *Bifidobacterium*) for another 10 weeks. Hepatic enzymes, triglycerides, cholesterol and histology were analysed, along with gene analysis of markers of liver function, lipid metabolism and inflammation. ***Results*:** Compared with HFD mice, the liver enzyme activity of alanine transaminase and aspartate transaminase, together with histology scoring for portal inflammation, Mallory's hyaline and steatosis were lower in mice fed a high fat diet with probiotics supplementation. Conversely, the expression of genes involved in lipid metabolism (e.g., peroxisome proliferator-activated receptor alpha and carnitine palmitoyltransferase 1) and proteins involved in iron uptake (e.g., transferring receptors 1 and 2) were higher in mice fed a high fat diet with probiotics supplementation compared with high fat fed mice. Probiotics supplementation also restored gene expression of liver fatty acid-binding protein (L-FABP) to a level similar to chow fed mice. ***Conclusion*:** Probiotics supplementation shows potential for protecting against NAFLD by increasing lipid metabolism and iron uptake and reducing fat deposits within the liver of mice fed a high fat diet.

4.2 Introduction

Non-alcoholic fatty liver disease is one of the most prevalent liver diseases in developed countries.[128, 129] Increased consumption of fat is among the major triggering factors of metabolic impairments and gut dysbiosis that can lead to obesity and NAFLD.[130, 131] A commonly accepted experimental model for studying NAFLD is to feed mice a high fat diet. If left untreated, progression of NAFLD to non-alcoholic steatohepatitis (NASH) may result in cirrhosis of the liver. The liver receives 70% of its blood supply from the gastrointestinal tract.[124] Consequently, disruption of the gastrointestinal tract can lead to increased inflammation of the liver, resulting in the development and/or progression of diseases such as NAFLD and NASH.[124, 132, 133] The functional interactions between the gastrointestinal tract and its microbiota lead to complex metabolic outcomes. Rescuing the gastrointestinal tract microbiome and its associated milieu is proposed to help re-regulate inflammation, attenuate intestinal permeability, and reduce the adverse effects resulting from pathogenic bacterial toxicity.[134, 135]

Mutation in the HFE gene is common in patients with NAFLD.[136, 137] This mutation disrupts iron absorption, resulting in increased extracellular iron.[138, 139] Iron is an essential element for all bacteria, with a few exceptions such as *B. burgdorferi*[140] (implicated in Lyme disease), and some lactic acid bacteria.[141] Elevated levels of extracellular iron may result in increased gastrointestinal tract pathogen toxicity[142] resulting in increased intestinal permeability and inflammation.[143-145] One of the body's first defenses for maintaining gastrointestinal tract health in the presence of invading pathogens is to withhold nutrients.[146, 147] One such involves transferrin preferentially binding extracellular iron to prevent pathogenic bacteria from accessing iron.[148] Transferrin is predominantly produced in the liver and is decreased in inflammation.[149] Inflammation is prevalent with NAFLD, which may limit transferrin production. NAFLD patients often present with iron overload and transferrin saturation.[138] This indicates an inability to bind extracellular iron adequately, which may allow pathogenic bacteria to prosper and associated inflammation to occur.

A commonly accepted model for studying hemochromatosis (iron overload) is the *hfe*$^{-/-}$ mouse model. The high iron environment created in hfe$^{-/-}$ mice increases the virulence of numerous pathogens in animal models.[147] The normal flora are unable to thrive in an environment high in iron, because they are 'out-competed' by the bacterial overgrowth of

pathogenic bacteria.[150, 151] The overgrowth of bacteria leads to increased intestinal inflammation and subsequently intestinal permeability[152, 153], which may in turn allow pathogens to reach the liver where they can initiate inflammation and NAFLD. Restoring the gastrointestinal tract environment may allow the natural gastrointestinal tract microbiome to prosper and restrict the growth of pathogenic bacteria.

Probiotics supplementation is touted as a possible therapy for rescuing the gastrointestinal tract environment.[154, 155] The implementation of a multi-strain probiotic blend, combining lactic acid bacteria (*Lactobacillus* and *Streptococcus*) with gram positive bacteria (*Bifidobacteria*) aimed to establish a gastrointestinal tract environment where the commensal microflora would favourably compete with pathogenic bacteria. By combining the *hfe*$^{-/-}$ mouse model with a high fat diet so as to induce NAFLD, it is possible to investigate the potential effects extracellular iron on NAFLD. Using this model, I investigated the effects of a multi-strain probiotic blend combining strains of *Bifidobacteria*, *Lactobacilli* and *Streptococcus* on inflammation and lipid metabolism in the gastrointestinal tract and liver in *hfe*$^{-/-}$ fed a high fat diet. I hypothesised that in an environment high in iron, the administration of a multi-strain probiotic would enable the resultant gastrointestinal tract microbial cohort to function better in the presence of high iron.

4.3 Method

Study Design

Mice were fed either a chow diet or a HFD for a total of 20 weeks. At the end of week 10, one HFD group were supplemented with probiotics in their drinking water for the remaining 10 weeks. At the end of week 20 all mice were euthanized, dissected and blood and tissue samples were stored for later analysis.

Animals

Four-week old *hfe*$^{-/-}$ mice (on a C57BL/6J background, originally supplied by Professor William Sly, St Louis University, MO) were randomly distributed into one of three groups: 1) a control group receiving a standard chow diet (n=9) for 20 weeks; 2) a group fed a high fat diet (HFD) (n=9) for 20 weeks; and 3) a HFD group for 10 weeks supplemented with probiotics (n=9; $1 \times 10^{8\text{-}9}$ CFU/mL) for another 10 weeks. All animals were housed in a SPF facility maintained at 20°C on a 12 hour light/dark cycle with access to clean water and food.

All procedures were carried out in accordance and with approval from The University of Queensland and Queensland Institute of Medical Research (QIMR) ethics committees.

Diet

The chow diet and HFD were purchased from Specialty Feeds (WA, Australia; HFD product no. SF03-020). The chow diet contained 4.8% total fat (monounsaturated fat 2%, polyunsaturated fat 1.8% and saturated fat 0.7%), providing 14 MJ/kg of energy. The HFD contained 23% total fat (monounsaturated fat 7.6%, polyunsaturated fat 2.0% and saturated fat 12.6%), providing 20 MJ/kg of energy.

Bacterial Strains

Nine strains of probiotics (*L. rhamnosus – 34.5%, Lactobacillus casei – 21%, Lactobacillus acidophilus – 16.5%, L. plantarum – 7%, Lactobacillus fermentum – 3%, Bifidobacterium lactis – 9%, Bifidobacterium breve – 3%, B. bifidum – 1%, S. thermophilus – 5%*), were kindly donated by Fit BioCeuticals (NSW, Australia). All strains were lyophilised, water soluble and added to the drinking water as a combination probiotic. Probiotics were added to fresh water every second day. The total concentration of probiotics added to the drinking water was $1 \times 10^{8\text{-}9}$ CFU/mL. This dosage was chosen because it represents a dose per kilogram of body mass equivalent for a human (65 kg) and the amount of water consumed (5 mL) by an average-sized mouse (25 g) per day.

Dissection

At the conclusion of week 20, all mice were anaesthetised using an intraperitoneal injection of pentobarbital and xylene. Once the mice were anaesthetised, blood was removed by cardiac puncture, and it was left to clot at room temperature before it was centrifuged to remove and store serum. The liver was removed and the mass was recorded before pieces (~50-100 mg) of liver tissue were snap frozen in liquid nitrogen or placed in formalin for histology. Tissue placed in formalin for fixing was transferred to a 70% ethanol solution after 24 hours.

Blood Biochemistry

Serum was analysed spectrophotometrically for the activity of the liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST), and the concentrations of albumin, glucose, total cholesterol and triglycerides. Analysis was performed using a Cobas Integra 400 auto-analyser, with reagents and calibrators supplied by Roche Diagnostics (NSW, Australia).

Hepatic Triglycerides

Prior to analysis, liver tissue was homogenised in a 1.5% potassium chloride solution (2.3 g KCl in 200 mL water). The homogenate (500 μl) was extracted using a 2:1 chloroform/methanol mixture. Extracts were dried and stored at -80°C until analysis. For analysis, samples were reconstituted using 2% triton-x with the aid of sonication. Samples were further diluted with 2% triton-x to a final concentration of 1:6 for analysis. Triglycerides were measured spectrophotometrically (Cobas Mira, Roche Diagnostics, Australia) using a kit and calibrators supplied by Novachem (Victoria, Australia).

Hepatic Isoprostanes

Isoprostanes were extracted from liver tissue using a previously reported method.[156] (Chapter 6, published in *J. Pharmacology and Biomedical Analysis*)

Hepatic Iron Concentration

Hepatic iron concentration (HIC) was measured as previously described.[157] Briefly, paraffin-embedded samples were deparaffinised, washed, dried, weighed and acid digested. HIC was measured using an atomic absorption spectrophotometer (Varian AAS 4) at a wavelength of 248.3 nm.

Protein

Liver protein concentration was measured as per the manufacturer's direction using a Pierce BCA protein assay kit supplied by Thermo Scientific (Victoria, Australia).

Histological Scoring

Histology was scored blindly on liver sections with H and E and oil red O staining. Sections were scored for diagnosis, NAFLD activity score, steatosis grade and percentage, portal

inflammation, lobular inflammation and ballooning, Mallory's hyaline (resulting from hepatocyte injury), fibrosis stage, portal score and centrilobular score.

RNA extraction and Quantitative Real-time RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Mount Waverley, Victoria, Australia), and then subjected to deoxyribonuclease I digestion and transcribed into cDNA using Superscript III according to the manufacturer's instructions (Invitrogen, Mount Waverley, Victoria, Australia). Quantitative gene expression was performed by real-time polymerase chain reaction (RT-PCR) (ViiA^{TM} 7, Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA) using Quantifast SYBR green as per the manufacturer's conditions (Qiagen, Chadstone Centre Victoria, Australia). Genes involved in lipid metabolism were assessed (Table 4.1), including liver fatty acid-binding protein (L-FABD), proliferator-activated receptor alpha and gamma (Ppar-α and Ppar-γ), carnitine palmitoyltransferase 1 (Cpt1A), adiponectin receptor 2 (AdipoR2), cluster of differentiation 36 (CD36), low-density lipoprotein receptor (LDLR), and acetyl-CoA carboxylase (ACC1). The expression of individual genes was normalised to the geometric mean of three house-keeper genes: basic transcription factor 3 (Btf-3), Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and β2-microglobulin (B2M). Oligonucleotides were custom synthesised by Sigma Genosys (Castle Hill, NSW, Australia).

Genes	Forward (5′→3′)	Reverse (5′→3′)
L-FABP	AAGTCAAGCCAGTCGTCAAGCT	TGAGTTCAGTCACGGACTTTATG
Ppar-α	CATGTGAAGGCTGTAAGGGCTT	TCTTGCAGCTCCGATCACACT
Ppar-γ	CATTGAGTGCCGAGTCTGTG	GCTTCAATCGGATGGTTCTT
Cpt1A	AGACCGTGAGGAACTCAAACCTA	TGAAGAGTCGCTCCCACT
AdipoR2	TACACACAGAGACGGGCAAC	TGGCTCCCAAGAAGAACAAG
CD36	ACAGACGCAGCCTCCTTTC	CAGATCCGAACACAGCGTAG
LDLR	GGACCTCAAGATTGGCTCTG	TGTAGCTGCCTTCCAGGTTC
ACC1	TGAAGACCTTAAAGCCAATGC	GTTGTTGTTGGGTCCTCCA
Reference Genes	Forward (5′→3′)	Reverse (5′→3′)
Btf3	TGGCAGCAAACACCTTCACC	AGCTTCAGCCAGTCTCCTTAAAC

B2M	CTGATACATACGCCTGCAGAGTTAA	ATGAATCTTCAGAGCATCATGAT
Gapdh	TCCTGCACCACCAACTGCTTAGC	GCCTGCTTCACCACCTTCTTGAT

Table 4.1: qRT-PCR gene primers.

Western blots

Supernatant or protein extracts (30 μg) from liver and isolated gut cell homogenates were electrophoresed in sodium dodecyl sulfate-10% polyacrylamide gel for 30 min at 100 V and blotted onto polyvinylidene fluoride membranes (Biorad, Hercules, CA). Following incubation with 5% skim milk to block nonspecific sites, membranes were immunostained with primary antibodies for transferrin receptor 1 (TfR1) and transferrin receptor 1 (TfR2; Zymed, life technologies, Vic, AUS). A horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (Santa Cruz) secondary antibody was added, and protein bands were visualised using a standard chemiluminescent kit (Supersignal West Femto, Thermo Scientific).

Statistical analysis

All data are presented as mean ± standard deviation, unless otherwise stated. Data were tested for normality of the distribution, and analysis was performed with the statistical software GraphPad Prism 6. Comparison between groups was carried out using either a 1-way analysis of variance with a Tukey's multiple comparison post hoc test or a Kruskal-Wallis test for non-parametric data with a Dunn's multiple comparisons post hoc test of significance between individual groups. Differences were considered significant when *P* was less than 0.05. Unless stated otherwise, all significant differences between groups were determined using the HFD group as the reference group.

4.4 Results

There were no significant differences in the initial body mass of the three groups (chow 23.4 ± 1.0 g; HFD 25.9 ± 1.0 g; HFD+Probiotics 24.6 ± 2.1 g) ($p = 0.9$). By week 20, the body mass of both HFD groups were ~25% greater than the chow group (chow 34.9 ± 2.7 g; HFD 46.8 ± 2.9 g; HFD+Probiotics 45.6 ± 3.2 g; $p<0.001$; Figure 4.1). Liver mass was also greater in the HFD groups (chow 1.7 ± 0.2 g; HFD 3.1 ± 0.8 g; HFD+Probiotics 2.6 ± 0.8 g; $p<0.05$; Figure 4.1). Histological examination of livers from HFD group showed steatosis with large fat droplets present. Chow fed mice showed no steatosis (Table 4.2) or fat accumulation.

Table 4.2: Liver histology grading

	Chow (n=9)	HFD (n=9)	HFD+Probiotics (n=10)
NAS	0.0[§] ± 0.0	6.1 ± 0.9	5.5 ± 2.0
Steatosis Grade	0.0[§] ± 0.0	3.0 ± 0.0	2.6 ± 1.1
Steatosis %	0.0[§] ± 0.0	95.5 ± 6.4	84.0 ± 32.5
Portal Inflammation	0.0[*] ± 0.0	0.44 ± 0.53	0.25 ± 0.38[*]
Lobular Inflammation	0.0[§] ± 0.0	1.6 ± 0.5	1.5 ± 0.6
Ballooning	0.0[§] ± 0.0	1.5 ± 0.5	1.4 ± 0.6
Mallory's Hyaline	0.0[§] ± 0.0	1.6 ± 0.5	0.00[§] ± 0.0

Data are mean ± SD. [*] = $p<0.05$; [§] = $p<0.001$; NAS – NAFLD activity score

Compared with the chow fed group, serum cholesterol concentration (Figure 4.3) and ALT and AST activity (Figure 4.2; $p<0.001$) were higher, whereas serum triglycerides (Figure 4.3; $p<0.001$) were lower in the HFD groups. Hepatic triglycerides ($p<0.05$) and hepatic protein ($p<0.001$) were also higher, whereas hepatic iron concentrations were lower (Figure 4.4 & 8; $p<0.01$) in the HFD groups compared with the chow fed group. Gene expression of PPAR- γ ($p<0.01$), CD36 ($p<0.001$) and L-FABP ($p<0.01$) were higher (Figures 5 & 6), whereas gene expression of LDLr (Figure 4.6; $p<0.05$) and AdiopR2 (Figure 4.7; $p<0.001$) and protein expression of TFR-1 (Figure 4.8; $p<0.001$) was lower in the HFD groups compared with the chow fed group.

Compared with the HFD group, serum triglyceride concentration was higher ($p<0.05$; Figure 4.3), whereas serum ALT and AST activity was lower ($p<0.05$; Figure 4.2) in the HFD+Probiotics group. The gene expression for PPAR-α, CPT1 and AdipoR2 was higher ($p<0.05$; Figures 5-7), whereas L-FABP gene expression was lower ($p<0.05$; Figure 4.6) in the HFD+Probiotics group compared with the HFD group. Lastly, hepatic isoprostanes

content ($p<0.05$; Figure 4.4) and protein expression of TFR-1 and TFR-2 ($p<0.001$; Figure 4.8) were higher in the HFD+Probiotics group compared with the HFD group.

4.5 Discussion

The aim of this study was to investigate the effects of a multi-strain probiotic blend combining strains of *Bifidobacteria*, *Lactobacilli* and a *Streptococcus* on inflammation and lipid metabolism in the gastrointestinal tract and liver in the *hfe*$^{-/-}$ mice fed a high fat diet. These results demonstrate that compared with the high fat diet group, a multi–strain probiotic blend increased the expression of genes that regulate lipid metabolism (PPAR-α, Cpt1A and AdipoR2) and improved liver function, as indicated by lower activity of the liver enzymes alanine transaminase and aspartate transaminase. These changes with probiotics supplementation were associated with partial reductions in portal inflammation and Mallory's hyaline, and increased expression of iron transport proteins. The results of this study suggest that probiotics may be beneficial for people with iron overload (e.g. haemochromatosis), dysregulated lipid metabolism and/or NAFLD.

Probiotic supplementation was effective for increasing liver enzyme activity, and reducing portal inflammation and Mallory's hyaline compared with the high fat diet group. Probiotics did not restore liver enzyme activity and inflammation to the same levels in the chow fed group. Nevertheless, these improvements suggest that probiotics improve liver function and reduce NAFLD resulting from a HFD. Although, improvements in steatosis grade and percentage in the probiotics group were not statistically significant, these variables showed a trend toward improvement. When non-responders (n=3) were removed, treatment with probiotics significantly improved steatosis grade and percentage. If the mice had been treated with probiotics earlier (i.e., after less than 10 weeks), then steatosis grade and percentage may have improved to a greater extent.

The availability of iron in the gastrointestinal tract directly affects the composition and metabolic state of the gut microbiota.[158, 159] This may partly explain how different dietary regimens (fat vs. protein vs. carbohydrate) alter the gut microbial phyla,[160, 161] In the present study, probiotics supplementation may not have reduced inflammation entirely because extracellular iron was already elevated and may have promoted pathogenic bacterial

toxicity.[150, 151] Compared with wild type mice, the faecal microbiota in *hfe*$^{-/-}$ mice has a greater proportion of *Enterococcus faecium* and species similar to Olsenella.[158] Changes to the microbiota milieu may lead to changes in epithelial cell functions and permeability[162] that in turn alter nutrient absorption and gastrointestinal tract inflammation.[163]

In humans, 80-95% of iron consumed in the diet passes through the gastrointestinal tract and is not absorbed.[164] This leaves high amounts of iron available for the pathogenic bacteria within the gastrointestinal tract. The amount of iron that is absorbed changes with different age, sex and strains (C57BL/6, DBA/2J, BALB/c & AKR) of mice.[165, 166] Changes to the amount of iron absorbed as humans age[167] may partially explain why the gut microbiota composition changes with age.[168] Reductions to the amount of iron absorbed with aging[167] makes more free iron available in the gastrointestinal tract for bacterial growth. This can alter the microbiota composition and concentration.[158] One mechanism that alters the amount of extracellular iron, is changes to receptors for transferrin bound iron. In the present study, HFD feeding reduced hepatic TFR-1 and TFR-2 protein expression compared with chow fed mice (Figure 4.8). TFR-1 and TFR-2 are required for iron delivery from transferrin, and mediate cellular uptake of transferrin-bound iron.[169] Probiotics supplementation partially increased TFR-1 protein expression, whereas it increased TFR-2 protein expression above that in chow fed mice. Increased TFR-1 and TFR-2 may reduce extracellular iron by increasing iron delivery for cell metabolism through the release of transferrin-bound iron. As evidence of increased cellular iron uptake, hepatic iron concentration was higher in mice supplemented with probiotics compared with the high fat diet group (Figure 4.8). A reduction in extracellular iron would further allow the natural microbiota to return to a regulated state.

Liver isoprostane content was lower in *hfe*$^{-/-}$ mice fed chow when compared wild type mice (Chapter 5). By contrast, probiotics increased liver isoprostane content ($p<0.05$) in *hfe*$^{-/-}$ mice to similar levels of chow fed mice (Chapter 5). Iron protects human endothelial cells against hydrogen peroxide-induced reductions in prostaglandin synthesis, possibly by initiating an extracellular reduction of free electrons available to reach the intracellular space (Fenton reaction).[170] In the presence of an overload with iron, free radicals may be neutralised, thereby blocking lipid peroxidation/redox signalling. In turn, this may alter cellular lipid metabolism. My data suggest that probiotics supplementation may increase/regulate lipid

peroxidation in the presence of high extracellular iron. Probiotics may exert these effects by reducing extracellular iron through increasing cell iron delivery transferrin receptor proteins.

Probiotic supplementation increased PPAR-α gene expression (Figure 4.5) compared to HFD and chow fed mice. This may serve to aid in fatty acid transport and lipid and glucose metabolism. Under normal circumstances PPAR-α and PPAR-γ gene expression is maintained in a regulated state, but in the presence of fatty acids, expression is increased.[171, 172] This may serve to mobilise fatty acids and prevent glucose accumulation / type II diabetes mellitus.[173] Consumption of a HFD also reduced AdipoR2 gene expression, whereas probiotics partially reversed this response (Figure 4.7). One potential explanation for why PPAR-α gene expression increased following probiotics supplementation, is that probiotics partially blocked the decrease in AdipoR2 gene expression that occurred in response to high fat feeding. Enhancement of AdipoR2 has been shown to improve NASH,[174] while a reduction in AdipoR2 results in reduced PPAR-α signalling.[174, 175]

Gene expression of CD36 (Figure 4.5) was increased with HFD feeding. In HFD fed mice, elevated CD36 gene expression may indicate dysregulated intestinal inflammation[176] and lipid accumulation[177] propagating NAFLD.[178-180] HFD also reduced LDLr gene expression (Figure 4.6), potentially resulting in accumulation of low-density lipoproteins and advancing NAFLD. However, Cpt1A gene expression was up regulated with probiotics supplementation (Figure 4.6), which may promote the transport and metabolism of long-chain fatty acids across cell membranes. The up-regulation of Cpt1A may therefore partially compensate for the down-regulation of LDLr. Probiotics also restored liver fatty acid-binding protein (L-FABP) gene expression to a similar level to that in chow fed mice (Figure 4.6). Probiotics supplementation increased lipid metabolism/signalling pathways (Cpt1A and PPAR-α) that may reduce lipids accumulating in the liver. This restored L-FABP expression to similar levels as in chow fed mice. Lipid metabolism may also be affected by changes in the expression of ACC1. There was no significant difference between the three groups for ACC1 gene expression. However, ACC1 gene expression tended to be lower following the high fat diet ($p=0.2$; Figure 4.7), which could have reduced fatty acid metabolism. By contrast, ACC1 gene expression tended to be higher following probiotics supplementation ($p=0.4$). These findings provide tentative evidence that probiotics may help to reverse (at least in part) the detrimental metabolic effects of high fat feeding.

In summary, probiotic supplementation reduced liver enzyme activity, liver histology scores for portal inflammation and Mallory's hyaline, whereas it increased the expression PPAR-α and Cpt1A genes and TFR-2 protein. By contrast, probiotics supplementation did not alter gene expression of CD36 or LDLr. The results of this study have beneficial implications for people with haemochromatosis, dysregulated lipid metabolism and/or reducing the progression of NAFLD alike. Future studies could investigate the effects of prophylactic treatment with probiotics over a longer period.

4.6 Figures

Figure 4.1: Body mass. HFD resulted in increased A) body mass and B) liver mass. Probiotics were able to partially prevent the liver mass gain.

Figure 4.2: Serum data. HFD feeding increased A) ALT and B) AST. Probiotics rescued this effect in part.

Figure 4.3: Serum data. A) Cholesterol was increased in both HFD groups B) Triglycerides was increased in the chow group C) Glucose was increased in the probiotics groups

Figure 4.4: Hepatic data. A) Isoprostanes were significantly increased in mice supplemented with probiotics. B) Triglycerides were elevated and C) protein reduced in both HFD groups.

Figure 4.5: RNA. A) Probiotics increased PPAR-alpha compared to chow fed mice. B) PPAR-gamma and C) CD36 levels were elevated in both groups of HFD fed mice.

Figure 4.6: RNA. A) LDLr was reduced in both HFD fed groups. B) Cpt1 was elevated compared to both HFD and chow fed mice. C) Lfabp was elevated in HFD fed mice, probiotics rescued this effect.

Figure 4.7: RNA. A) Acc1 B) AdipoR2 significantly reduced in HFD fed mice. Probiotics partially rescued this effect.

Figure 4.8: RNA. A) Tfr1 was significantly reduced in both HFD groups. B) Tfr2 was increased with probiotics supplementation. C) HICs was decreased in both HFD fed groups. Probiotics in part rescued this effect.

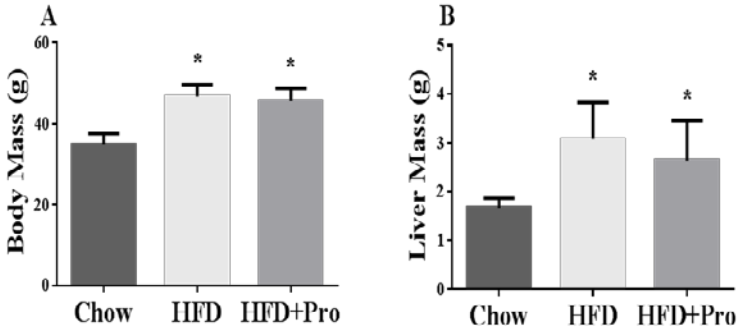


Figure 4.1

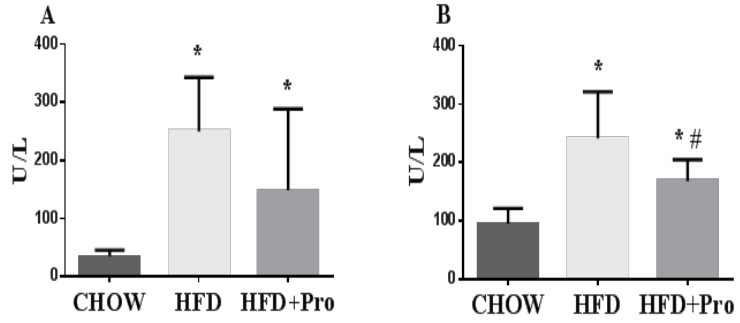
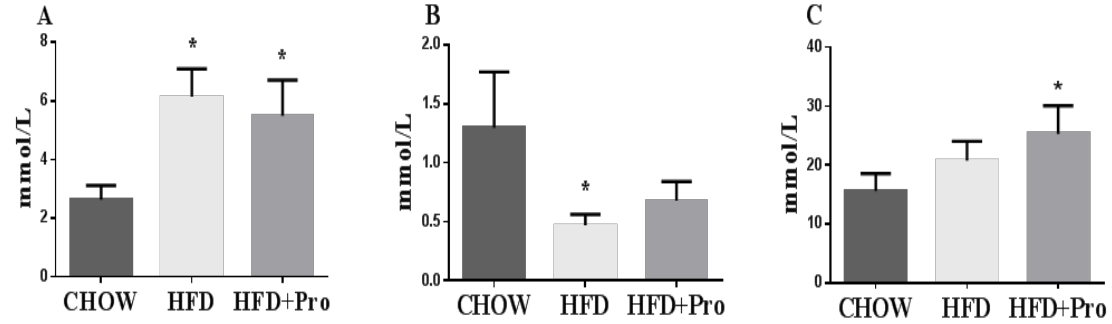


Figure 4.2



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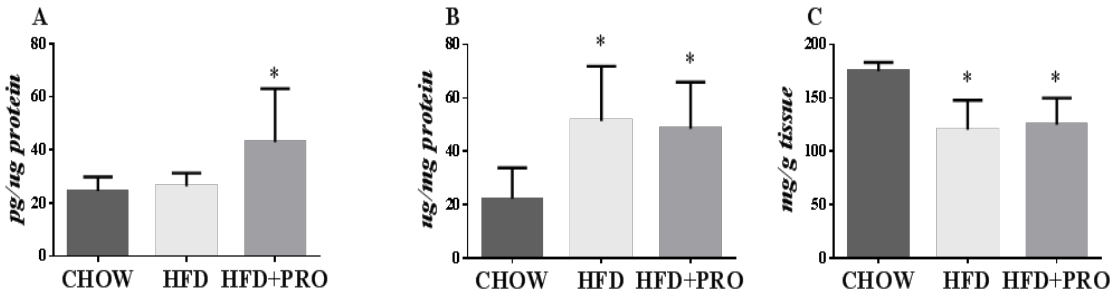

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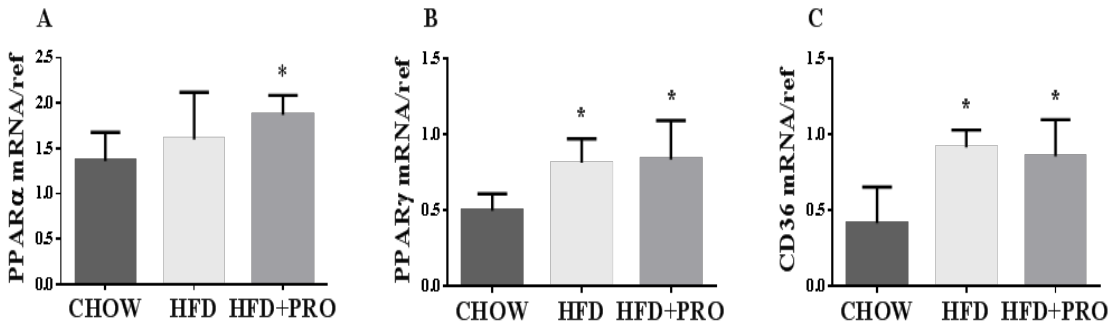


Figure 4.5

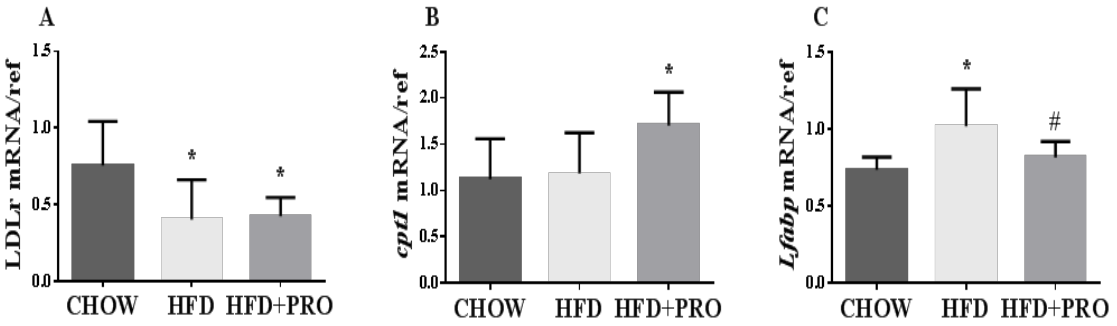


Figure 4.6

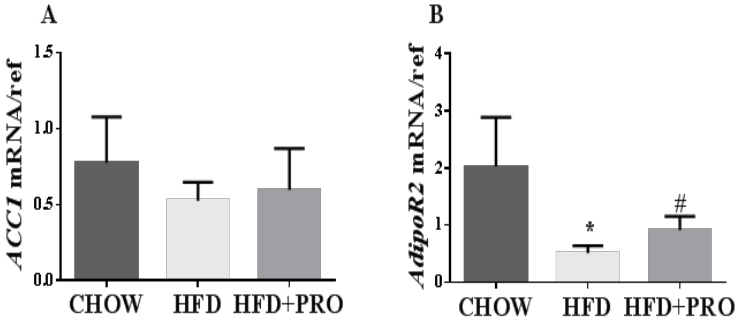
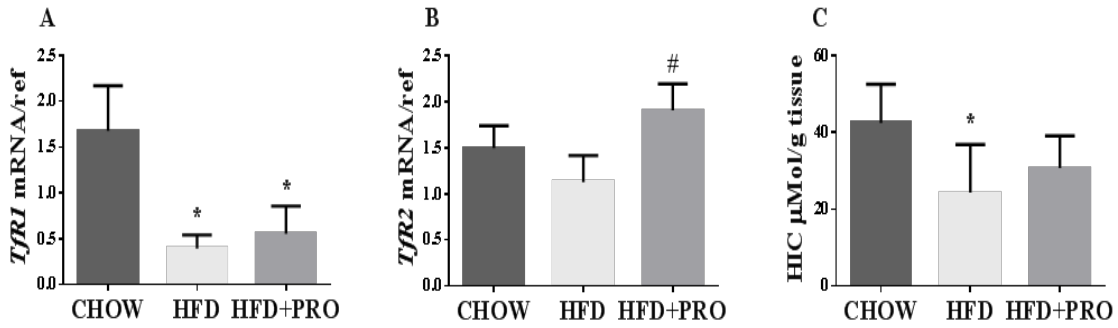


Figure 4.7


A
TfR1 mRNA/ref
2.5
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2.5
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#
CHOW
HFD
HFD+PRO
C
HIC µMol/g tissue
60
40
20
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CHOW
HFD
HFD+PRO

Figure 4.8

Chapter 5

The data presented in this chapter is a continuation of the data collected from mice in chapter 3. This data was intended to support the posit that probiotics could mitigate the adverse effects of a HFD on the liver. It was hypothesised that this data would show probiotics capable of preventing excessive oxidation of hepatic lipids. However because the findings were opposite to what was expected, the data did not fit with the structure or theme of chapter 3. Rather these findings require a standalone chapter to explain the interesting findings.

Lipid peroxidation serves as a lipid signalling pathway regulating lipid metabolism: do probiotics have a role?

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5.1. ABSTRACT

Quantification of lipid peroxidation has been a long–standing measure of cellular oxidative damage. Isoprostanes are formed through a reactive oxygen species reaction that is characterised by the oxidative degradation of arachidonic acid. Isoprostanes are currently considered the "gold standard" marker of oxidative damage. Whether the gastrointestinal tract and probiotics supplementation can affect lipid peroxidation is still unknown. ***Aim*****:** Investigate the effects of a high fat diet +/- probiotics on lipid peroxidation in the liver. ***Methods*****:** Mice were fed either a chow diet, high fat diet or a high fat diet with probiotics for 20 weeks. At sacrifice, all animals were weighed and liver samples were collected for lipid peroxidation analysis. ***Results*****:** Isoprostanes and malondialdehyde content of the liver was significantly lower in mice fed a high fat diet for 20 weeks compared with chow fed mice. Isoprostanes content correlated with malondialdehyde content and fat pad weight in the chow fed group. Malondialdehyde correlated weakly with fat pad weight in all groups. Probiotics supplementation had no effect on lipid peroxidation. ***Conclusion*****:** Probiotics supplementation proved ineffective at altering lipid peroxidation in mice fed a HFD. Reduced isoprostanes and malondialdehyde concentrations following a HFD may indicate a potential role of lipid peroxidation in lipid metabolism and accumulation.

5.2. INTRODUCTION

Reactive oxygen species (ROS) and their role in the development / progression of chronic diseases and aging have had a long theoretical history.[181, 182] The production of ROS is not spontaneous as is widely published[183], but instead is part of a regulated process.[184] Recently it has been posited and scientifically supported that intracellular generation of ROS are important signalling molecules essential for the normal functioning of the human metabolome[183, 185, 186] and have beneficial immune functions that protect against infective agents.[179, 182] However, dysregulation of ROS production can cause tissue damage. Under normal, healthy conditions, several enzymatic mechanisms prevent this event from occurring[183]. [184, 187]

Lipid peroxidation is thought to be an important factor in the pathophysiology of a number of diseases[181] and has gained interest as a marker for quantifying the purported damage due to ROS. F_2-isoprostanes have been established as an accurate and stable marker for measuring

lipid peroxidation *in vivo.*[188, 189] F_2-isoprostanes are produced from the oxidative degradation of arachidonic acid, and are considered the "gold standard" marker of lipid peroxidation. Quantification of F_2-isoprostanes in biological samples is difficult however. Increasing evidence supports the notion that isoprostanes have strong biological effects.[188, 190, 191] The pathophysiological role isoprostanes and other lipid peroxidation compounds have in certain diseases still remains relatively unknown.

In recent decades, a number of molecular mechanisms have been found to contribute to the development of NAFLD and its progression to non-alcoholic steatohepatitis (NASH). Studies have reported that the pathogenesis of NAFLD and NASH is due to the "two hit" hypothesis. This depicts a state where one physiological mechanism is responsible for the initial development of NAFLD, and a second mechanism is responsible for the progression of the disease to NASH. Within the two hit theory, oxidative damage, dysregulated cytokine production and other inflammatory mediators may each play a role, with lipid peroxidation promoted as a leading causative factor.[192, 193] Hence, ROS are extensively reported to promote lipid peroxidation, leading to detrimental effects on hepatocytes and other hepatic cells.[192] ROS are also reported to increase cellular production of pro-inflammatory cytokines, promoting cell death, inflammation and fibrosis.[194]

It has been reported that there is a differential effect on ROS activity with diets comprised predominantly of fat over a balanced diet.[195-198] Also diets rich in saturated fat content can be very stable to oxidation, whereas diets rich in polyunsaturated fatty acids (PUFAs), specifically 18:2n-6, are prone to oxidation.[199] There is also published literature on how diet can alter the composition of the intestinal microbiota.[200-204] Diets rich in carbohydrate, fat or protein alters the microbiota population to favour a specific enterotype (*Bacteroides, Prevotella or Ruminococcus*).[205-207] Changes to the gastrointestinal microbiota as a result of an unbalanced diet can promote dysbiosis increasing the risk inflammatory diseases.[160, 161]

The aim of this study was to investigate the effects of lipid peroxidation in response to a high fat diet with a secondary aim to examine whether probiotics altered this response. I hypothesised that a diet high in fat would cause increased lipid peroxidation and that probiotics could rescue the altered lipid peroxidation by modifying the gut microbiota and re-regulating the gut-liver-axis.

5.3. METHODS

Study design

Mice were fed either a chow diet or a HFD for a total of 20 weeks. At the end of week 10, probiotics were added to the drinking water of one group for the remaining 10 weeks. At the end of week 20 all mice were euthanized, dissected and samples stored for later analysis.

Animals

Eight week old C57B1/6J mice (Animal Resources Centre, Perth, WA, Australia) were randomly assigned into one of three groups: 1) a control group, receiving a standard laboratory chow diet (chow; n=9); 2) a high fat diet group (HFD) (n=10); and 3) HFD fed group supplemented with probiotics (probiotics; n=10; $1 \times 10^{8\text{-}9}$ CFU/mL). All animals were housed in a SPF facility with *ad libitum* access to clean food and water. Housing conditions were maintained at 19°C to 23°C with 50% humidity on a 12 hour light/dark cycle. All animals received humane care under the guidelines and approval of the Queensland Institute of Medical Research and the University of Queensland Animal Ethics Committees.

Bacterial strains

Nine strains of probiotics (*L. rhamnosus – 34.5%,* Lactobacillus casei – 21%, *Lactobacillus acidophilus – 16.5%, L. plantarum – 7%,* Lactobacillus fermentum – 3%, Bifidobacterium lactis – 9%, *Bifidobacterium breve – 3%, B. bifidum – 1%, S. thermophilus – 5%*), were kindly donated by Fit BioCeuticals (NSW, Australia). All strains were lyophilised, water soluble and added to the drinking water as a combination probiotic. The total concentration of probiotics added to the drinking water was $1 \times 10^{8\text{-}9}$ CFU/mL. This dosage was chosen because it represents a dose per kilogram of body mass equivalent for a human (65 kg) and the amount of water consumed (5 mL) by an average-sized mouse (25 g) per day.

Diet

The standard laboratory chow and HFD were purchased from Specialty Feeds (WA, Australia; HFD product SF03-020). The chow diet contained 4.8% total fat (mono unsaturated fat 2.0%, polyunsaturated fat 1.8% and saturated fat 0.7%) providing 14MJ/Kg of energy while the HFD contained 23% total fat (mono unsaturated fat 7.6%, polyunsaturated fat 2.0% and saturated fat 12.6%) providing 20MJ/Kg of energy.

Dissection

At the conclusion of week 20, mice were anaesthetised using an intraperitoneal injection of pentobarbital and xylene. Once anaesthetised, animals were killed via cardiac puncture for blood collection. The blood was allowed to clot at room temperature before serum was removed and stored at -80°C. The liver was removed, weighed, cut into small pieces and snap frozen in liquid nitrogen for determination of oxidative compounds as detailed below.

Isoprostanes

Tissue extraction

Isoprostanes were extracted from liver tissue using a previously published method.[156] Samples were weighed and 1mL of methanolic NaOH added. Samples were incubated at 42 °C for 30 minutes, homogenised and 400 µl transferred to a screw-capped glass tube. A further 400 µl of methanolic NaOH was added together with 800 µl of distilled water and spiked with 400 pg/mL of 8-iso-$PGF_{2\alpha}$-d_4 internal standard (Cayman Chemicals, USA). The resulting solution was vortex mixed, capped and incubated at 42°C for 60 minutes.

Once removed from incubation, samples were placed on ice for 10 minutes then acidified to pH 3 with 450 µl of 3 M hydrochloric acid and vortex mixed. Hexane was added (3.0 mL), gently rotated for 10 minutes and centrifuged at 3000 × g; hexane supernatant was removed and discarded. The remaining solution was then extracted with ethyl acetate (3.0 mL); with extracts transferred to a clean tube and dried under nitrogen. Once dry, the samples were reconstituted with 200 µl of acetonitrile, vortex mixed, transferred into 400µl silinised glass inserts and dried for derivatization.

Using a derivatization method based on that by Mori and colleagues[208], 40 µl pentafluorobenzylbromide (PFBBr, 10% in acetonitrile) and 20 µl Diisopropylethylamine (DIPEA, 10% in acetonitrile) were added to the dried extract, which was then incubated at room temperature for 30 minutes. Samples were then dried under nitrogen and treated with 10 µl of Pyridine and 20 µl of Bis(trimethylsilyl)trifluoroacetamide + Trimethylchlorosilane (BSTFA + TMCS 99:1) and incubated at 45°C for 20 minutes. Following incubation, 60 µl of hexane was added to each sample, mixed and placed on the auto sampler. Samples were analysed using a Varian 320 MS/MS with a Varian 450 gas chromatograph (Varian, Australia). The compounds analysed were *m/z* 569.3/299.3 and *m/z* 573.3/303.3 for isoprostanes and its internal standard, respectively.

Malondialdehyde (MDA)

HPLC was used to determine plasma malondialdehyde (MDA) using the method of Sim et al. (2003). The principle of this method is that malondialdehyde contained in plasma is derivatised with 2,4-dinitrophenylhydrazine, which forms stable hydrazones that can be easily separated by HPLC using diode array detection (Shimadzu, Kyoto, Japan).

Protein

Protein was measured as per manufacturer's direction using a Pierce BCA protein assay kit supplied by Thermo Scientific (Victoria, Australia)

Statistical analysis

All data are presented as mean ± standard deviation unless otherwise stated. Data were tested for normality of the distribution, and analysis was performed with the statistical software GraphPad Prism 6. Comparison between groups was carried out using a 1-way analysis of variance with a post hoc test of significance between individual groups. Differences were considered significant when p was less than 0.05. All significant differences between groups were determined using the HFD group as the reference group.

5.4. RESULTS

Ingestion of a high fat diet reduced the formation of liver isoprostanes and MDA compared to the chow fed group (Figure 5.1). No differences in MDA or isoprostanes concentrations were evident between the HFD and HFD + probiotics groups. Isoprostanes concentration correlated with MDA levels in the chow fed group only ($R^2 = 0.605$; Figure 5.2). In the chow group, MDA and isoprostanes showed positive correlations with fat pad mass (Figure 5.3 & 5.4) and isoprostanes negatively correlated with liver protein concentration (Figure 5.5). There was no correlation between MDA or isoprostanes with either liver mass (Figure 5.6) or body mass (Figure 5.7). The chow group weighed less than both the HFD groups ($p<0.01$; data not shown), but there was no difference in body mass between two HFD groups (data presented in chapter 3, Figures 9 and 10).

5.5. DISCUSSION

Lipid peroxidation has long been postulated as a marker of oxidative damage. Accumulation of lipid peroxidation compounds is a sign of dysregulated lipid peroxidation that can lead to the development or progression of diseases. I hypothesised that mice fed a high fat diet would increase lipid peroxidation indicated by increased accumulation of isoprostanes and MDA in the liver, and that probiotics would reduce this lipid peroxidation. On the contrary, however, high fat feeding actually reduced lipid peroxidation in the liver (Figure 5.1), while probiotics did not alter this response. These findings challenge the popular notion that high fat feeding and obesity cause increased ROS production and lipid peroxidation.[184, 187]

The findings of this study were unexpected. Other literature has reported that lipid peroxidation increases in mice fed a high fat diet.[209, 210] One factor that may account for this disparity is the fat composition of diets.[211, 212]. A diet high in polyunsaturated fat may cause differing degrees of lipid peroxidation compared with a diet that is high in saturated fat. The high fat diet used in the present study was predominantly saturated fat (12.6%), which is less susceptible to oxidation.[213] Studies investigating the effects of mono unsaturated, polyunsaturated and saturated fat on NAFLD, lipid peroxidation and fat accumulation should be considered for future studies.[214-216]

The reduction in lipid peroxidation may be a protective response to help prevent cirrhosis of the liver. The amount and type of lipid the tissue is exposed to may also play an important factor in the disease state. Lu[126] demonstrated that hepatic stellate cells exposed to varying concentrations of triglycerides or VLDL, had modified activation of stellate cells. A high dose of triglycerides (400 mg/L) inhibited stellate cell activation, while a low dose of triglycerides (12.5 mg/L) and high levels of VLDL (50 – 100 mg/L) promoted activation. This supports the importance of the type and amount of lipids the liver is exposed to and may help explain why some people progress from NAFLD to NASH. In the presence of increased triglycerides, if the stellate cells continue to be activated it may lead to cirrhosis of the liver.

Reduced lipid peroxidation in HFD fed mice may be a response to prevent cirrhosis of the liver through deactivation of stellate cells. Stellate cell activation, rather than lipid peroxidation, may be the second hit in the '2-hit theory' that defines whether an individual progresses from NAFLD to NASH. If this pathway is dysregulated, stellate cells may remain

activated, causing excessive fibrosis progressing to cirrhosis and NKT cells to activate. Hepatic stellate cells and hepatocytes are also necessary for lipid mobilization.[217, 218] While the liver may prevent cirrhosis by reducing stellate cell activation, it also prevents mobilisation of the lipids. Whether lipid peroxidation has a roll in this effect is not known. Lipid peroxidation has been linked with hepatic stellate cell proliferation.[219-221] Montosi[222] demonstrated that oxidative compounds anticipate stellate cell activation. As stellate cells are deactivated, so too oxidative compounds reduce. I speculate that exposure to high levels of triglycerides prevents the activation of stellate cells and subsequent lipid peroxidation and lipid metabolism.

Another difference between results reported are the assays used. ELISA based assays for determination of lipid peroxide compounds are not selective enough often over estimating concentrations and having poor reproducibility.[223, 224] The length of the study may also be an important factor. I fed my mice a HFD for 20 weeks allowing NAFLD to develop. Shorter feeding periods may not reach the same disease state where certain metabolic changes take effect.[225, 226] Shorter feeding periods may not allow the liver sufficient time to accumulate the required lipids to deactivate the stellate cells and oxidative compound formation. Gaemers[226] demonstrated that the effects of the diet can fluctuate over the course of a study. Possible future work may involve measurement of the lipid content, hepatic stellate cell activation and lipid peroxide accumulation at regular intervals over a 20 week period.

These results do not exclude isoprostanes and MDA as a measure of oxidative damage. However, it may be necessary to consider the population and / or individual when interpreting data for isoprostanes and MDA. High levels of isoprostanes and MDA may still indicate oxidative damage, but only above certain concentrations. Another point to consider when looking at these results is that isoprostanes are typically measured in plasma or serum. Due to the sample volume required it was not possible to measure serum isoprostanes in this study. As a substitute, liver isoprostanes were measured as a direct indicator of lipid peroxidation within the liver as opposed to systemic production of isoprostanes. This creates another question, does the liver concentration and plasma concentration of isoprostanes correlate? The present data show for the first time that liver isoprostanes and MDA are correlated, but only in chow fed mice. The lack of correlation in the HFD groups appears to be due to the small amount of isoprostanes and MDA produced in HFD mice.

The mice were housed in a SPF facility with a tightly controlled pathogen population. Housing animals in an environment exposed to more or different bacteria may influence the microbiota population and subsequently the gastrointestinal health and disease formation. The microbiota has been shown to have a dramatic effect on disease formation.[227-229] If mice are exposed to different pathogens that alter the gut-liver axis, this may alter lipid peroxidation and fat metabolism.

Probiotics were proposed to re-regulate fat-induced changes to the microbiota population and subsequent changes to lipid peroxidation. The lack of effect may be due to a number of reasons. The dose of probiotics may have been too low. Despite administering a dose based on body mass and the recommended dose for humans, the amount administered may have been too low to account for the changes caused by the high fat diet. Under the extreme circumstances, a higher dose may be required to rescue lipid peroxidation. The duration supplementation may have been too short, or were introduced too late into the study. By administering the probiotics 10 weeks into the study, the disease state may already be too established for short term supplementation to be effective. Administering the probiotics earlier, possibly as a prophylactic, may alter the results. Despite probiotics having little effect on lipid peroxidation, it is possible supplementation had beneficial effects elsewhere. As a consequence, it could be that a multi-strain probiotic administered in sufficient doses or as a prophylactic can rescue a dysbiotic gastrointestinal tract microbiome and in turn affect fat metabolism in the liver thereby possibly reducing its progression to fatty liver disease. Future studies will investigate other possible benefits of probiotics supplementation within a high fat feeding model as well as investigate signalling pathways that reduce lipid peroxidation.

In summary, lipid peroxidation as determined by isoprostanes and MDA concentrations were reduced in mice fed a HFD for 20 weeks. Reduced lipid peroxidation in HFD fed mice may be a response to prevent cirrhosis of the liver via stellate cell deactivation. Stellate cell activation, rather than lipid peroxidation, may be the second hit in the 2-hit theory that defines whether an individual progresses from NAFLD to NASH. If this pathway is not regulated, stellate cells may remain activated causing excessive fibrosis progressing to cirrhosis and NKT cells to activate. Can isoprostanes therefore be used as a possible marker for NAFLD and NASH severity?

5.6. FIGURES

Figure 5.1: Lipid peroxidation markers of A) liver isoprostanes and B) malondialdehyde

Figure 5.2: Comparison of isoprostanes and MDA

Figure 5.3: Comparison of MDA and fat pad weight

Figure 5.4: Comparison of isoprostanes and fat pad weight

Figure 5.5: Comparison of isoprostanes and liver protein

Figure 5.6: Comparison of liver weight and MDA

Figure 5.7: Comparison of body weight and isoprostanes

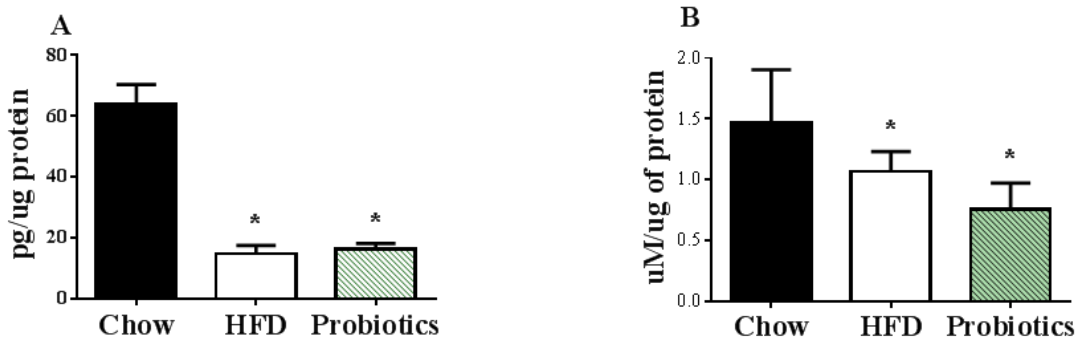
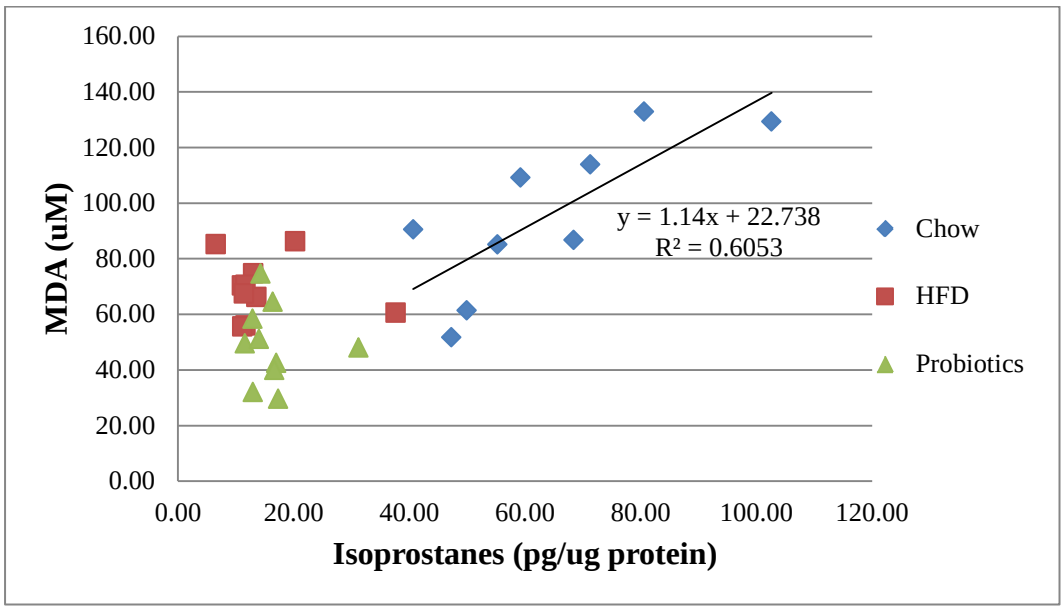


Figure 5.1



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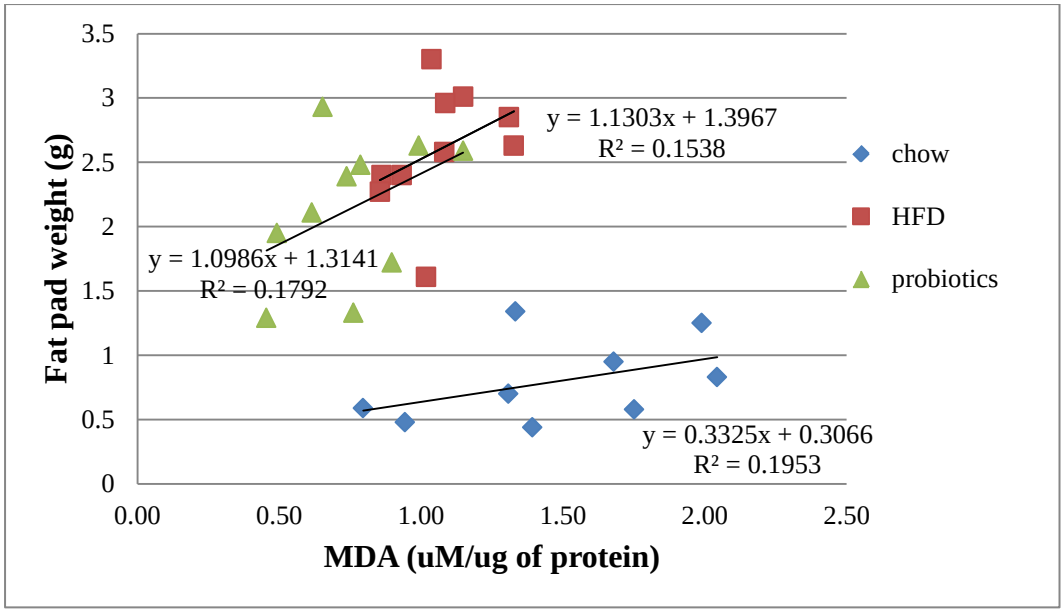

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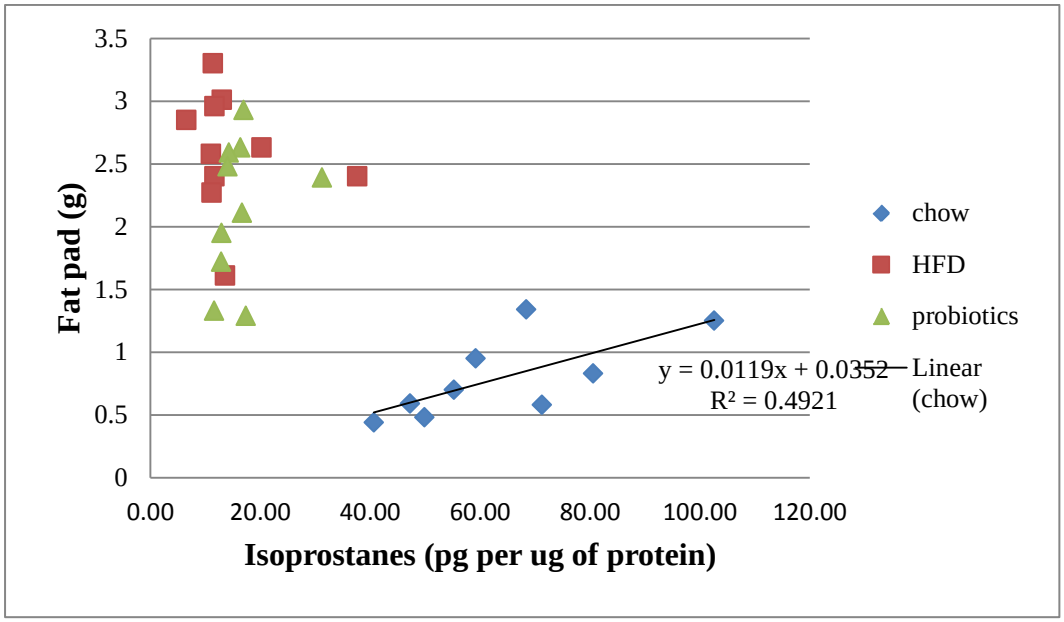

Figure 5.4

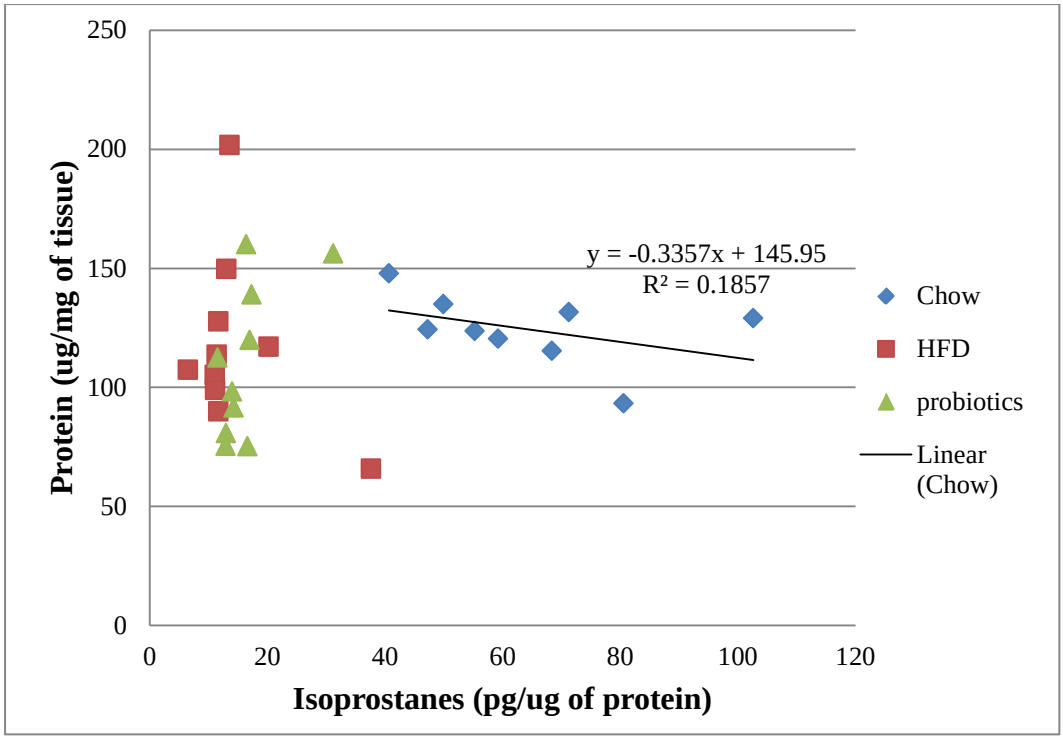

Figure 5.5

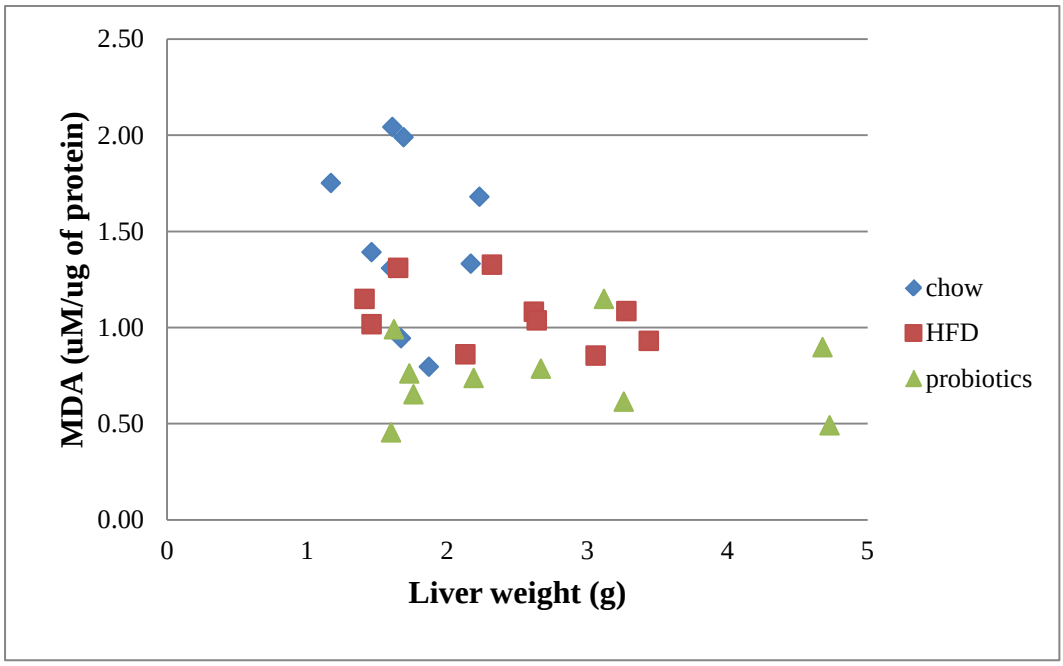

Figure 5.6

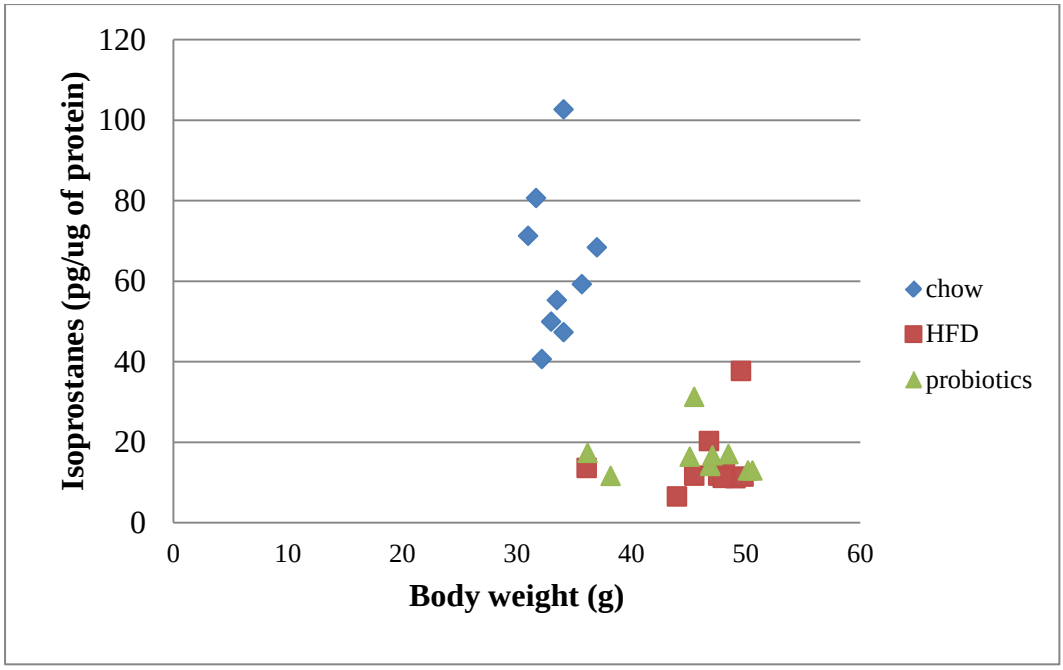
120
100
80
60
40
20
0
Isoprostanes (pg/ug of protein)
0
10
20
30
40
50
60
Body weight (g)
chow
HFD
probiotics

Figure 5.7

Chapter 6

Optimised Method for Quantification of Total F_2-isoprostanes Using Gas Chromatography-Tandem Mass Spectrometry

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Optimized method for quantification of total F_2-isoprostanes using gas chromatography–tandem mass spectrometry

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abstract

F_2-isoprostanes are produced from the oxidative degradation of arachidonic acid and are considered the gold standard marker of lipid peroxidation in biological samples. We developed a liquid–liquid extraction method for the determination of total isoprostanes using negative chemical ionization gas chromatography–tandem mass spectrometry in plasma and tissue homogenates. Incorporating liquid–liquid extraction allows for greater sample through-put than current approaches. Here we describe the protocol and include numerous trouble-shooting suggestions. The method found healthy individuals with 150–250 pg of isoprostanes per ml of plasma and end stage kidney disease patients to have the highest measured values of up to 1100 pg/ml. This assay has an accurate working linear range of 40–1000 pg of isoprostanes (100–2500 pg/ml) and an average coefficient of variance of 7%. Tissue values for healthy mice liver were 50–70 pg/j.tg protein. This method provides increased ion selectivity and detection capabilities with economical sample through-put.

1. Introduction

Oxidative stress is involved in the development and progression of numerous chronic diseases including kidney disease [1], cardiovascular disease, neurodegenerative conditions and malignancies [2]. Markers of lipid peroxidation are the most commonly used determinants of oxidative stress and F_2-isoprostanes are established as the most accurate and stable marker for measuring lipid peroxidation and oxidative damage in vivo [3,4]. Regarded as the "gold standard" for assessing oxidative damage in humans, F_2-isoprostanes are generally measured in plasma and urine but have also been measured in tissue homogenate [5,6]. F_2-isoprostanes are prostaglandin-like compounds formed from the free-radical catalyzed peroxidation of arachidonic acid. There are four groups of F2-isoprostanes regioisomers formed from arachidonic acid (5-, 8-, 12- and 15-series isoprostanes), each containing 8 possible diastereomers making 32 different F2-isoprostanes possible [7,8].

Due to the complex methodologies and expensive equipment required to separate F_2-isoprostanes from other prostaglandins and their metabolites, measurement of F_2-isoprostanes in biological samples is difficult. There are numerous approaches for quantifying F_2-isoprostanes including enzyme-linked immunosorbent assay (ELISA) [9,10], gas chromatography–mass spectrometry (GC/MS) [6,10,11], gas chromatography–tandem mass spectrometry (GC/MS/MS) [12,13], liquid chromatography–mass spectrometry (LC/MS) [14,15] or liquid chromatography–tandem mass spectrometry (LC/MS/MS) [16–18]. The majority of published studies measuring isoprostanes in vitro use the ELISA approach and while it is economical and reproducible, it provides vastly different and varied results compared to a GC/MS approach [19]. The mean and median ELISA results were 30-fold greater than results from GC/MS results (range 9–138-fold). The differences were speculated to be due to the non-specific binding properties of the ELISA plate compared to the specificity and separation of mass spectrometer. The comparison between ELISA kits has also been shown to be unreliable, with correlations between different ELISAs being very poor [20]. GC/MS and LC/MS are accurate but often require elaborate extraction methods leading to low through-put. The GC/MS approach also requires additional derivatization steps and has difficulty separating isomers compared to LC/MS. Advancements in technology have led to greater affordability and availability of tandem mass spectrometry (MS/MS). Although still expensive, MS/MS gives increased selectivity of compounds compared to MS, allowing for improved isolation and quantification of F_2-isopostanes.

Despite the technology in use for detecting isoprostanes, the majority of limitations for quantifying F_2-isoprostanes remain with the extraction from biological samples. Current methods incorporate solid phase extraction (SPE) [11,18,21–23], liquid–liquid extraction (LLE) [6,17], thin-layer chromatography (TLC) [24,25],

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affinity column [14], silica gels [21] and reverse-phase cartridges [11]. These methods are time consuming with low sample through-put making them expensive. The method presented here uses LLE that uses inexpensive reagents overall allowing for greater sample through-put (50 extractions per day). The greatest limitation to sample through-put with this method is the GC/MS/MS run time.

Our laboratory spent considerable time unsuccessfully attempting to develop GC/MS, LC/MS and LC/MS/MS methods with and without SPE. This led us to GC/MS/MS and the refinement of previous methods from Taylor et al. [17] and Mori et al. [11] and the use of negative chemical ionization and LLE. We now have a protocol that has high sample through-put capacity, is economical, sensitive and very reliable. Here we describe the protocol along with a discussion of important issues encountered during method development to allow for ease of replication and trouble-shooting.

2. Methods

2.1. Reagents and standards

Chemicals were sourced from Labscan (ethylacetate; Gliwice, Poland), LiChrosolv (methanol, hexane, acetonitrile and hydrochloric acid; Merck, Darmstadt, Germany) and Chem-Supply (sodium hydroxide pellets; Gillman, SA, Australia). Derivatization reagents N,O-bis-(trimethylsilyl)trifluoroacetamide 1% trimethylchlorosilane (BSTFA + TMCS, 99:1) were purchased from Supelco and pentafluorobenzylbromide (PFBBr), N,N-diisopropylethylamine (DIPEA) and pyridine purchased from Sigma–Aldrich (NSW, Australia). Standards used (8-iso prostaglandin F_{2a} and 8-iso prostaglandin F_{2a}-d_4) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Methanolic sodium hydroxide was made as per previously published by Taylor et al. [17]. Briefly, 12 g of NaOH was dissolved in 28 ml of water and 160 ml of methanol added. Hydrochloric acid was diluted to 3 M using deionised water.

2.2. Plasma collection

Samples were collected by drawing blood into a vacutainer containing ethylenediaminetetraacetic acid (EDTA) and centrifuged immediately or placed on ice and centrifuged within 30 min of collection at 2000 × g at 4 °C. Aliquots of samples (>850 j.tl) were placed into tubes containing 10 j.tl of 100 mM butylated hydroxytoluene (BHT) and stored at −80 °C until required. Serum is also suitable for use. The protocol below describes plasma extraction, but would be the same for serum.

2.3. Tissue collection

Liver tissue was collected from mice at the time of termination, cut into small pieces (~50 mg pieces) and snap frozen using liquid nitrogen. Samples were stored at −80 °C until required.

2.4. Extraction and derivatization

Table 1 contains the protocol for extraction and derivatization of plasma and tissue samples.

2.5. Gas chromatography–tandem mass spectrometry analysis

Samples were analyzed for total isoprostanes concentration using a Varian 320 MS/MS, with a Varian 450 gas chromatograph equipped with a CP8400 auto sampler. Data was analyzed using the Varian MS Workstation-System control software version 6.9.2 (Varian, Australia). 1 j.tl of sample was introduced in splitless mode using a 10 j.tl Hamilton syringe. After 1 min the injector port was switched to a 1:20 split. The injector operated at 250 °C with a

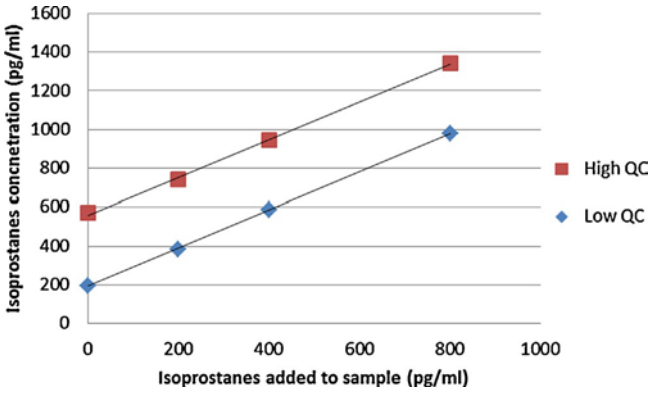

Fig. 1. Two quality control plasma samples spiked with 0, 200, 400 or 800 pg/ml of isoprostanes. Recovery of isoprostanes from spiked plasma sample showed a recovery of >90% and an accuracy of >96%.

Varian FactorFour Capillary Column (VF5 MS 30 m × 0.25 mm ID, DF = 0.25) using helium as the carrier gas at a flow rate of 1.0 ml/min. The column oven was started at 160 °C and held for 1 min, then increased at 20 °C/min to 300 °C, and held for 10 min. The total column oven run time is 18 min with the isoprostane peak eluted around 9–10 min (Fig. 1).

Running in negative chemical ionization mode (NCI) at 70 eV, the chemical ionization gas was argon run at an ion source pressure of 4.20 Torr and the collision gas was argon run at 2.00 mTorr. The mass spectrometer was operating at a transfer line temperature of 250 °C, ion volume temperature of 200 °C and collision energy of 17 V. The detector operated at 1700 V. Isoprostanes peak identification of a sample was established by comparing the retention time and fragmentation pattern of a standard and deuterated standard. The mass size and transition of isoprostanes and the respective deuterated standard were established at m/z 569.3/299.3 and 573.3/303.3 respectively.

2.6. 8-iso-$PGF_{2_{\alpha}}$ standard preparation

Three different standard concentrations were made by adding 80, 160 or 320 pg of isoprostanes to a silanized vial inserts from a concentrated stock solution of 500 j.tg/ml of 8-iso-PGF_{2a} (diluted using methanol). Standards were made in batches, immediately dried under nitrogen, capped and stored at −80 °C for later use. Upon removal from the freezer for use, the vial was allowed to equilibrate to room temperature before the same amount of deuterated standard as added to the samples (400 pg/ml equivalent), was added. These standards were derivatized along with the samples and gave a final concentration equivalent to 200, 400 and 800 pg/ml when 400 j.tl of sample was extracted.

2.7. Intra- and inter-assay variability and accuracy

Intra-assay variability was determined by the average coefficient of variance of over 1000 different samples analyzed in duplicate over 12 months. Inter-assay variability was assessed by the inclusion of high level (pooled samples from sick individuals) and low level (pooled samples from young, healthy individuals) quality control (QC) samples, extracted in conjunction with study samples each day.

Accuracy was assessed by comparing plasma samples spiked with known amounts of isoprostanes. Known amounts of 8-iso-PGF_{2a} (0, 200, 400 and 800 pg/ml) were added to our QC plasma samples and analyzed in duplicate on multiple occasions. F_2-isoprostane concentrations were separately quantified in these

Table 1
Summarized protocol for extraction and derivatization of plasma or tissue samples for F2-isoprostanes measurement using GC–MS/MS.

Plasma extraction	Tissue extraction
• In a 10 ml screw cap glass tube add 160 pg of 8-iso-PGF_{2a}-d_4 + 400 j.tl sample + 800 j.tl methanolic NaOH for saponification + 800 j.tl dH_2O and vortex for 5 s	• Place 30–100 mg of tissue in a microfuge tube containing 1 ml of methanolic NaOH
• Incubate in a water bath at 42 °C for 60 min	• Incubate in water bath at 42 °C for 30 min
• Place on ice for 10 min	• Homogenize the tissue
• Precipitate proteins out of solution by adjusting to pH 3 using 450 j.tl of 3 M HCl – vortex for 5 s	• In a 10 ml screw cap glass tube add 160 pg of 8-iso-PGF_{2a}-d_4 + 400 j.tl tissue homogenate + 400 j.tl methanolic NaOH for saponification + 800 j.tl dH_2O and vortex for 5 s
• Remove neutral lipids by adding 3 ml hexane	• Incubate in a water bath at 42 °C for 45 min
• Gently mix (end over end at 20–30 rpm) for 10 min, centrifuge for 10 min (3000 × g) at room temperature and remove the supernatants and discard using glass Pasteur pipette	• Place on ice for 10 min
• Extract isoprostanes with 3 ml ethyl acetate and vigorously shake for 10 s	• Precipitate proteins out of solution by adjusting to pH 3 using 450 j.tl of 3 M HCl – vortex for 5 s
• Gently mix (end over end at 20–30 rpm) for 10 min, centrifuge for 10 min (3000 × g) at room temperature and remove the supernatants using glass Pasteur pipette to clean glass tube	• Remove neutral lipids by adding 3 ml hexane
• Dry extracts under dry nitrogen stream at 40 °C (approximately 60 min)	• Gently mix (end over end at 20–30 rpm) for 10 min, centrifuge for 10 min (3000 × g) at room temperature and the supernatants remove and discard using glass Pasteur pipette
• Reconstitute in 200 j.tl acetonitrile, vortex for 5 s and transfer into 400 j.tl silanized glass inserts	• Extract isoprostanes with 3 ml ethyl acetate and vigorously shake for 10 s
• Dry under nitrogen stream at 40 °C ready for derivatization (about 15–20 min)	• Gently mix (end over end at 20–30 rpm) for 10 min, centrifuge for 10 min (3000 × g) at room temperature and the supernatants remove using glass Pasteur pipette to clean glass tube
	• Dry extracts under dry nitrogen stream at 40 °C (approximately 60 min)
	• Reconstitute in 200 j.tl acetonitrile, vortex for 5 s and transfer into 400 j.tl silanized glass inserts
	• Dry under nitrogen stream at 40 °C ready for derivatization (about 15–20 min)

Derivatization [11]

• Add 40 j.tl of pentafluorobenzylbromide (PFBBr, 10% in acetonitrile – 4 j.tl of PFBBr and 36 j.tl of ACN) and 20 j.tl di-isopropylethylamine (DIPEA, 10% in acetonitrile – 2 j.tl DIPEA and 18 j.tl of ACN) and vortex for 5 s
• Incubate at room temperature for 30 min
• Dry for 30 min under nitrogen
• Add 10 j.tl anhydrous pyridine and 20 j.tl bis-(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA + TMCS, 99:1), cap and vortex for 5 s
• Incubate for 20 min at 45 °C
• Add 60 j.tl of anhydrous hexane
• Cap with PTFE lined cap in autosampler carrier vial
• Place on auto sampler rack for analysis

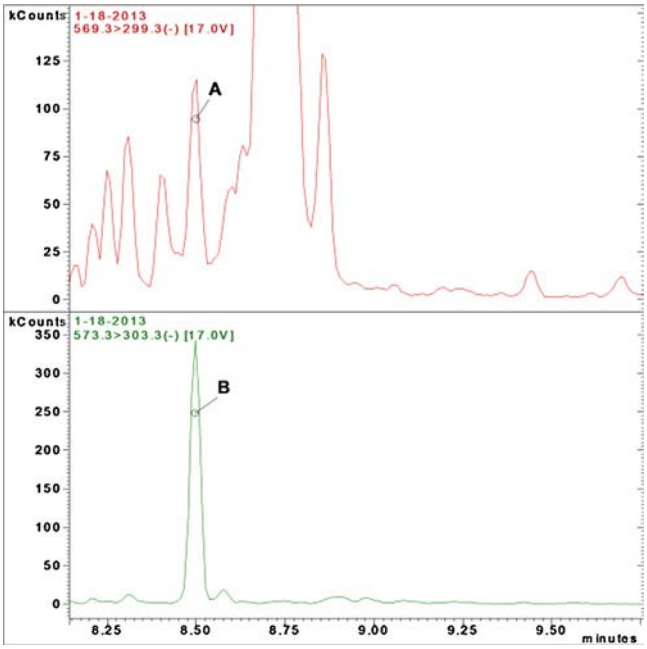

Fig. 2. Plasma QC sample from a healthy individual. (A) Total isoprostanes peak representative of 200 pg/ml of plasma and (B) internal standard peak.

samples, unspiked samples and aqueous standards (Fig. 1a). Regression lines were calculated using Microsoft Excel.

2.8. Calculations

A standard curve was developed by injecting known amounts of F2-isoprostane (200, 400 and 800 pg/ml) and F2-isoprostanes-d_4 (internal standard). By comparing the ratio of internal standard to isoprostanes in both the aqueous standards and biological samples allows for quantification of the total isoprostanes. The percentage recovery of the internal standard added to biological samples compared to the aqueous standards determines the efficiency of the extraction process. Internal standard addition allows for the variability in recovery of every sample to be accounted for. Our recovery data is based on multiple data sets where a sample and standard were injected sequentially.

2.9. Protein analysis

Protein concentration was analyzed using a Pierce BCA protein assay kit (Thermo Scientific Australia). Briefly, 40 j.tl of homogenized tissue (described earlier) was diluted in 1 ml of distilled water. Of this solution, 15 j.tl was added to a well of a 96 well plate and 200 j.tl of BCA reagent added. Protein concentration was determined by a standard curve using the protein standard provided in the kit.

3. Results

3.1. Range

Samples were analyzed from young (20–35 years) healthy individuals (Fig. 2) and patients with chronic kidney disease (n = 1040), end stage kidney disease (n = 172) or metabolic syndrome (n = 41). The isoprostanes concentration ranged from levels found in healthy subjects of 100–250 pg/ml (typically 150–200 pg/ml) to the highest concentrations found in end stage renal disease patients reaching 1100 pg/ml (typically >400 pg/ml).

Pooled QC samples were collected from young, healthy individuals (low QC) or from surplus plasma collected from a local hospital (high QC). The QC material was a plasma mixture from de-identified waste samples from a pathology laboratory and from our own venepuncture laboratory. As such, ethics approval was not necessary. The low QC gave a mean of 189.7 pg/ml (95% CI 187.1–192.0 pg/ml) and the high QC gave a mean of 567.2 pg/ml (95% CI 557.8–579.1 pg/ml).

Samples collected from mice showed a healthy mouse liver to have a concentration of 50–70 pg/j.tg of protein.

3.2. Sample recovery and accuracy

When the chemical ionization gas was methane, there was degradation over time in the mass spectrometers signal/sensitivity. This made it difficult to be accurate with our recovery data. With argon as our chemical ionization gas [26], the signal remained stable and recovery and accuracy data was able to be determined. Our extraction method consistently recovers >90% (typically >95%) of added internal standard with an accuracy of >96% (Fig. 1a).

3.3. Inter- and intra-assay variability

The inter-assay variability of the low and high QC's was 7.6 and 5.9% respectively with an intra-assay variability from more than 1000 samples of 7.0%.

4. Discussion

Following failed attempts to replicate previously published methods for measuring isoprostanes, we felt the need to develop and refine a reliable, sensitive and reproducible method capable of high sample through put. Modifying methods by Taylor et al. [17] and Mori et al. [11], we developed a LLE method that gives consistent and reliable results.

Previous methods rely on a combination of TLC, SPE, HPLC prep columns and/or LLE. These are time consuming with limited sample through-put capability and expensive with low recoveries. Our method only uses LLE requiring inexpensive reagents, and allowing for greater sample through-put (50 samples per day). The greatest limitation to sample through put with this method is the run time on the mass spectrometer.

Previous methods have commonly measured isoprostanes using a single quadrupole MS [6,10,11,14,15], only capable of running in selective ion monitoring (SIM) mode. Our method incorporates tandem MS giving increased selectivity capable of pairing a precursor ion with a product ion through fragmentation. This allows accurate measurement of specific compounds and may explain why our reported value for healthy controls (150–200 pg/ml) is often lower than other published values (400 pg/ml) [23].

4.1. Purification

Once we established an adequate methodology, we proceeded to further purify the extract. One attempt to purify the chromatograms was to remove the supernatant following the precipitation of the proteins. Once the samples had been saponified, the samples were placed on ice and centrifuged allowing us to extract the supernatant into a clean tube leaving the proteins behind. However, extraction of the supernatant resulted in very poor recoveries (~10%). We concluded that the majority of isoprostanes were still isolated in the protein pellet leaving only the free isoprostanes in the supernatant. When the proteins were left in the bottom of the tube, recovery returned to >90% of total isoprostanes.

To maximize the recovery of isoprostanes, tests were carried out to check the recovery of one, two and three ethyl acetate extractions. Results showed that the first ethyl acetate extraction recovered >90% of the total isoprostanes, the second extraction recovered <5% and the third extraction recovered <1%. However, the second and third extractions carried over more impurities, resulting in a reduction in the signal to noise ratio (data not shown). It was therefore deemed that one ethyl acetate extraction is the most efficient for recovering the majority of isoprostanes and giving the best signal to noise ratio.

As a further means to reduce impurities and maximize sample volumes, 200 j.tl and 300 j.tl aliquots of sample were assayed. The use of 200 j.tl and 300 j.tl of sample increased the detection limit from 50 pg/ml for 400 j.tl of sample to 200 pg/ml and 100 pg/ml respectively due to a reduction in signal to noise ratio resulting in poor peak resolution. Therefore, 400 j.tl of sample was deemed optimal. Using an instrument with greater sensitivity may enable the use of less sample volume.

4.2. Emulsion formation

During development of this method, numerous complications were faced. During sample extraction, when hexane was added to the acidified solution an emulsion interphase between the aqueous and organic phase occurred, resulting in the formation of a gel like layer that is unable to be separated. A number of things were attempted to reduce the emulsion including: replacing the methanolic NaOH with methanolic potassium hydroxide (KOH), altering the amount of water added for hydrolysis, altering the concentration and volume of NaOH added, altering the volume of hexane added, adjusting the pH of the solution, adding surfactants and changing the temperature and/or duration for saponification and protein precipitation. Of these, the only effective changes that prevented emulsion formation was to increase the temperature for saponification (from 37° to 42°) and cooling the samples prior to protein precipitation. The emulsion formation was even more pronounced when a second hexane extraction was attempted, regardless of the pre-treatment conditions. Due to this, only one hexane extraction was incorporated.

4.3. Isoprostanes bond to glass

Early on we noted a great deal of variability in our results. The recovery of our internal standard from extracted samples would at times exceed 100% of our internal standard added to the standard vials. Upon investigation, we discovered the glass vial inserts we used were the cause of the problem. From different batches of vials, differing amounts of both isoprostanes and internal standard would bond to the glass surface, causing variability in the results. This problem became even more pronounced when standards were dried in vial inserts and stored. This problem was no longer evident once we switched to silanized vial inserts. Storing standards in silanized inserts for 6 months at −80 °C has had no effect on the stability of the standards to date.

4.4. Hydrolysis

Of the whole extraction process, the crucial step is at derivatization. If adequate drying time between derivatization steps (PFBBr and TMCS) was not provided, moisture would remain within the vial causing irreversible hydrolysis of the sample. This reduced the chromatogram quality and altered the yield of isoprostanes. While investigating the glass vial inserts for isoprostanes bonding, silanized limited volume inserts (tapered) were trialed as silanized narrow flat bottom inserts, as previously used were unavailable. However, due to the shape of the insert, drying the sample proved difficult with samples often unable to be dried completely, even after >3 h under nitrogen. This led to a great deal of samples hydrolysing and the sample unable to be analyzed. Therefore 400 j.tl silanized flat bottom inserts were used with a greater volume of hexane added to make up volume adequate volume for an injection.

4.5. CI gas/NCI mode

One major downfall with GC/MS/MS for isoprostanes analysis is the requirement to operate in NCI mode where it is common practice to use methane as the CI gas. We started out using methane as the CI gas and found the instrument rapidly loss sensitivity requiring calibration, cleaning or maintenance on a daily basis. The signal degradation would often result in periods of no signal. This resulted in our peaks of interest having part of the peak fall below the baseline with no way to confidently quantify the peak. Upon continued investigation, it was discovered that our degradation was primarily due to the ion volume becoming contaminated and affecting ion generation. After changing to a clean ion volume and re-tuning, the sensitivity would return to 100%. However, within the first 5–10 injections the sensitivity would decrease by up to 90% and continue to gradually decline. When operating with methane as the CI gas, re-tuning once the ion volume had become contaminated was not possible. However, based on the publication of Eckstein et al. [26], we switched to argon as the CI gas and re-calibrating following numerous injections was possible. While calibrating returned some of the sensitivity, a complete change of the ion volume was still the only way to fully restore the instruments sensitivity. Currently we are capable of injecting >60 samples before the need to change the ion volume or re-tune. Our particular instrument does not require venting (no down-time) in order for the ion volume to be changed. Changing to argon as the CI gas also provided other benefits: methane is highly flammable and therefore a safety hazard and methane is an expensive gas so changing to argon provided an economical alternative.

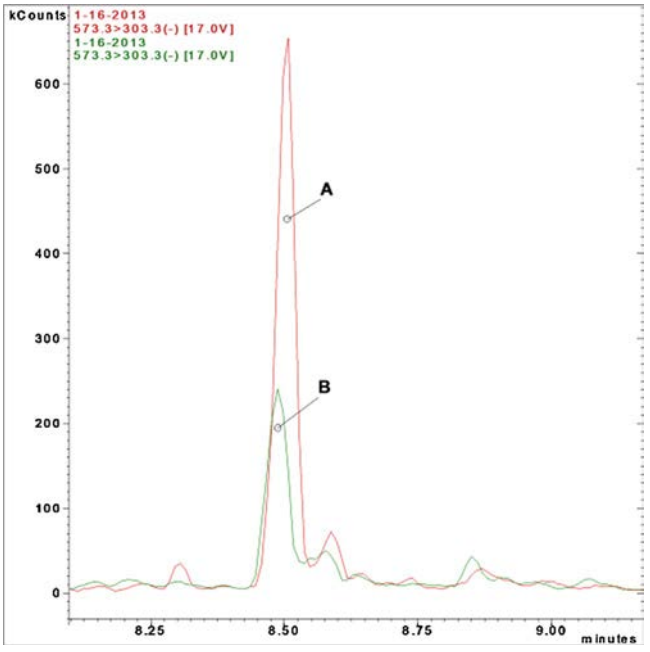

Fig. 3. A single plasma sample from an end stage renal disease patient showing the presence of an unidentified interfering compound. (A) Sample extracted with the addition of 400 pg/ml of the F2-isoprostanes-d_4 internal standard and (B) sample extracted without the addition of any internal standard.

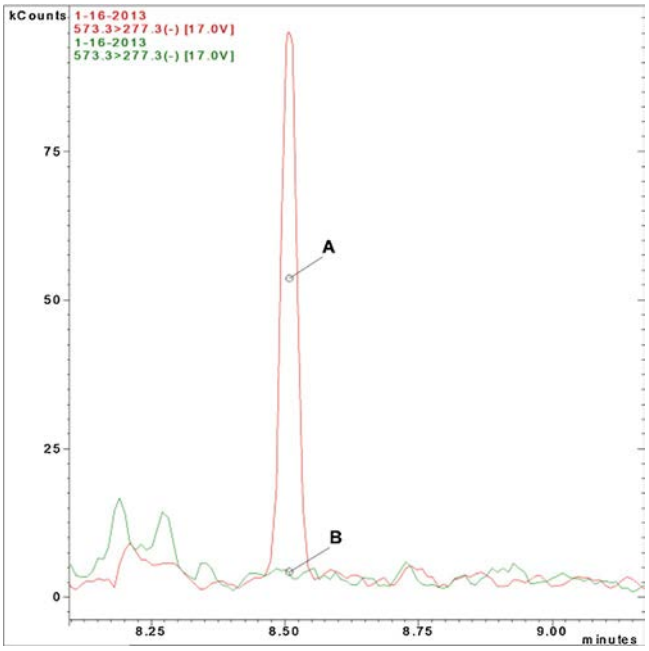

Fig. 4. A single plasma sample from an end stage renal disease patient using m/z 573/277 as the transition mass. (A) Sample extracted with the addition of 400 pg/ml of the F2-isoprostanes-d_4 internal standard and (B) sample extracted without the addition of any internal standard.

4.6. Interfering compound

Mas et al. [27] has previously shown there is a compound found in urine that interferes with the isoprostane internal standard peak. We have found that there is a compound in the blood of end stage renal disease patients that also interferes with the isoprostane internal standard peak when m/z 573.3/299.3 is used (Fig. 3). To overcome this problem we introduced a second transition at m/z 573.3/277.3. The interfering compound was not identified at this mass but gave a reduced signal to noise ratio (Fig. 4). The interfering compound was not identified but is potentially a uremic toxin or a drug, treatment or supplement common to this population. This observation was not seen in any other population.

4.7. Free versus total isoprostanes

Total isoprostanes, as measured in this paper, is the quantification of both free (non-esterified) and esterified isoprostanes. By removing the methanolic NaOH incubation step from the extraction, free isoprostanes can also be measured. Free isoprostanes are the biologically active compound, however both free and esterified isoprostanes are the result of oxidation. Therefore to quantify oxidation, both the free and esterified isoprostanes need to be quantified. Morrow et al. [28] demonstrated that while esterified isoprostane levels are significantly greater than free isoprostane levels in plasma, both levels increased and decreased at a similar rate.

5. Summary

We have developed a simple method for the accurate measurement of total isoprostanes using liquid–liquid extraction. This method allows for high through-put of both plasma and tissue samples. Results of the new method are generally below current published normative values due to the specificity gained by incorporating GC/MS/MS analysis.

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Chapter 7

Combined curcumin and vitamin E treatment attenuates diet-induced steatohepatitis in wild type and *Hfe*$^{-/-}$ mice

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7.1 ABSTRACT

Hfe$^{-/-}$ mice fed a high calorie diet develop steatohepatitis and fibrosis. Therapy with curcumin or vitamin E has proved beneficial in nonalcoholic steatohepatitis. I investigated the synergistic properties of these agents by using them in combination in an *Hfe*$^{-/-}$ high calorie diet model of steatohepatitis. Wild-type C57BL/6J and *Hfe*$^{-/-}$ mice were fed either laboratory chow or a high calorie diet for 20 weeks. In treatment groups, mice were fed a high calorie diet for 10 weeks followed by 10 weeks of either of the following diets: chow; or a high calorie diet in combination with; 1% curcumin; 1.5% vitamin E; 1% curcumin + 1.5% vitamin E. *Hfe*$^{-/-}$ mice fed a high calorie diet for 20 weeks developed steatohepatitis. Minimal residual steatosis was observed in those mice switched to the chow diet. Combination treatment resulted in a greater reduction of macro- and micro-vesicular steatosis and lobular inflammation than either vitamin E or curcumin therapy alone. Pericentral fibrosis was present in *Hfe*$^{-/-}$ mice fed a high calorie diet with upregulation of fibrosis-related genes. Combination therapy in this group resulted in significant reduction in monocyte chemoattractant protein-1 mRNA expression, and lower expression of α_1(I)-procollagen and tissue inhibitor of metalloproteinase. Wild-type and *Hfe*$^{-/-}$ mice fed curcumin and vitamin E showed an upregulation in carnitine palmitoyl transferase 1A, peroxisome proliferator-activated receptor α and *adiponectin receptor 2* mRNA expression which was consistent with an upregulation of fatty acid oxidation pathways. *Hfe*$^{-/-}$ mice fed a high calorie diet developed steatohepatitis and fibrosis which was absent in mice switched back to chow from the high calorie diet. Combination treatment of curcumin and vitamin E decreased liver injury indicating that this treatment may be of therapeutic value in nonalcoholic fatty liver disease.

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in Western countries [230, 231]. NAFLD encompasses a range of hepatic pathologies from simple steatosis to the more aggressive lesion nonalcoholic steatohepatitis (NASH).[232] The *HFE* gene regulates intestinal iron absorption and systemic iron balance, and homozygosity for the C282Y mutation in the *HFE* gene is the most common cause of hereditary hemochromatosis (HH).[233] Steatosis is common in patients with HH[234] and is associated with increased hepatic fibrosis, and conversely, heterozygosity for the C282Y mutation in *HFE* is common in patients with NAFLD.[235] Even mild increases in hepatic iron concentration (HIC) may play an important role in the transition from simple steatosis (a benign lesion), to the pathologically more significant lesion, NASH. *Hfe*$^{-/-}$ mice fed a high calorie diet develop NASH, impaired anti-oxidant activity and accelerated liver injury.[236] Thus animal and human clinical studies

suggest a relationship between HFE, altered iron metabolism and the development of NAFLD, and the basis of this association probably includes increased oxidative stress. This relationship could be the target for therapeutic strategies that may attenuate disease progression in NASH. Curcumin is a widely available plant product which has antioxidant, anti-inflammatory and iron chelating properties.[237] Curcumin has been shown to improve insulin sensitivity in obese mice.[238] Enhancement of insulin sensitivity and inhibition of gluconeogenesis has been implicated in the effects observed with curcumin.[239] Several studies have shown benefit of vitamin E administration in liver diseases associated with high levels of oxidative stress, and vitamin E supplementation has been associated with improvements in liver injury in animal models of liver disease and in humans with NAFLD.[240, 241]

Both of these agents have been used individually in experimental models of NASH and curcumin has been shown to have some therapeutic benefit,[242] however these agents have not been tested in combination. I hypothesised that these two agents may have synergistic activity in attenuating disease progression in NASH viz. the *Hfe*$^{-/-}$ - high calorie diet animal model. To date, these agents have mostly been used as preventative treatment which does not replicate the usual clinical situation of ongoing exposure to hepatic toxins in the setting of underlying liver injury. In the present study, I have investigated the potential use of a combination of curcumin and vitamin E in the treatment of wild type and *Hfe*$^{-/-}$ mice with established liver injury induced by a high-fat, high-carbohydrate or high calorie diet, and with ongoing exposure to the diet. My results show that combination treatment of curcumin and vitamin E decreased liver injury suggesting that this treatment may be of therapeutic value in nonalcoholic fatty liver disease.

7.2 Methods

Animals.

All animals received humane care under the guidelines and approval of the Queensland Institute of Medical Research and the University of Queensland Animal Ethics Committees, detailed in the Australian Code of Practice. Eight week old male C57BL/6J wild-type (WT) mice (Animal Resources Centre, Perth, WA, Australia) and *Hfe*$^{-/-}$ mice (on a C57BL/6J background, originally supplied by Professor William Sly, St Louis University, MO) [243], were fed either a standard laboratory control chow diet (n = 9) for 20 weeks or a high fat diet

(HFD) (SF03-020, Speciality Feeds, Glen Forrest, Australia) (n = 109) for a period of 10 weeks. The mice fed the HFD diet were then randomly assigned to receive either HFD alone (n=9), chow (n=9) or HFD plus 1% curcumin (n=9-10), HFD and 1.5% vitamin E (n=7-10) or HFD and a combination of 1% curcumin and 1.5% vitamin E (n=9-10) for a further 10 weeks. All treatment diets were custom made by Specialty Feeds, Glen Forrest, Australia and the Curcumin C^3 complex was supplied by Sabinsa Corporation, Sami Labs Ltd, India. The fat-derived caloric contribution for HFD was 43%; the cholesterol content was 0.19% and sucrose content was 405 g/kg. The chow diet contained 6% fat and the sucrose content was 7g/kg. Animals were allowed *ad libitum* access to diets and drinking water and were maintained on a 12hr light/dark cycle, at a temperature of 19°C to 23°C and at a humidity of 50%. After 20 weeks of dietary treatment, animals were sacrificed under anesthesia following cardiac puncture for blood collection. The livers were removed and portions were snap-frozen in liquid nitrogen and stored at -80°C for determination of markers of oxidative stress and inflammation as listed below. Separate portions were collected for histology or dried for the determination of hepatic iron concentration. Epididymal fat pads were weighed and stored at -80^0C. Proximal small intestines were harvested immediately and either snap frozen for RNA extractions or fixed in 10% formalin for histology or kept cold in ice for the extraction of duodenal and jejunal enterocytes as described previously.[244]

Histopathological analysis.

Liver samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin (H&E). Histological parameters were staged and graded according to accepted criteria by a specialist liver pathologist in a blinded fashion.[245-247] Steatosis was graded according to the percentage of steatotic hepatocytes (grade 0, < 5% affected; grade 1, 5 - 33% affected; grade 2, 34 - 66% affected; and grade 3, > 66% affected) and necroinflammatory activity was graded according to severity (0, none; 1, mild; 2, moderate; and 3, severe) according to criteria established by Brunt *et al*.[245]

Lobular inflammatory activity was scored based on the number of inflammatory foci per 200x field (0, none; 1, < 2 seen; 2, 2 - 4 seen; and 3, > 4 seen) and ballooning was scored based on the degree of hepatocyte ballooning (0, none; 1, few; and 2, many) according to established criteria.[247] Portal inflammation was also scored according to severity (0, none; 1, mild; 2, moderate; and 3, severe) according to established criteria.[245] Activity was also scored

using NAFLD activity score (NAS) established by the NASH Clinical Research Network (CRN), which is an unweighted sum of scores for steatosis (0-3), lobular inflammation (0-3) and ballooning (0-2).[247]

Fibrosis stage was assessed following Picro Sirius red staining for collagen according to the criteria established by Brunt et al[245] (stage 1, zone 3 perisinusoidal only (1a, 1b) or portal/peri-portal only (1c); stage 2, zone 3 perisinusoidal and portal fibrosis; stage 3, bridging fibrosis; and stage 4, cirrhosis). Additionally, the centrilobular and portal components of fibrosis were also each independently scored according to the extent of fibrosis (0, none; 1, < 50% of central or portal areas; 2, > 50% of central or portal areas; 3, bridging fibrosis; and 4, cirrhosis).

Serum biochemistry and hepatic triglyceride assay.

Serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured on a Cobas Integra 400 Chemistry Automated Analyser as per manufacturer's instructions (Roche Diagnostics, Castle Hill, NSW, Australia). Triacylglycerol was extracted from liver homogenates as previously described[248, 249] and assayed using a commercial kit (Wako diagnostics, Virginia, USA). Serum ferritin was measured using a commercial available ELISA (Kamiya Biomedical, Seattle, WA, USA).

Hepatic antioxidant enzyme assay.

Total cellular glutathione peroxidase (GPx), reduced (GSH) and oxidised glutathione (GSSG) and catalase and mitochondrial manganese superoxide dismutase (MnSOD) activities were measured on homogenised liver tissue using commercial assay kits as per manufacturer's instructions (Cayman, MI, USA).

RNA extraction and Quantitative Real-time Polymerase Chain Reaction.

Total RNA was extracted using TRIzol reagent (Invitrogen, Mount Waverley, Victoria, Australia), subjected to deoxyribonuclease I digestion and transcribed into cDNA using Superscript III according to the manufacturer's instructions (Invitrogen). Quantitative gene expression was performed by real-time polymerase chain reaction (RT-PCR) (ViiA^{TM} 7, Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA) using Quantifast SYBR green as per manufacturer's conditions (Qiagen, Chadstone Centre,

Victoria, Australia). The expression of individual genes were normalised to the geometric mean of three house-keeper genes: basic transcription factor 3 (*Btf-3*), Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh)* and β2-microglobulin (*β2-m*). Oligonucleotides were custom synthesised by Sigma Genosys (Castle Hill, NSW, Australia). Mouse primer sequences for genes investigated are shown in Supplementary Table 1.

Statistical analysis.

Normally distributed data were analysed using one-way ANOVA with a Bonferroni multiple comparison post-test using GraphPad Prism 5 software (GraphPad Inc, San Diego, CA) and expressed as the mean + standard error of the mean (SEM). All other variables were analysed using Kruskal-Wallis ANOVA with a Dunn's multiple comparison post-test as appropriate.

7.3 RESULTS

Effects on Tissue Weight.

Feeding a HFD caused a significant increase in total body weight in WT and *Hfe*$^{-/-}$ mice compared to chow-fed mice (42%, $p<0.001$ and 38%, $p<0.001$ respectively) (Figure 7.1A and 1D). WT and *Hfe*$^{-/-}$ mice which were switched back to chow after 10 weeks of feeding HFD had similar end of study weights to chow fed controls (Figure 7.1A and 7.1D). WT and *Hfe*$^{-/-}$ mice fed the combination of curcumin and vitamin E had lower total body weight than HFD fed mice. However, there was no difference in total body weights between groups of animals fed either curcumin, vitamin E or the combination treatment (Figure 7.1A and 7.1D). Visceral adipose tissue (VAT) and liver weight were increased in WT and *Hfe*$^{-/-}$ mice fed HFD compared to chow (Figure 7.1B, 7.1C, 7.1E and 7.1F). VAT and liver weight were similar in all treatment groups for WT and *Hfe*$^{-/-}$ mice.

Severe steatosis and lobular inflammation following hfd feeding is attenuated by curcumin and vitamin e combination therapy

WT and *Hfe*$^{-/-}$ chow-fed mice showed no evidence of steatosis or liver injury (Table 1, Figures 7.2A, 7.2D, 7.3A and 7.3D). WT HFD-fed mice developed moderate to severe steatosis with hepatocyte ballooning and necroinflammation present in 67% of mice meeting criteria for steatohepatitis. The remaining 33% were classified as simple steatosis (Table 1 and Figure 7.2B). *Hfe*$^{-/-}$- mice fed the HFD developed severe micro- and macro-vesicular steatosis with hepatocyte ballooning and inflammatory cell infiltration in all mice (Table 7.1

and Figure 7.3B). WT and *Hfe*$^{-/-}$ mice switched from HFD to chow had normal liver pathology in almost all cases. NAS, steatosis grade and percent steatosis were all lower in the HFD to chow group compared to HFD-fed mice (Table 1 and Figures 7.2C and 7.3C). Treatment with either curcumin or vitamin E had minimal effects on hepatic histology (Table 7.1). Combination treatment with curcumin and vitamin E reduced micro- and macro-vesicular deposits throughout the liver, NAS and percent steatosis in WT and *Hfe*$^{-/-}$ mice (Table 7.1 and Figure 7.3F).

HFD feeding in of WT and *Hfe*$^{-/-}$ mice led to increases in ALT (5 fold, $p < 0.001$; 7.5 fold, $p < 0.001$ respectively) and AST (2 fold, $p < 0.01$; 2.5 fold, $p < 0.01$ respectively) compared to chow-fed mice (Figure 7.4). Transaminases were significantly decreased in the WT and *Hfe*$^{-/-}$ HFD to chow fed mice. Combination treatment in WT mice resulted in transaminase levels similar to HFD to chow fed levels. *Hfe*$^{-/-}$ mice fed combination treatment had significantly lower ALT (5 fold, $p < 0.001$) and AST (1.8 fold, $p < 0.001$). Curcumin treatment in WT mice significantly reduced ALT (2.2 fold, $p < 0.01$) but not AST concentration, while *Hfe*$^{-/-}$ mice had significantly lower ALT (3 fold, $P < 0.001$) and AST (1.7 fold, $P < 0.01$) concentrations. Vitamin E treatment in WT mice significantly reduced ALT (2.2 fold, $P < 0.01$) but not AST levels while in *Hfe*$^{-/-}$ mice significantly reduced ALT 2 fold ($P < 0.01$) and AST 1.4 fold ($P < 0.01$).

Centrilobular (stage 1) fibrosis (as detected by Sirius red staining) was observed in the majority of *Hfe*$^{-/-}$ mice fed the HFD. The prevalence of fibrosis was reduced in all treatment groups (Figure 7.5). In WT mice, only minimal fibrosis was present (Supplementary Figure 7.S1).

Gene expression of monocyte chemoattractant protein-1 (*Mcp-1*), (a product of activated stellate cells),[250] was elevated in WT and *Hfe*$^{-/-}$ mice fed HFD and was significantly reduced with combination treatment ($p<0.01$ respectively) (Figure 7.6A and 7.6D). Treatment with either agent reduced *Mcp-1* expression in WT mice only ($p<0.05$) (Figure 7.6A).

Expression of α_1(I)-procollagen (*Col1a1*) and tissue inhibitor of metalloproteinase *(Timp-1)* (genes related to hepatic fibrogenesis) were significantly elevated in WT and *Hfe*$^{-/-}$ HFD-fed mice compared to chow-fed mice (5 fold, $P < 0.001$ and 9 fold, $P < 0.001$ respectively). The

HFD to chow WT- and *Hfe*$^{-/-}$ fed mice had levels similar to those seen in the chow-fed only groups (Kruskal-Wallis, $P < 0.003$ and $P < 0.0001$ respectively for all treatment comparisons) (Figure 7.6B and 7.6E). Combination therapy reduced *Col1a* and *Timp-1* expression in WT and *Hfe*$^{-/-}$ - fed mice however this change did not reach statistical significance.

Dysregulated lipid signalling pathways due to curcumin and vitamin e combination therapy

HFD feeding in both WT and *Hfe*$^{-/-}$ mice led to increased serum glucose and serum cholesterol compared to CH fed mice (1.5 fold, $p<0.05$; 1.3 fold, $p<0.05$ respectively and 2 fold, $p<0.0001$ and 2.3 fold, $p<0.001$ respectively) and decreased serum triglyceride (2 fold, $p<0.01$; 2.7 fold, $p<0.001$ respectively). An increase in serum glucose implies an increase in hepatic lipogenesis through insulin signalling resulting in increased TG storage in the liver. Indeed, HFD feeding resulted in an increase in hepatic TG content in both WT and *Hfe*$^{-/-}$ mice (10 fold, $p<0.01$; 4 fold $p<0.01$ respectively) compared to chow fed mice. However, in WT and *Hfe*$^{-/-}$ mice, HFD feeding resulted in the downregulation of the expression of lipogenic genes such as *Srebp-1c*, and *Fas* and an increase in the phosphorylation of ACC1 protein which are consistent with downregulation of fatty acid synthesis. It is possible this reflects a dysresgulation in the ability of the liver to respond effectively to insulin. Also contributing to increased hepatic TG levels is an increase in FA uptake as reflected by an increase in serum NEFA….. and an increase in *CD36* mRNA expression in WT and *Hfe*$^{-/-}$ mice compared to CH fed mice (3 fold, $p<0.01$ and 2 fold, $p<0.001$ respectively.

Treatment with curcumin or vitamin E did not alter serum cholesterol or triglyceride levels in both WT and *Hfe*$^{-/-}$ mice compared to HFD feeding and caused a slight elevation in serum glucose levels in *Hfe*$^{-/-}$ mice compared to HFD fed mice. In contrast to HFD mice however, hepatic TG levels were significantly reduced in both WT and *Hfe*$^{-/-}$ mice fed a combination of curcumin and vitamin E (2 fold, $p<0.05$; 4 fold, $p<0.01$ respectively). This suggests that pathways such as FA synthesis and FA oxidation pathways are affected by treatment with curcumin and vitamin E. Western blot analysis on the rate limiting enzyme ACC in FA synthesis indicates a suppression on FA synthesis and lipogensis in treated WT and *Hfe*$^{-/-}$ mice compared to HFD fed mice (Fig….) There was also a return to normal CH fed levels of *Srebp-1c* and *Fas* mRNA expression in all WT treatment groups (3-4 fold, Kruskal-Wallis, $p<0.01$ for all comparisons compared with HFD alone) and a return to normal CH fed levels

of *Srebp-1c* expression in *Hfe*$^{-/-}$ mice fed a combination diet (2 fold, $p<0.01$). Similar to HFD fed mice however, treatment with curcumin, vitamin E and co-treatment increased fatty acid uptake to the liver as evidenced by elevated *CD36 mRNA* expression compared to CH fed mice. In *Hfe*$^{-/-}$ mice this increase was significantly higher than HFD fed mice, implying there is additional FA uptake by the liver. Combined with a decreased TG content this suggests the FA are channelled into β-oxidation pathways by mitochondria and peroxisomes.

Switching the mice back to chow after 10 weeks resulted in reducing serum cholesterol back to CH fed levels in both WT and *Hfe*$^{-/-}$ mice ($p<0.01$ for both WT and *Hfe*$^{-/-}$ comparisons) but no change in serum glucose. Serum triglyceride levels in these mice returned to normal CH fed levels in WT mice and increased compared to that in HFD fed mice (2 fold, $p<0.05$) approaching 50% of normal CH fed levels. Hepatic TG levels followed a very similar pattern with a 4 fold reduction seen in the WT mice switched back to chow compared to HFD fed mice and a return to normal CH fed levels in *Hfe*$^{-/-}$ mice. The slight reduction in FA uptake as seen by reduced *CD36* mRNA expression shows one mechanism by which TG storage has been reduced in this group in contrast to treatment groups. Also in contrast to treatment groups, no changes were seen in the expression of FA synthesis genes, *Srebp-1c*, *Fas* or *Scd1* to account for this reduction in hepatic TG content.

Enhanced gene expression of fatty acid oxidation pathways with curcumin and vitamin e combination therapy

Treatment of WT mice with curcumin, vitamin E and combination therapy resulted in a significant increase in hepatic adiponectin receptor 2 (*AdipoR2)* mRNA expression compared to the HFD control group (Figure 7.7A). Treatment of *Hfe*$^{-/-}$ mice with vitamin E and combination therapy also significantly increased hepatic *AdipoR2* mRNA expression (Figure 7.7D). There was an increase in both peroxisome proliferator-activated receptor α (*Pparα)* and carnitine palmitoyl transferase 1A (*Cpt1a)* mRNA expression in WT mice with curcumin, vitamin E and combination treatment compared to HFD alone (Figure 7.7B and 7.7C). *Cpt1a* expression was increased in *Hfe*$^{-/-}$ mice with vitamin E treatment and *Hfe*$^{-/-}$ mice with combination treatment and *Pparα* mRNA expression was increased in *Hfe*$^{-/-}$ mice with combination therapy compared to HFD alone (Figure 7.7F).

Effects on hepatic antioxidant enzyme activities and mitochondrial function

There was no difference in GPx activity between both WT- and *Hfe*$^{-/-}$-HFD fed mice and chow fed mice (data not shown). Hepatic mitochondrial manganese superoxide dismutase (MnSOD) activity was similar across all groups (data not shown). There was no significant change in GSH/GSSG ratio for WT and *Hfe*$^{-/-}$ mice and therefore no indication of oxidative stress (data not shown).

Effects on hepatic iron

Hfe$^{-/-}$ mice fed the chow diet had mild iron overload with a mean HIC of 42 µmol/g (dry weight) and as shown previously,[236] the HFD- fed mice had a significantly lower mean HIC of 24 µmol/g ($p< 0.01$) (Supplementary Figure 7.2). Interestingly, in the group of mice that were switched from HFD to chow, the mean HIC increased 3-fold to 59 µmol/g (ANOVA, $p<0.001$) when compared to HFD- fed mice. Hepcidin anti-microbial peptide 1 *(Hamp1)* mRNA levels were similar across all groups (data not shown). All other treatments did not affect the amount of iron in the liver and were similar to the levels found in the HFD-fed mice.

7.4 DISCUSSION

In this study, it was demonstrated that the combination of curcumin and vitamin E therapy attenuated steatosis and steatohepatitis in wild type and *Hfe*$^{-/-}$ mice fed a HFD. WT mice fed a HFD for 20 weeks developed steatosis and 67% had histological features consistent with steatohepatitis. The histological injury was more severe in the *Hfe*$^{-/-}$ mice, with all animals showing features of steatohepatitis, and the majority of these showing centrilobular fibrosis. Monotherapy with either vitamin E or curcumin was associated with an improvement in some components of steatohepatitis, but combination therapy markedly reduced micro- and macrovescicular steatosis and percent hepatic steatosis as well as lobular inflammation, ballooning degeneration and fibrosis.

The administration of HFD for 20 weeks caused a marked elevation of ALT and AST, and increased gene expression of *Mcp-1*, *Col1a1* and *Timp-1* in WT and *Hfe*$^{-/-}$ animals. Hepatic collagen deposition, as assessed by Sirius red staining, was not increased in wild type animals, but a characteristic ‘chicken wire’ appearance seen in NASH developed in *Hfe*$^{-/-}$ animals fed a HFD. Previously it was demonstrated similar changes after 8 weeks of feeding of HFD to *Hfe*$^{-/-}$ animals.[236] In the current study a group of animals fed a HFD for 10 weeks

were switched back to a chow diet for a further 10 weeks before sacrifice. This HFD to chow group provided an excellent comparator group and in these animals there was minimal to no hepatic fibrosis, and serum ALT and AST concentrations as well as *Col1a1*, *Mcp-1* and *Timp-1* gene expression all normalised. Combination therapy with curcumin and vitamin E was not as effective as the HFD to chow switch, but virtually every measure of liver injury was improved with dual treatment and the improvement with combination treatment was greater than with either monotherapy. This implies that vitamin E and curcumin exert a synergistic effect over that provided by each individual therapy.

Curcumin is the yellow pigment of the plant *Curcuma longa* (turmeric) and has potent antioxidant and chemo preventative effects. The administration of curcumin has been shown to produce beneficial effects in many animal models of liver diseases, including alcohol–related injury,[251, 252] carbon tetrachloride-induced injury[253] and the cholestatic injury seen in *Mdr2*$^{-/-}$ mice.[254] Curcumin ameliorates the early stages of experimental steatohepatitis and limits the development and progression of fibrosis in mice fed a methionine choline deficient diet.[255] Several studies have shown benefit of vitamin E administration in liver diseases associated with high levels of oxidative stress,[256] and vitamin E supplementation has been associated with improvements in liver injury in animal models of liver disease and in humans with NAFLD.[241] The mechanism by which the combination therapy of vitamin E and curcumin offered therapeutic advantage was explored in this study. I did not find evidence of increased oxidative stress in both wild type and *Hfe*$^{-/-}$ animals fed a HFD and there was no effect of either monotherapy with either vitamin E or curcumin, or combination therapy on hepatic mitochondrial manganese superoxide dismutase (MnSOD) activity, catalase activity or GSH:GSSG ratio. Thus the improved histology in animals fed a combination of vitamin E and curcumin appears to be independent of antioxidant properties. Higher curcumin concentrations are achievable *in vitro*, where the primary effect is through its antioxidant action. However, recent research has suggested that the effects of curcumin at lower concentrations which are achieved *in vivo* are independent of its antioxidant properties and are via signal transduction and gene expression.[257, 258] Indeed, treatment was associated with changes in the gene expression of proteins involved in fatty acid oxidation. Of interest, the gene expression of *AdipoR2* was attenuated in *Hfe*$^{-/-}$ mice fed a HFD consistent with previous observations. Combination therapy resulted in a 2.5 fold increase in *AdipoR2* mRNA expression and this was accompanied by significant increases in *Ppar-α* and *Cpt1a* gene

expression consistent with increased mitochondrial uptake of free fatty acids and increased *β*-oxidation in the treated animals. WT animals treated with combination therapy also demonstrated increased gene expression of *AdipoR2, Ppar- α and Cpt1*, but this was also seen in monotherapy treatment groups. *AdipoR2* has been shown to activate *Ppar-α* and fatty acid oxidation genes, as well as inhibit lipogenesis.[175] These results might imply that combination therapy is effective due to *AdipoR2* upregulation resulting in increased gene expression of fatty acid oxidation pathways and therefore protection against steatohepatitis. However, it is worth noting that the change in *AdipoR2* expression in mice treated with either monotherapy alone was similar to that achieved by combination therapy. This would suggest that additional mechanisms are involved in the combination treatment groups.

Curcumin has also been reported to have iron chelating properties,[237] but the HIC of the treated animals was not different from the animals that received HFD, thus iron chelation seems an unlikely explanation for the observed benefit. Regardless, the change in the hepatic iron concentration of the *Hfe*$^{-/-}$ animals fed a HFD was of interest. *Hfe*$^{-/-}$ fed chow for 20 weeks had an HIC of 42 µmol/ g and the back-to-chow group had a final HIC 59 µmol/g, whereas *Hfe*$^{-/-}$ animals fed a HFD for 20 weeks had a significantly lower HIC. This finding remains difficult to explain but it confirms previous findings, and it has been suggested by others that a HFD alters intestinal iron absorption.[259] In my animals, *Hamp1* mRNA levels were similar across all groups suggesting that any effect on iron absorption is independent of hepcidin gene expression.

The principal difficulty with oral administration of curcumin is low systemic bioavailability due to poor oral absorption; therefore alternative methods of administration may need to be developed before these observations can be translated into clinical treatments. The dose of curcumin administered in the current study was sufficiently high to ensure absorption, although serum concentrations of curcumin were not analyzed.

The high prevalence of obesity and the metabolic syndrome means that many patients with liver disease of varying etiologies will have co-existent nonalcoholic fatty liver disease. Indeed contemporary clinical practice in hepatology is often characterised by the need to address multiple co-toxins in one patient. The results of the present study are an excellent illustration of the concept of co-toxic liver disease since slight increases in HIC as seen in the

Hfe$^{-/-}$ animals fed a HFD were associated with necroinflammation and hepatic fibrosis. Because of the beneficial effect of combination therapy, I believe vitamin E and curcumin should be investigated in other animal models of NASH, and could be moved rapidly into human studies if a beneficial effect is demonstrated, and if appropriate dosing strategies can be developed.

7.5 Figures

Figure 7.1. Body Weights. Effects of diets on (A) total body, (B) liver, and (C) visceral adipose tissue (VAT) weights in wild-type (WT) mice, (D) total body, (E) liver and (F) VAT weight in *Hfe*$^{-/-}$ mice. Values are means + SE. *P < 0.05 compared to HFD alone. $^{\#}$P < 0.05 compared to Chow alone. *N* = 7-10 per group.

Figure 7.2. Assessment of liver injury and fibrogenesis in Wild-Type mice. Representative liver histology stained with hematoxylin and eosin (H&E) on paraffin-embedded sections (original magnification x 20).

Figure 7.3. Assessment of liver injury and fibrogenesis in *Hfe*$^{-/-}$ mice. Representative liver histology stained with hematoxylin and eosin (H&E) on paraffin-embedded sections (original magnification x 20).

Figure 7.4. Assessment of liver injury. Serum alanine aminotransferase (ALT) (A) and serum aspartate aminotransferase (AST) (B) levels in wild-type (WT) mice and ALT (C) and AST (D) in *Hfe*$^{-/-}$ mice. *P < 0.05 compared to HFD alone. *N* = 7-10 per group.

Figure 7.5: Hepatic collagen deposition as assessed by Picro-Sirius red staining in *Hfe*$^{-/-}$ mice. (original magnification x 20). Representative liver histology stained with Picro-Sirius red on paraffin liver sections.

Figure 7.6. Assessment of fibrogenesis. Reverse transcriptase-polymerase chain reaction (RT-PCR) for hepatic fibrogenic pathway gene expression of α_1(I)-procollagen (*Col1a1*), monocyte chemoattractant protein-1 (*Mcp-1*); tissue inhibitor of metalloproteinase -1 (*Timp-1*). *P < 0.05 compared to HFD alone. *N* = 7 - 10 per group.

Figure 7.7. Assessment of hepatic lipid pathways. RT-PCR for hepatic expression of fatty acid oxidation genes adiponectin receptor 2 (*AdipoR2*), peroxisome proliferator-activated receptor α (Pparα), carnitine palmitoyl transferase 1A (*Cpt1a*). *P < 0.05 compared to HFD alone. *N* = 7-10 per group.

Table 7.1. Results of H&E on liver sections for Wild-Type and *Hfe*$^{-/-}$ mice. Steatosis was graded (0 to 3) and lobular inflammation (0 to 3) on H&E staining. Values are expressed as the median (range). Nonalcoholic Steatohepatitis Activity Score (NAS) is expressed as median values (range). *N* = 7 - 10 per group.

Supplementary Figure 7.S1. Hepatic collagen deposition as assessed by Picro-Sirius red staining in Wild-Type mice. (original magnification x 20). Representative liver histology stained with Picro-Sirius red on paraffin liver sections.

Supplementary Figure 7.S2. Hepatic iron concentration (HIC). Hepatic iron concentration (HIC) in livers from (A) wild-type (WT) mice and (B) *Hfe*$^{-/-}$ mice. *P < 0.05 compared to HFD alone. *N* = 7 - 10 per group.

Supplementary Table 7.1. Mouse primer sequences for genes used in RT-PCR.

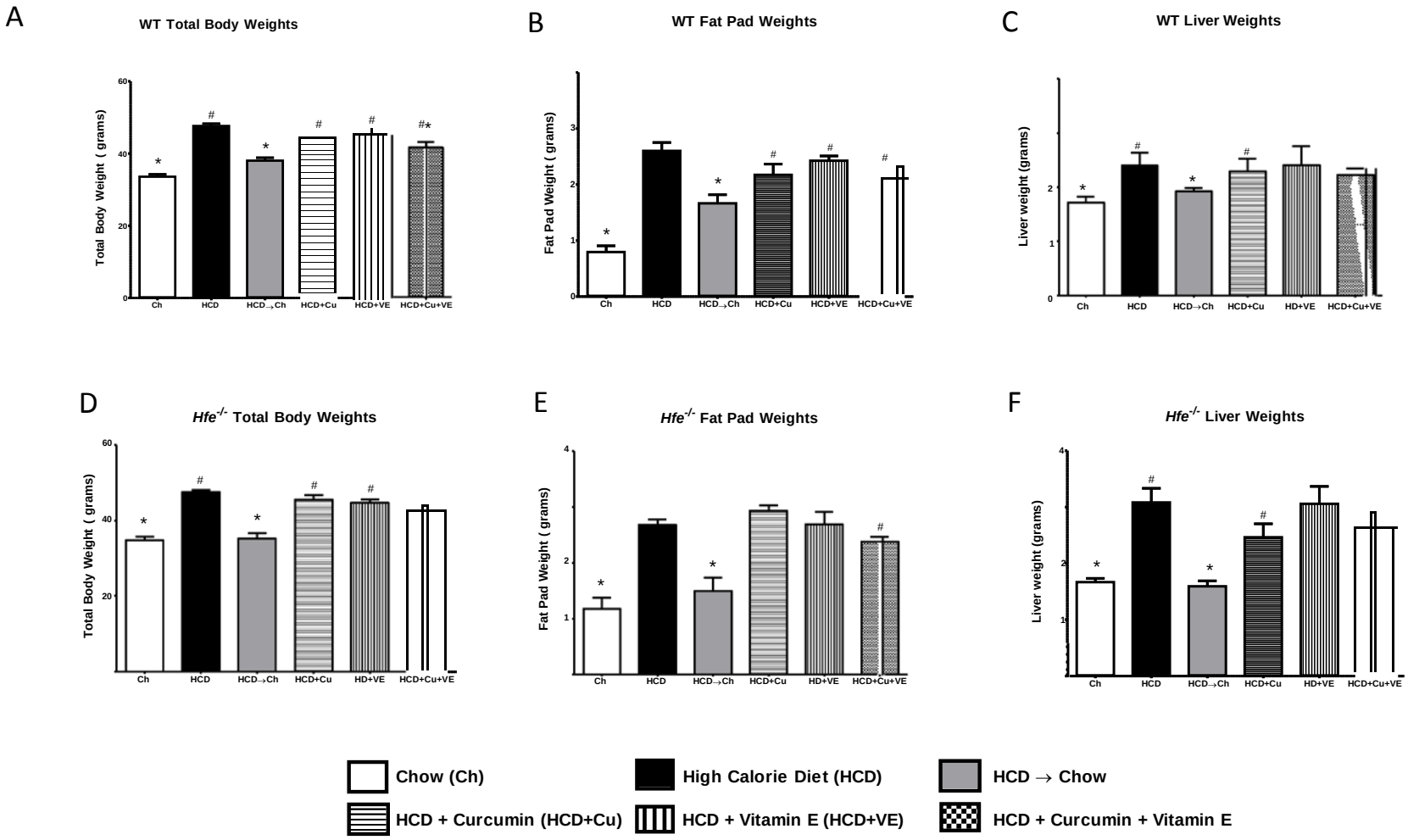

A
WT Total Body Weights
Total Body Weight (grams)
B
WT Fat Pad Weights
Fat Pad Weight (grams)
C
WT Liver Weights
Liver weight (grams)
D
Hfe-/- Total Body Weights
Total Body Weight (grams)
E
Hfe-/- Fat Pad Weights
Fat Pad Weight (grams)
F
Hfe-/- Liver Weights
Liver weight (grams)
Ch
HCD
HCD→Ch
HCD+Cu
HD+VE
HCD+Cu+VE
Chow (Ch)
High Calorie Diet (HCD)
HCD → Chow
HCD + Curcumin (HCD+Cu)
HCD + Vitamin E (HCD+VE)
HCD + Curcumin + Vitamin E

Figure 7.1

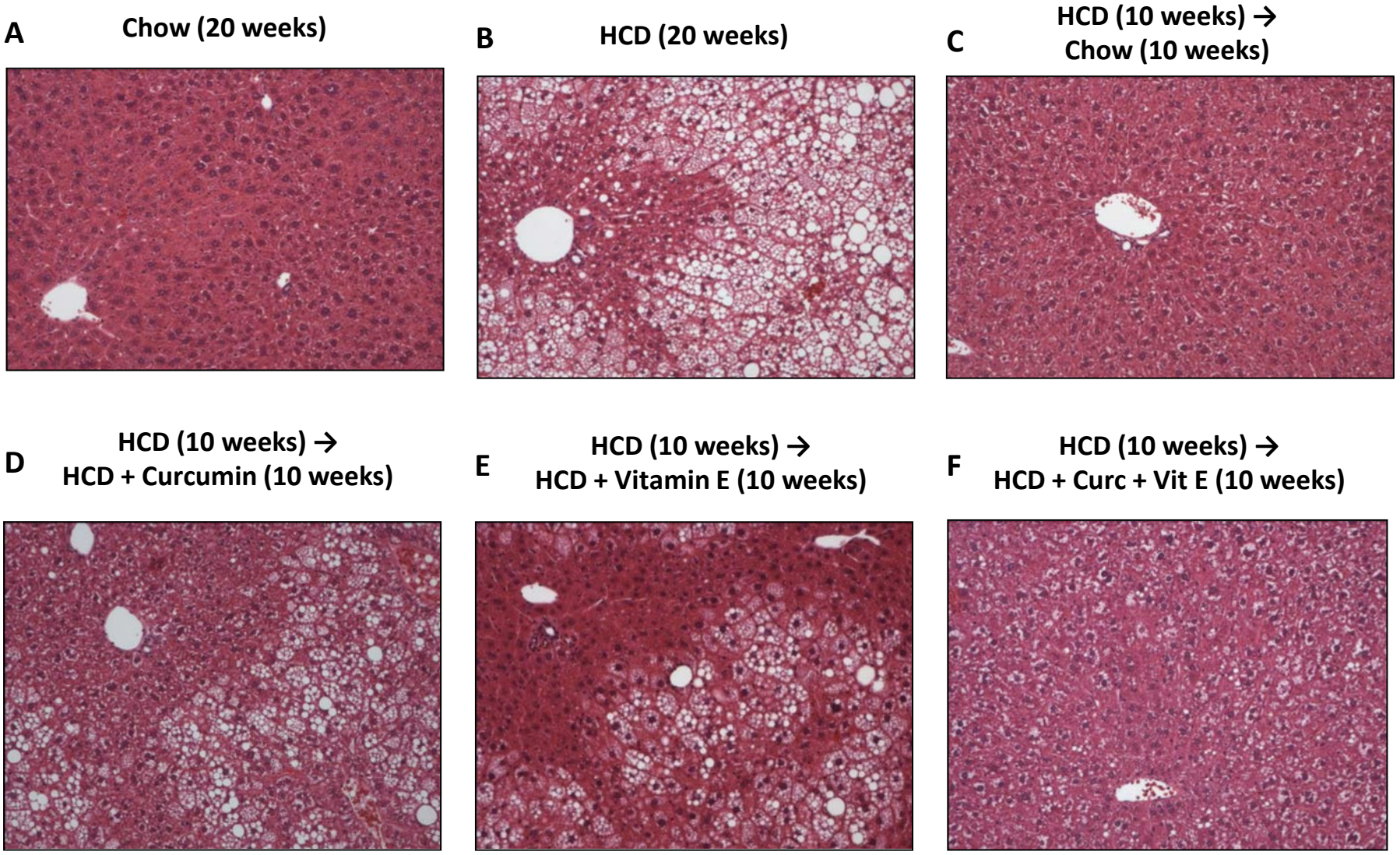

A
Chow (20 weeks)
B
HCD (20 weeks)
C
HCD (10 weeks) →
Chow (10 weeks)
D
HCD (10 weeks) →
HCD + Curcumin (10 weeks)
E
HCD (10 weeks) →
HCD + Vitamin E (10 weeks)
F
HCD (10 weeks) →
HCD + Curc + Vit E (10 weeks)

Figure 7.2

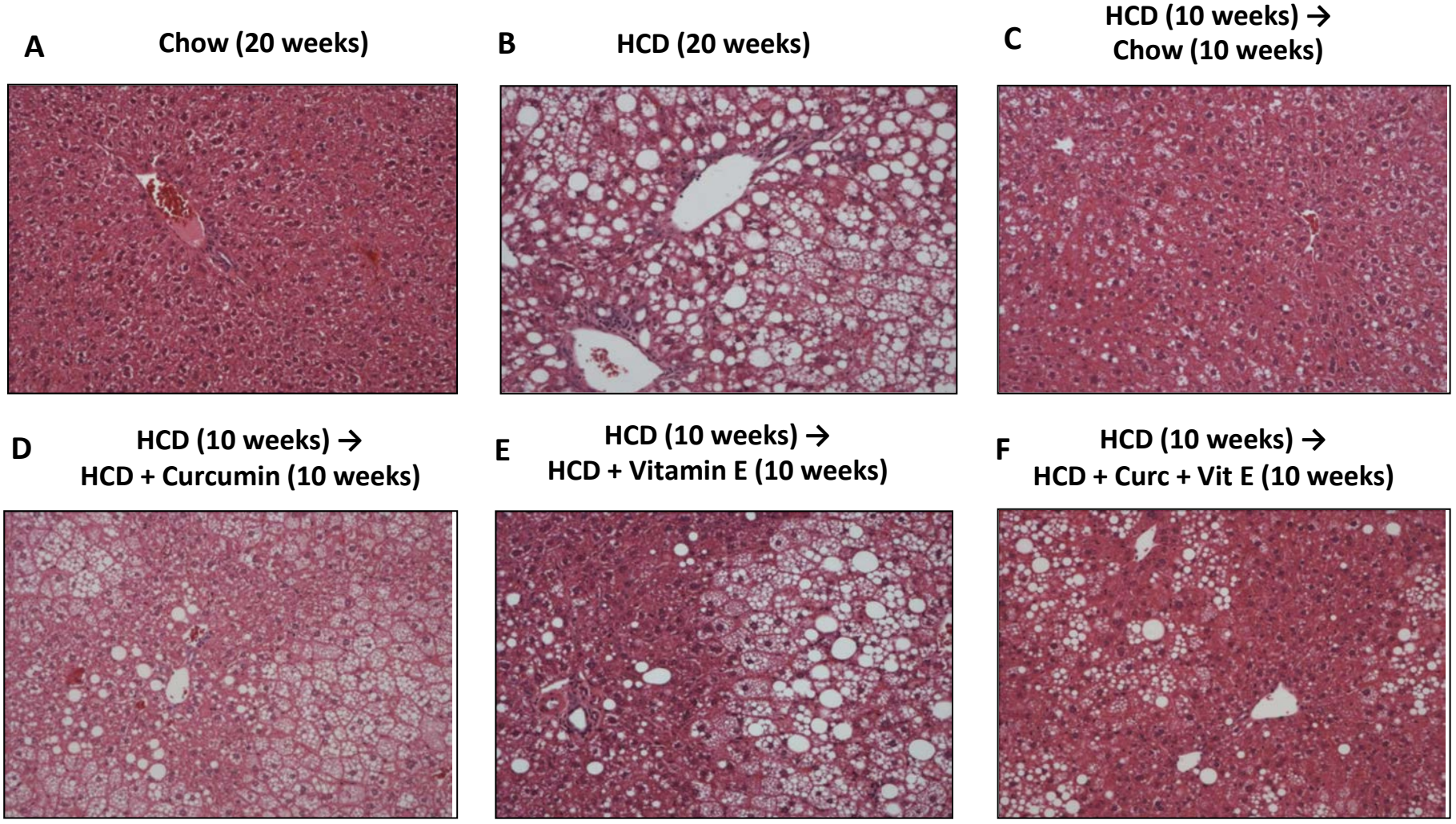

A
Chow (20 weeks)
B
HCD (20 weeks)
C
HCD (10 weeks) →
Chow (10 weeks)
D
HCD (10 weeks) →
HCD + Curcumin (10 weeks)
E
HCD (10 weeks) →
HCD + Vitamin E (10 weeks)
F
HCD (10 weeks) →
HCD + Curc + Vit E (10 weeks)

Figure 7.3

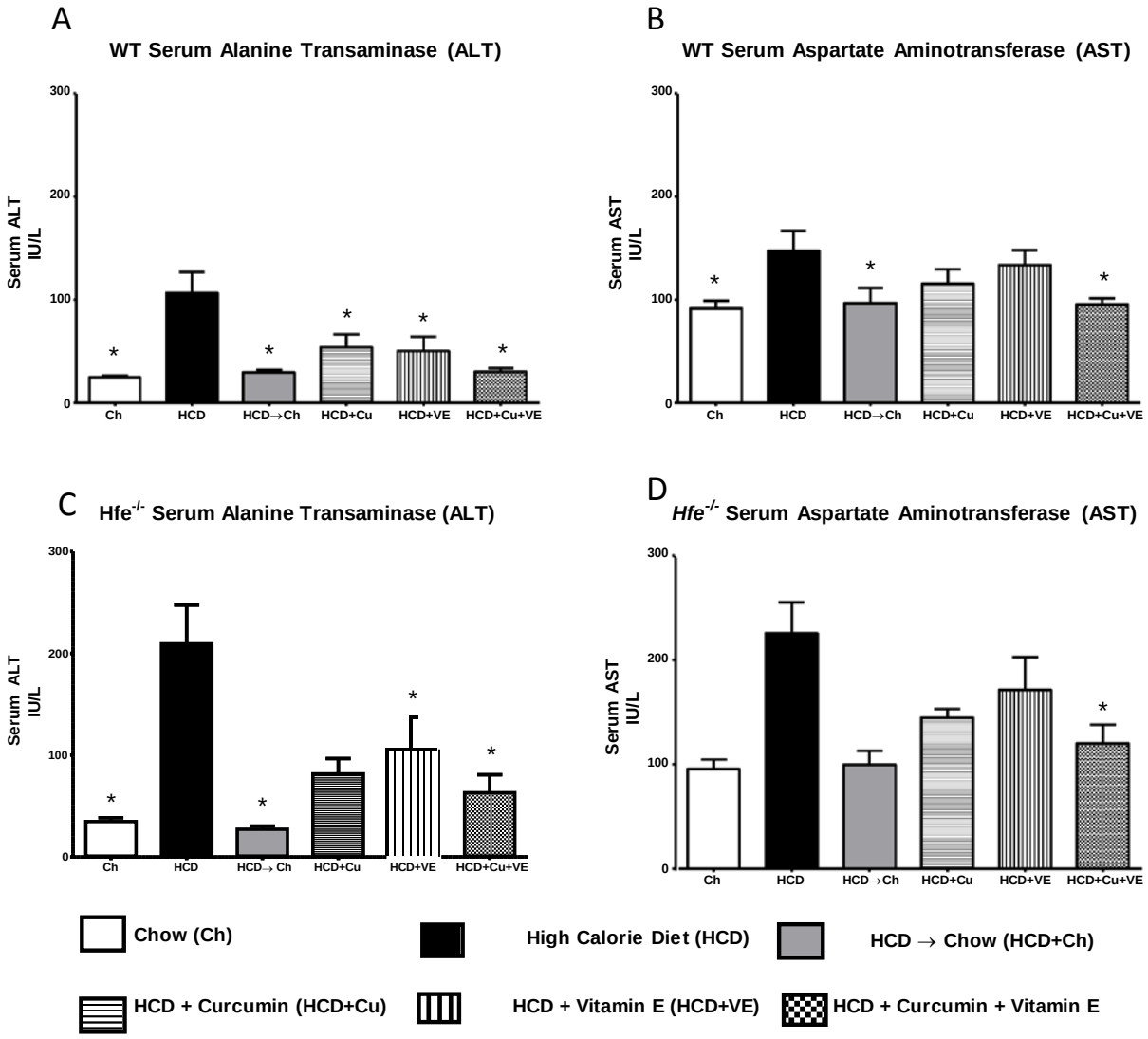

A
WT Serum Alanine Transaminase (ALT)
Serum ALT IU/L
300
200
100
0
Ch
HCD
HCD→Ch
HCD+Cu
HCD+VE
HCD+Cu+VE
B
WT Serum Aspartate Aminotransferase (AST)
Serum AST IU/L
C
Hfe-/- Serum Alanine Transaminase (ALT)
D
Hfe-/- Serum Aspartate Aminotransferase (AST)
Chow (Ch)
High Calorie Diet (HCD)
HCD → Chow (HCD+Ch)
HCD + Curcumin (HCD+Cu)
HCD + Vitamin E (HCD+VE)
HCD + Curcumin + Vitamin E

Figure 7.4

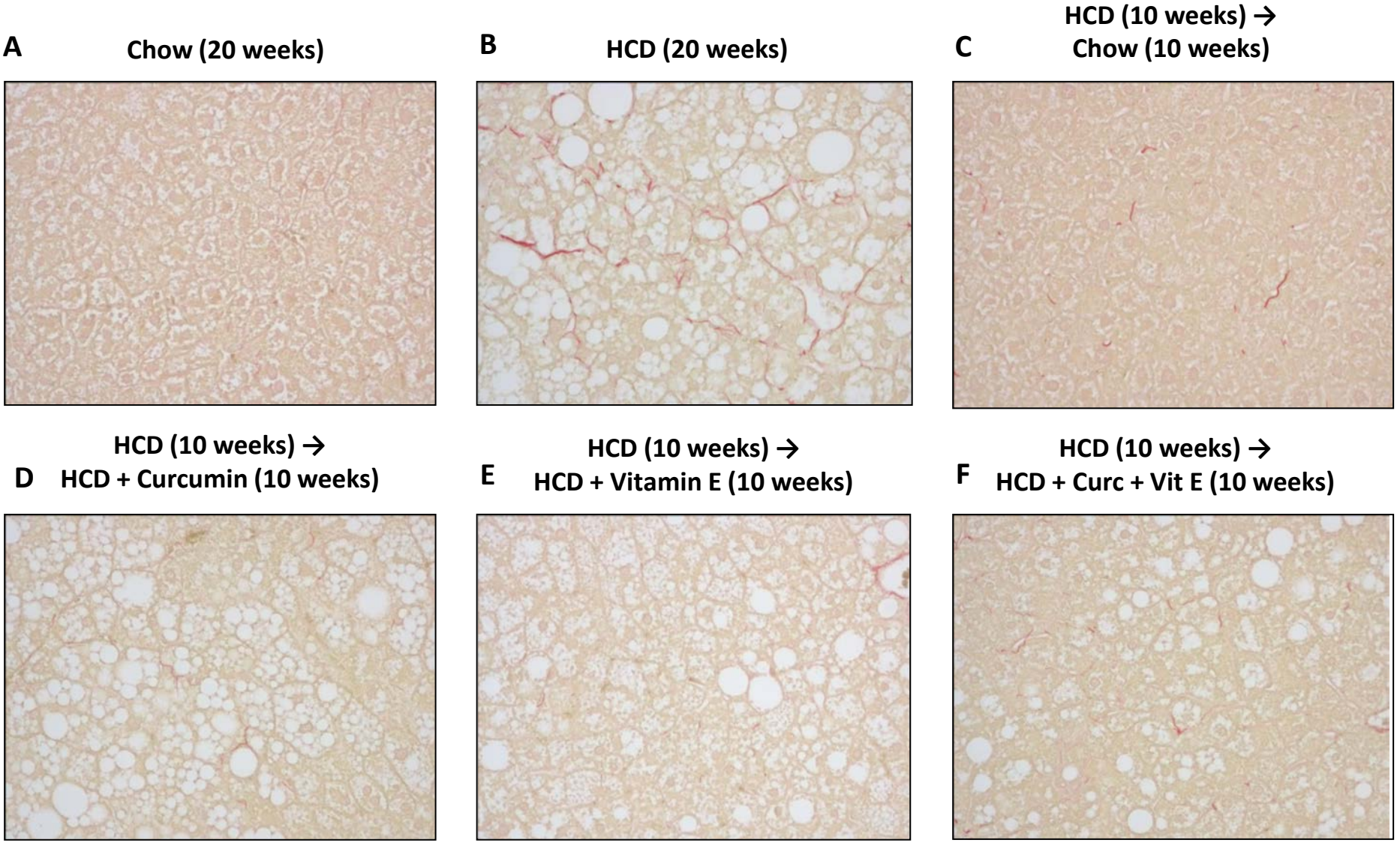

A
Chow (20 weeks)
B
HCD (20 weeks)
C
HCD (10 weeks) →
Chow (10 weeks)
D
HCD (10 weeks) →
HCD + Curcumin (10 weeks)
E
HCD (10 weeks) →
HCD + Vitamin E (10 weeks)
F
HCD (10 weeks) →
HCD + Curc + Vit E (10 weeks)

Figure 7.5

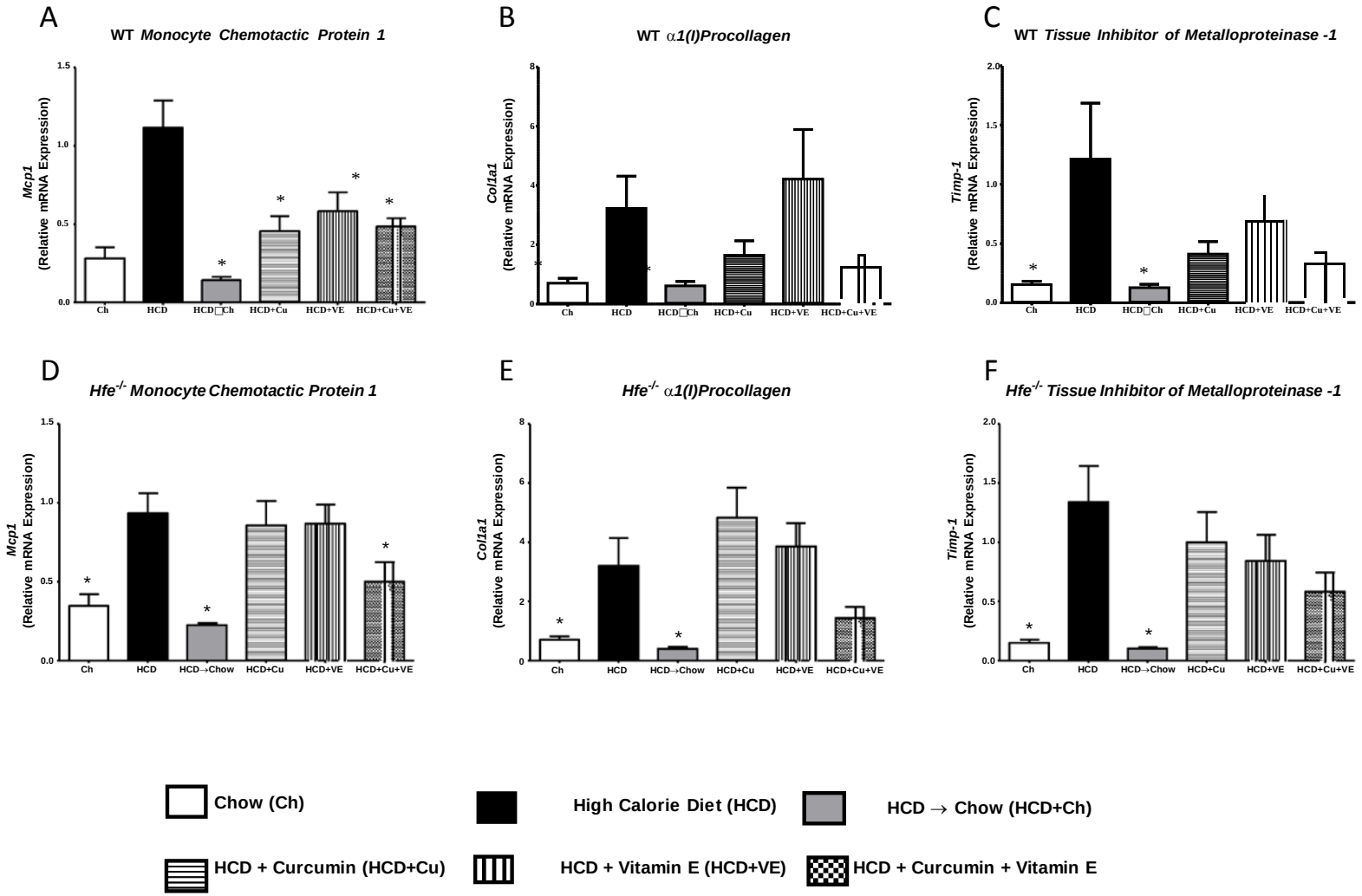

A
WT Monocyte Chemotactic Protein 1
Mcp1 (Relative mRNA Expression)
B
WT α1(I)Procollagen
Col1a1 (Relative mRNA Expression)
C
WT Tissue Inhibitor of Metalloproteinase -1
Timp-1 (Relative mRNA Expression)
D
Hfe-/- Monocyte Chemotactic Protein 1
Mcp1 (Relative mRNA Expression)
E
Hfe-/- α1(I)Procollagen
Col1a1 (Relative mRNA Expression)
F
Hfe-/- Tissue Inhibitor of Metalloproteinase -1
Timp-1 (Relative mRNA Expression)
Ch
HCD
HCD→Ch
HCD→Chow
HCD+Cu
HCD+VE
HCD+Cu+VE
Chow (Ch)
High Calorie Diet (HCD)
HCD → Chow (HCD+Ch)
HCD + Curcumin (HCD+Cu)
HCD + Vitamin E (HCD+VE)
HCD + Curcumin + Vitamin E

Figure 7.6

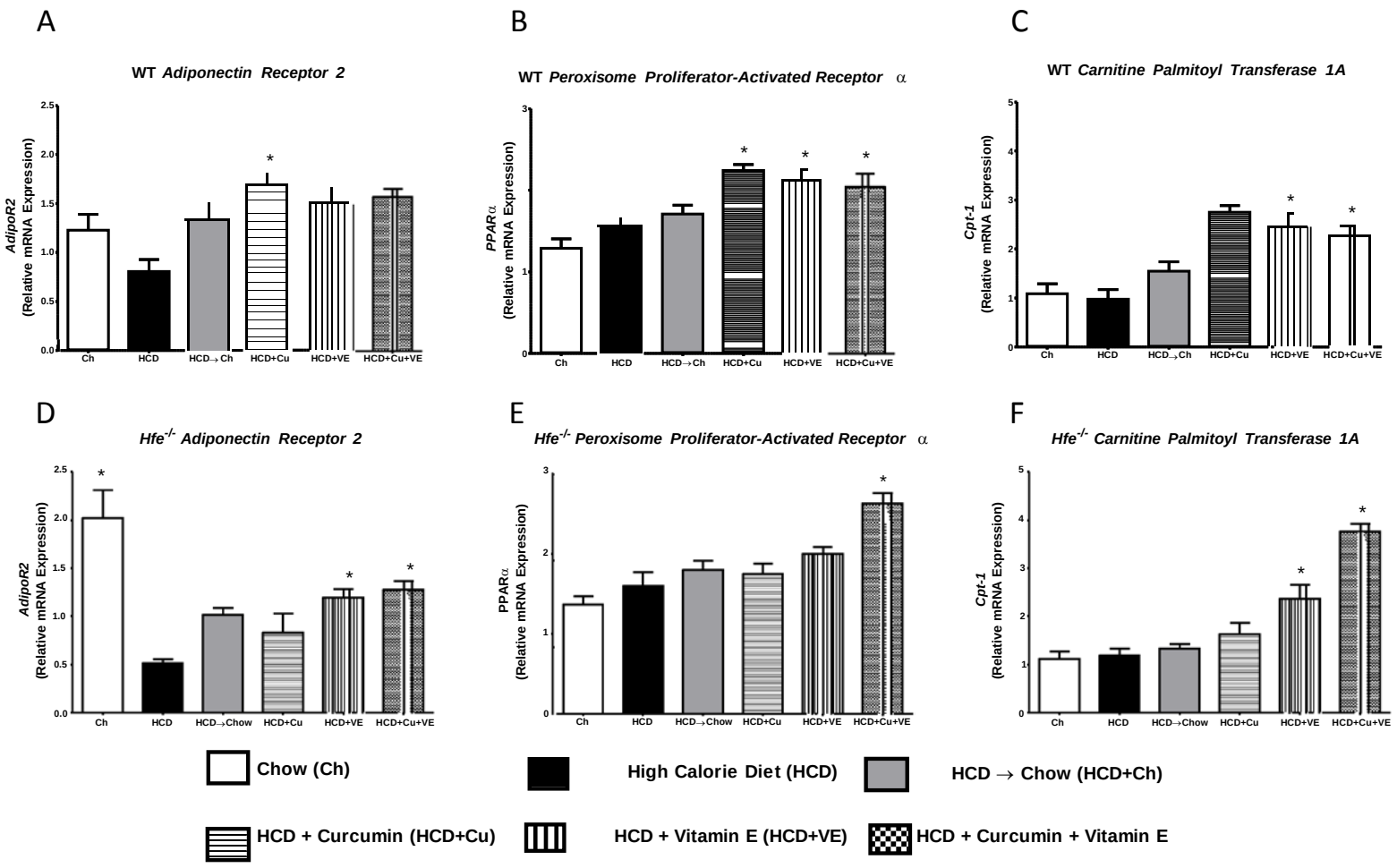
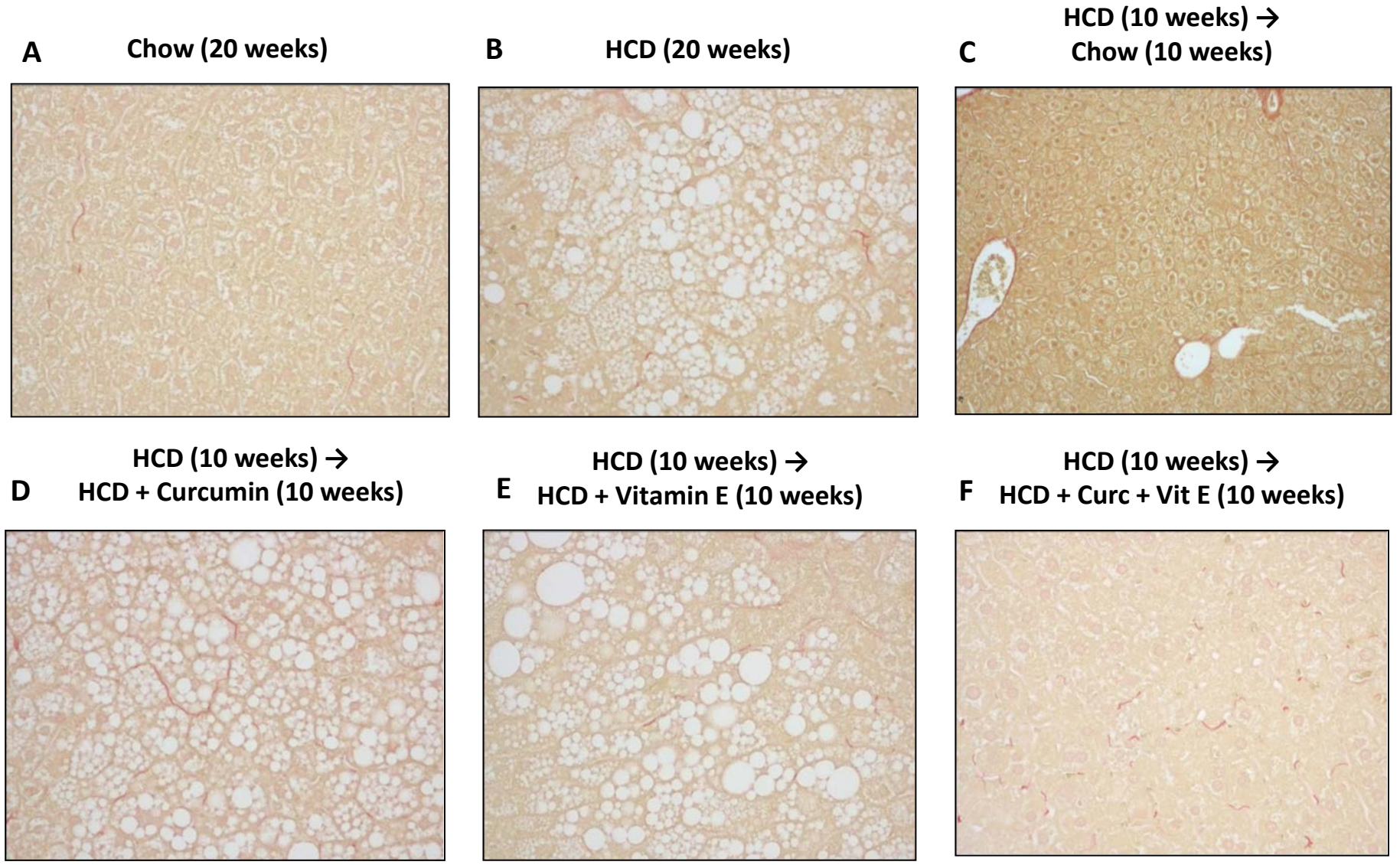
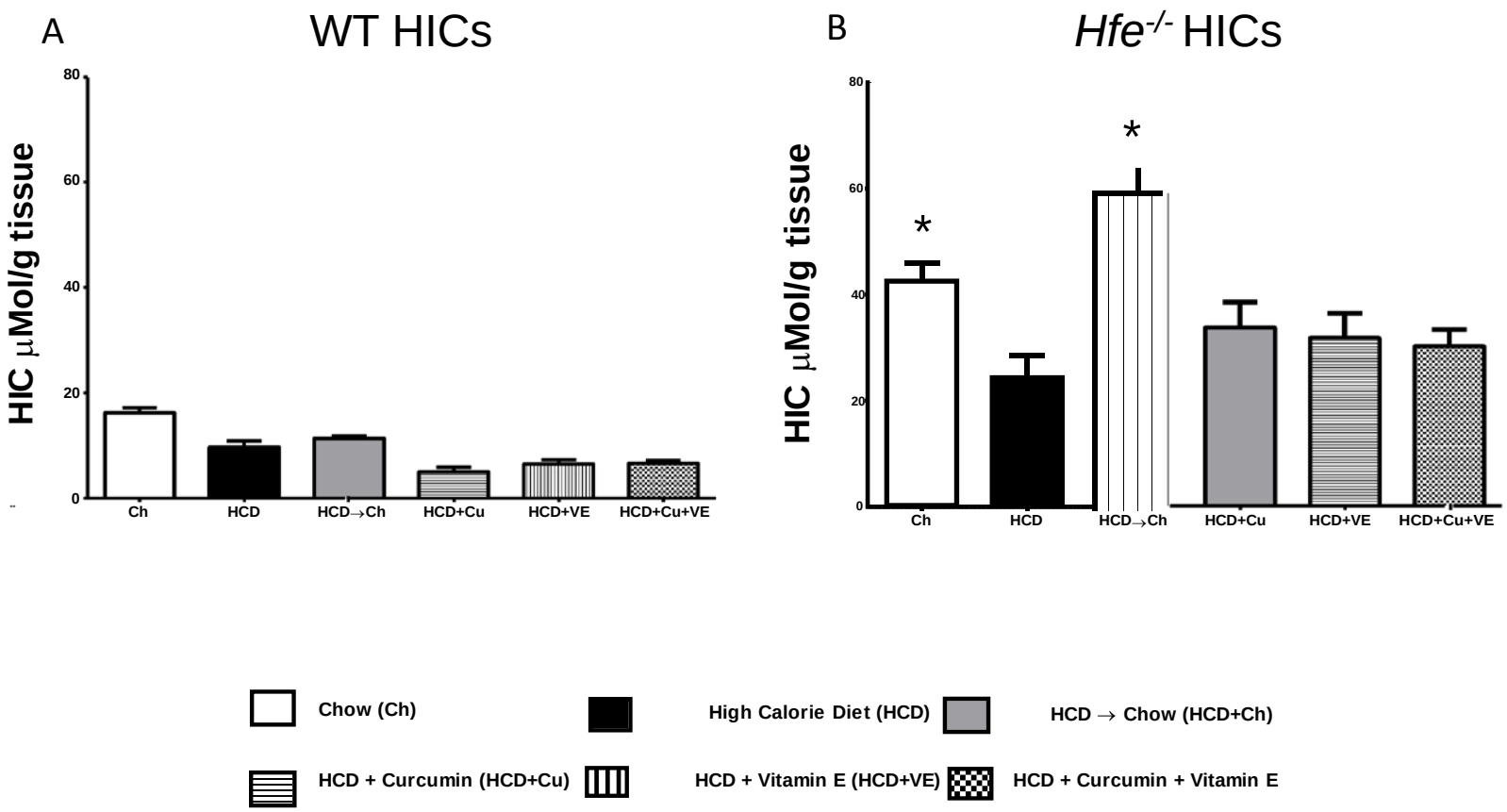

A
WT Adiponectin Receptor 2
AdipoR2 (Relative mRNA Expression)
Ch
HCD
HCD→ Ch
HCD+Cu
HCD+VE
HCD+Cu+VE
B
WT Peroxisome Proliferator-Activated Receptor α
PPARα (Relative mRNA Expression)
HCD→Ch
C
WT Carnitine Palmitoyl Transferase 1A
Cpt-1 (Relative mRNA Expression)
HCD→Ch
D
Hfe-/- Adiponectin Receptor 2
HCD→Chow
E
Hfe-/- Peroxisome Proliferator-Activated Receptor α
F
Hfe-/- Carnitine Palmitoyl Transferase 1A
Chow (Ch)
High Calorie Diet (HCD)
HCD → Chow (HCD+Ch)
HCD + Curcumin (HCD+Cu)
HCD + Vitamin E (HCD+VE)
HCD + Curcumin + Vitamin E

Figure 7.7



Supplementary Figure 7.S1



Supplementary Figure 7.S2

Chapter 8

Continuing with investigations into the role of probiotics in chronic diseases, this study was designed to show that probiotics are capable of providing beneficial effects in disease and animal models that are not conventionally used. Observations made in the laboratory led us to investigate the influence of bacteria and probiotics on tumour development. Unfortunately due to unforeseen changes, this study had to be terminated and no further progression of the study hypothesis could be made.

Probiotics administered to $atm^{-/-}$ mice

The effects of probiotics on tumour development and survival times in $Atm^{-/-}$ mice

8.1 Abstract

Background: Ataxia telangiectasia (AT) is a rare autosomal recessive genetic disorder. Modification of the ataxia telangiectasia mutated (atm) gene provides a model to study AT. $atm^{-\backslash-}$ mice spontaneously develop tumours and when housed in a non SPF facility, tumour development has been noted to be accelerated and life expectancy is reduced. ***Aim***: The main aim of this study was to prolong the life expectancy of $atm^{-/-}$ mice housed in a "dirty" environment through supplementation with probiotics. ***Methods/Results***: Following 6 months of supplementation with probiotics, neither the control nor the experimental group showed signs of tumour development and the study was terminated. ***Discussion***: Recent removal of other animal species from the animal house and the introduction of filter top cages reduced the pathogens that the mice were exposed to. This is suspected to have prevented the previously noted acceleration in tumour development when $atm^{-/-}$ mice were housed in a non SPF facility.

8.2 Introduction

Ataxia telangiectasia (AT) is a rare autosomal recessive genetic disorder that affects many parts of the body causing severe disability. It is estimated that AT affects 1 in 40,000-100,000 people worldwide[260]. Individuals with AT have a several hundred fold increased risk of developing cancers, particularly leukaemia and lymphomas.[261, 262] Other disorders associated

with AT are neurodegeneration with progressive ataxia, variable immunodeficiency, premature ageing and recurrent infections. AT has also been referred to as an immunodeficiency disorder, a chromosomal instability disorder or a DNA repair disorder.

Ataxia telangiectasia mutated (ATM) is a protein kinase activated by DNA double strand breaks, initiating the phosphorylation of several key proteins. Among the proteins phosphorylated, activation of the tumour suppressor protein p53 results in cell-cycle arrest and DNA repair restoring cellular and genetic stability. In the event of irreparable DNA damage, the cell undergoes apoptosis. Damage to the ATM gene product changes the cell-cycle checkpoint control leading to accumulation of DNA damage. An accumulation of DNA damage can destabilise the genome and result in the formation of cancer.

A large problem faced by patients with AT is recurrent autoimmune disorders. Patients with AT production fewer T-cells[263, 264] and have an increased T-cell activation compared with the healthy population.[265, 266] T-cells originate in the thymus as naive T-cells and migrate around the body. Once a T-cell comes into contact with an antigen through an antigen-presenting cell, the T-cell differentiates into either a TH_1 or TH_2 phenotype. Following differentiation, the T-cell produces cytokines to help facilitate an immune response. A high percentage of T-cells activation leads to a high rate of cytokine production and subsequent immune activation. Continuous activation of the immune system leaves the host susceptible to new infections and a higher degree of inflammation. Respiratory tract infections are amongst the more prevalent infections that occur in patients with AT.[267]

Currently there are no treatments specifically for AT. Rather treatments are directed at the specific symptoms presented. Studies have reported that increasing the dietary intake of various foods, such as fish oils, helps reduce the development of some cancers (colon, prostate and breast).[264, 268] Reduced levels of antioxidant micronutrients are commonly reported in immunodeficiency diseases and cancer.[269, 270] AT patients often present with elevated levels of reactive oxygen species (ROS) and low levels of reactive-scavenger enzymes (antioxidants).[271] This imbalance leads to oxidative damage, resulting in cell damage. Supplementing $Atm^{-/-}$ mice with antioxidants retards the formation of thyoma tumours.[272, 273]

In an attempt to help understand and develop treatments for AT, an $Atm^{-/-}$ mouse model has been developed. Among the observations made using $Atm^{-/-}$ mice, is that $Atm^{-/-}$ mice housed in a 'dirty' environment rapidly develop tumours and typically require euthanasia around five months of age. In contrast, $Atm^{-/-}$ mice housed in a 'clean' environment tend to live significantly longer. One possibility that might explain the greater longevity of animals living in a 'clean' environment is the lack of pathogenic bacteria present. Pathogenic bacteria invade the gastrointestinal tract initiating an immune response that can result in dysregulated inflammation causing problems varying from muscular pain, fatigue and diarrhoea to tumour and cancer development.[274]

Probiotic therapy may help to modulate the immune response of AT. Probiotics have been shown to have immunomodulating effects, with the capability to alter cytokine production, systemic antibody responses, tight junction protein distribution and dendritic cell distribution.[6, 33, 35, 36, 61, 62] The beneficial effects of probiotics are not only limited to the gastrointestinal tract, however. Probiotics have also been shown to help with liver function[275], dermatological conditions[276, 277] and respiratory tract infections.[278]

The main aim of this study was to prolong the life expectancy of $Atm^{-/-}$ mice by regulating the immune system and inflammation. A subsequent aim was to map the metabolic profile of $Atm^{-/-}$ mice to find significant changes in metabolite production. The primary hypothesis was that administering probiotics would significantly increase the quality of life and delay the time to euthanasia of $Atm^{-/-}$ mice by enhancing the beneficial bacteria content of the gastrointestinal tract, subsequently reducing inflammation and immune activation.

8.3 Methods

Probiotics

Four strains of water soluble lyophilised probiotics (*B. bifidum, B. longum, L. rhamnosus* or *S. thermophilus*) were provided by BioCeuticals. These strains were administered as a multi-strain blend to the drinking water at a total concentration of $1 \times 10^{8\text{-}9}$ cfu/mL.

Animals

Probiotics or a placebo were administered to $Atm^{-/-}$ mice in drinking water for the duration of the study. Freely available food and water were changed at least twice a week. Mice were monitored daily at the start of the project for signs of distress using an animal welfare monitoring sheet. Animal welfare scores were assessed daily initially and reduced to weekly

following consistent low scores. If the welfare scores of the animals increase, the frequency of monitoring were adjusted accordingly. Any animal scoring high were considered under severe stress and were euthanized. Faecal matter and blood were collected at regular intervals and stored for later analysis.

$Atm^{-/-}$ and wild type mice were supplied by QIMR and housed in a 'dirty' environment at the Mt. Gravatt facility. The animals were housed under conventional conditions (light cycle, temperature and atmospheric conditions) and have free access to food (standard chow) and water. The animals were split into 3 groups: 1) $Atm^{-/-}$ mice receiving a combination of four probiotic strains (n=13); 2) $Atm^{-/-}$ mice receiving a placebo (n=10); and 3) wild type litter mates with no intervention (n=6). Three animals from group 1 were euthanized when the majority of the animals in group 2 are considered to be under severe stress for later comparison.

8.4 Results

No evidence of tumour development was present after 6 months. The animals were terminated as per instructed by the animal ethics committee.

8.5 Discussion

In this study, I aimed to show probiotics were capable of prolonging the life expectancy of $Atm^{-/-}$ mice by regulating the immune system and inflammation. This aim was based on observations from previous studies that observed when $atm^{-\backslash-}$ mice were housed in a "clean" SPF facility, tumour development was delayed and the life expectancy of the mouse was as long as 1-2 years. However, when the animals were housed in a "dirty" non-SPF facility, tumour development was accelerated and the life expectancy was reduced to as little as 3 months.

Following 6 months of housing $atm^{-/-}$ mice in a "dirty" animal house I failed to observe any tumour development and ethics requested I terminate the study. It was later learnt that recent changes to the animal house facility had made the facility as "clean" as a SPF facility. These changes included removing other animal species so only mice remained and introducing filter top cages. These changes resulted in a pathogen report comparable to a SPF facility. With the tighter control over pathogens, the mice were no longer exposed to pathogens that may aggravate the intestinal tract initiating an inflammatory response and subsequently an

immune response. This may therefore allow the immune system to focus on suppressing tumour development, allowing the mice to live longer.

Future studies would benefit by housing $atm^{-/-}$ mice in an environment where they are exposed to a wide variety of pathogens. This would ensure the mice have the maximal exposure to pathogens that may initiate inflammation of the intestinal tract and accelerate tumour development. Unfortunately such a facility is difficult to find in today's society that minimises bacteria growth and exposure.

Chapter 9
Thesis summary and Conclusion

The GIT and its microbiota population are commonly overlooked organs of the body. With a growing prevalence of food intolerances and inflammatory conditions of the GIT (IBD & IBS), there is an increasing emphasis on the health of the GIT. As such, new products (probiotics, prebiotics, synbiotics) are being introduced to the market. One area with still limited research is the link between the GIT/microbiota and other organs and systems of the body. Receiving 70% of its blood supply from the GIT, the liver is influenced by the health of the GIT. Increased consumption of 'Western' high calorie diets, particularly high in saturated fat, is a major contributor to obesity and its comorbidities. NAFLD is one such outcome. Due to the multitude of products on the market today, and the limited data in the area, this thesis set out to explore the link between a popular commercial probiotic blend and chronic diseases derived from intestinal inflammation. The primary focus of the thesis was on lipid metabolism associated with over nutrition and NAFLD. The initial investigation involved searching the literature to identify strains of probiotics that have been studied, in what medium were they studied in (cells, animals or human) and to summarise the research findings. The findings of this investigation are discussed in great length in chapter 1.

Study 1 (chapter 3)

This study examined the effects of high-fat feeding with or without probiotics on the development of NAFLD. It was hypothesised that probiotics supplementation is capable of reducing the effects of a HFD and the development of NAFLD by altering the inflammatory profile of the GIT and liver subsequently decreasing intestinal permeability. The reduction of HFD-induced steatosis by a multi–strain probiotic supplement supports the posit that multi–strain probiotics may assist with lipid disposal from a HFD by reducing the accumulation of fat deposits in the liver. Following the consumption of a HFD, expression of tight junction proteins ZO-1 and ZO-2 was reduced. Probiotics supplementation increased the expression of the tight junction proteins, indicating a potential mechanism through which probiotics supplementation may protect against the development of NAFLD. The increased liver mass that occurred following probiotics supplementation warrants further studies. Theoretically, an enlarged liver is not a healthy response. However, some of the larger livers from the probiotics fed group showed little or no sign of NAFLD or fat deposits. Therefore, the larger

liver mass may be a healing response to the initial HFD injury. Once the body mass of mice reached approximately 45g, the mice seemed to store most of their additional mass in the liver. This response may be related to HSC activation altering the lipid metabolism within the liver. The cellular mechanisms responsible for the observed lipid clearance from the liver when probiotics were administered are uncertain. Nevertheless, responses that may help to identify cellular mechanisms were found. The findings from this study indicated that in chow fed mice, as the liver triglyceride content increased, so too did the serum triglyceride concentration. By contrast, in the HFD fed mice, as the liver triglyceride content increased, the serum triglycerides decreased. Following the signalling pathway back to find mechanisms responsible for the removal of lipids from the liver, and the subsequent appearance of triglycerides in the serum, may allow us to identify mechanisms that reduce NAFLD. By targeting these compounds/signalling molecules, it may be possible to increase lipid clearance from the liver, and restore a balanced lipid profile within the liver.

Study 2 (Chapter 4)

High extracellular iron and/or mutation in the HFE gene are common in patients with NAFLD.[136, 137] Mutation to the HFE gene dysregulates iron absorption, resulting in increased extracellular iron.[138, 139] Increased extracellular iron alters the microbiome within the GIT. Probiotics have been proposed as a potential therapeutic option that may rescue a dysbiotic GIT by readjusting the local commensal bacterial cohort and its environment. This study investigated the effects on the GIT and liver of a multi-strain probiotic blend combining strains of *Bifidobacteria*, *Lactobacilli* and a *Streptococcus* in $hfe^{-/-}$ mice fed a HFD so as to induce NAFLD. The findings from this study showed that compared to the HFD group, a multi–strain probiotic blend increased the expression of genes involved in lipid metabolism (PPAR-α, Cpt1A and AdipoR2), increased liver function (ALT and AST), and partially attenuated portal inflammation and Mallory's hyaline. Probiotics also increased the gene expression of iron transporters (TFR-1 and TFR-2) removing additional iron from the GIT and allowing the natural microbiota to be restored. The results of this study show that probiotics have potential for increasing lipid metabolism and iron uptake and reducing fat deposits within the liver. The findings of this study have implications for both clinical and non-clinical populations. Individuals who suffer from NAFLD, iron overload, GIT symptoms or lipid metabolism dysregulation may all benefit from probiotics supplementation. This

study not only offers evidence of the beneficial effects probiotics, but also provides novel insights into how iron levels influence the GIT and the metabolism of lipids.

Study 3 (Chapter 5)

Quantification of lipid peroxidation has been a long–standing measure of cellular oxidative damage. Isoprostanes are formed by the oxidative degradation of arachidonic acid and is the "gold standard unit" marker of oxidative damage. The production of ROS is not spontaneous as is widely published,[183] but rather part of a regulated process. Recently it has been proposed, and scientifically supported, that intracellular generation of ROS are important signalling molecules essential for the normal functioning of the human metabolome.[183, 185, 186] The aim of this study was to investigate the effects of lipid peroxidation in response to a high fat diet with a secondary aim to examine whether probiotics altered this response. It was hypothesised that a diet high in fat would cause increased lipid peroxidation, and that probiotics could rescue the altered lipid peroxidation by modifying the gut microbiota and re-regulating the gut-liver-axis. Mice fed a high fat diet for 20 weeks had significantly lower liver isoprostanes and malondialdehyde content compared to chow fed mice. Probiotics supplementation did not significantly either of these markers of lipid peroxidation. Reduced lipid peroxidation in HFD fed mice may be a response to prevent cirrhosis of the liver through deactivation of stellate cells. Stellate cell activation, rather than lipid peroxidation, may be the second hit in the '2-hit theory' that defines whether an individual progresses from NAFLD to NASH. If this pathway is dysregulated, stellate cells may remain activated, causing excessive fibrosis progressing to cirrhosis and NKT cells to activate.

Study 4 (Chapter 7)

Single therapy with either curcumin or vitamin E, agents which have antioxidant, iron chelation and anti-inflammatory properties has proven beneficial in liver disease.[279, 280] This study investigated the synergistic properties of these agents by using them in combination in the *Hfe*$^{-/-}$ model of NAFLD. The findings from this study demonstrated that the combination of curcumin and vitamin E therapy attenuated steatosis and steatohepatitis in wild type and *Hfe*$^{-/-}$ mice when concomitantly fed a HFD. Histological injury was more severe in the *Hfe*$^{-/-}$ mice compared to wild type mice, with all animals showing features of steatohepatitis, and most animals showing evidence of centrilobular fibrosis. Monotherapy with either vitamin E or curcumin was associated with an improvement in some components of steatohepatitis, but combination therapy markedly reduced micro- and macrovescicular steatosis and percent

hepatic steatosis as well as lobular inflammation, ballooning degeneration and fibrosis. The results of the present study lend support to the use of a combination therapy, because slight increases in HIC as seen in the *Hfe*$^{-/-}$ animals fed a HFD were associated with necroinflammation and hepatic fibrosis. Combination therapy of vitamin E and curcumin should be investigated in varying combinations and doses in further animal models of NAFLD. This would allow rapid progression into human studies if a beneficial effect is demonstrated.

Study 5 (Chapter 8)

This study investigated the potential importance of the microbiota in reducing or preventing tumour growth. Specifically, this study examined whether probiotics supplementation prolonged the life expectancy of atm$^{-/-}$ mice when housed in a non SPF 'dirty' facility by regulating their immune system and inflammation. This aim was based on observations from previous studies that noted when atm$^{-\backslash-}$ mice were housed in a 'clean' SPF facility, tumour development was delayed compared to mice housed in a non SPF facility prolonging life expectancy of the mouse. However due to unannounced changes in the non SPF animal housing conditions the mice were no longer exposed to the level of pathogens as when previous observations were made. These changes resulted in a lack of tumour development even after 6 months of housing. Despite the lack of tumour development, the fact the mice lived for 6 months in a facility where prior to the environmental changes tumours developed resulting in euthanasia by 3 month indicates the relationship between bacteria and tumour development warrants further investigation.

General Limitations

All of the studies herein are limited by the relatively small sample size, due to the availability of animal housing space, time constraints to use additional experimental animals at the same time, and the expense of conducting animal studies. The use of mice rather than humans is also not ideal, because this approach assumes that the microbiota is comparable in mice versus humans, and that humans will react to probiotics interventions the same as mice. Sample analysis was restricted due to the amount of tissue that could be obtained from the mice. The environment in which the animals were housed in is also a limitation, because a SPF facility limits the exposure of mice to bacteria. This may alter the microbiota composition of each mouse, and affect the effectiveness of each strain of probiotic. In addition, the pathogens may differ between animals house facilities, making it difficult to

compare the results of experiments performed in different animal houses.

Further Research Recommendations

Further directions for the future would be to investigate the prophylactic versus the therapeutic effects of these therapies on NAFLD. The majority of individuals consume products as part of a therapeutic treatment. However for those at risk of NAFLD or haemochromatosis, a prophylactic therapy may be ideal. Further investigation into the role that lipid peroxidation compounds play in lipid signalling/metabolism would also be warranted. The relationship between isoprostanes and HSC in lipid metabolism and cirrhosis would be of interest also. If lipid peroxidation can be maintain at suitable levels while reducing HSC activation it may allow for increased lipid metabolism/clearance while preventing cirrhosis from developing. The relationship between serum triglycerides and hepatic triglycerides concentrations also warrants further investigations. If these questions can be successfully answered in an animal model, transition into human trials and dosing trials will follow.

Thesis Summary

Probiotics were effective at maintaining tight junction proteins and subsequently maintaining the epithelial barrier integrity. This prevents pathogens from crossing from the intestinal tract in to the circulatory system where they can induce inflammation in the liver. Probiotics were also shown to prevent the development of steatosis and hepatic triglyceride accumulation by rescuing lipid peroxidation and serum triglyceride concentrations. A relationship between the concentration of hepatic triglycerides and serum triglycerides was noted which requires further investigation. In the presence of high iron probiotics were also proven to have a beneficial effect. Probiotics increased the expression of genes involved in lipid metabolism (PPAR-α, Cpt1A and AdipoR2), increased liver function (ALT and AST), and partially attenuated portal inflammation and Mallory's hyaline. A combination therapy of Vitamin E and curcumin demonstrated beneficial properties when given in conjunction with a HFD. The combination therapy markedly reduced micro- and macrovescicular steatosis and percent hepatic steatosis as well as lobular inflammation, ballooning degeneration and fibrosis above either individual therapy. In conclusion, this thesis shows neutraceutical compounds are capable of reducing the severity of a HFD and the resulting NAFLD. Overall I can conclude that while further investigation is required, probiotics and a combination therapy of Vitamin

E and curcumin is capable of at least in part attenuating the effects of a HFD and reducing the rate of development/progression of NAFLD. These results have implications for millions of individuals worldwide who suffer from NAFLD, are at risk of NAFLD, have lipid metabolism imbalances or elevated iron concentrations due to genetic or dietary reasons.

References

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