The effects of dietary eicosapentaenoic acid and arachidonic acid on gene expression changes in a mouse model of human inflammatory bowel diseases

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy
in
Nutritional Science

at Massey University
Palmerston North, New Zealand

Bianca Knoch

2010

ABSTRACT

Nutrigenomics studies the genome-wide influence of nutrients to understand the association between nutrition and human health. Studies in animal models and humans have demonstrated that dietary n-3 polyunsaturated fatty acids (PUFA) from fish oil may be beneficial in inflammatory bowel diseases (IBD).

This thesis aimed to test the hypothesis that dietary n-3 PUFA eicosapentaenoic acid (EPA) reduced and n-6 PUFA arachidonic acid (AA) increased colitis in the interleukin-10 gene-deficient (*Il10*$^{-/-}$) mouse model of IBD, and that these PUFA altered the intestinal bacteria community during colitis development using genome-wide expression and bacterial profiling.

Using a combined transcriptomic and proteomic approach, the time-course study defined the onset and progression of colitis in *Il10*$^{-/-}$ mice. Histopathology, transcript and protein changes before and after colitis onset involved in innate and adaptive immune responses suggested delayed remodelling processes in colitic *Il10*$^{-/-}$ mice and 11 weeks of age as suitable time point to study the effects of dietary PUFA on colitis development. Comparing the transcriptome and proteome profiles associated with colon inflammation of mice fed with the AIN-76A or oleic acid (OA) diet showed that OA was an appropriate control for unsaturated fatty acids in multi-omic studies. The PUFA intervention study indicated that dietary EPA-induced lipid oxidation might have a potential anti-inflammatory effect on inflamed colon tissue partially mediated through activation of peroxisome proliferator-activated receptor alpha (PPARα). Unexpectedly, dietary AA decreased the expression of inflammatory and stress colonic genes in *Il10*$^{-/-}$ mice. Altered intestinal bacteria community observed in *Il10*$^{-/-}$ mice before and after colitis onset was associated with the lack of IL10 protein led to changes in intestinal metabolic and signalling processes. Interestingly, dietary EPA and AA seemed to change intestinal bacteria profiles during colitis development. The role of PPARα in the colon was further examined in a concluding study which identified vanin1 as a likely new PPARα-target gene which may also be involved in lipid metabolism.

These findings using a state-of-the-art approach combining transcriptomics, proteomics and physiology provide a basis for future research on molecular mechanisms underlying

the effects of dietary PUFA, and might contribute to the development of fortified foods that improve intestinal health and wellness.

ACKNOWLEDGEMENTS

This study was part of Nutrigenomics New Zealand, a collaboration between AgResearch, Plant & Food Research and The University of Auckland, primarily funded by the New Zealand Foundation for Research, Science and Technology (FRST). I acknowledge AgResearch for funding my PhD Fellowship and research project within the Nutrigenomics New Zealand partnership and for providing excellent research facilities.

I thank Prof Warren McNabb for entrusting me with this interesting topic in the early days of Nutrigenomics New Zealand, for his supportive and instructive guidance and for clarifying the big picture to me. I express my sincere gratitude to Dr Nicole Roy for her kind-hearted support and tremendous help at any time and appreciation especially for her expertise and efficiency in preparing scientific papers. To my other supervisor Dr Scott Knowles I express my warm thanks for valuable discussions and advice. I am grateful for their time and their collaborative efforts mentoring me all the way through.

I am grateful to Assoc Prof Sander Kersten for giving me the opportunity to conduct my last research project in the Nutrition, Metabolism & Genomics Group at Wageningen University in the Netherlands, for his treasured training and guidance. Thanks to all members from this lab and especially to Shohreh Keshtkar for assistance with the final laboratory analyses. Special thanks also to Prof Cuzzocrea and his team at the University of Messina in Italy for providing their samples.

I thank my collaborators Drs Matthew Barnett, Katia Nones, Yvonne Dommels, Janine Cooney and William Laing from Nutrigenomics New Zealand for their excellent teamwork. I also thank Diane Barraclough for exceptional contribution to the proteomics analysis. I am also very thankful to Dr Shuotun Zhu and Kelly Armstrong for valuable assistance with histological examination, and Kate Broadley and Graham Naylor for technical support. Thanks to Zaneta Park, Alan McCulloch, Anar Khan, Paul MacLean and Dongwen Luo from the AgResearch Bioinformatics, Mathematics and Statistics Section.

The reviewers of some manuscripts and chapters; Dr Adrian Cookson, Dr Peter Janssen, Dr Mark McCann and Dr Sandra Kittelmann are gratefully acknowledged for their expert advice and Bruce Sinclair and Denise Martin for their valuable suggestions to improve publications and this thesis.

I am grateful to all my friends in New Zealand, Germany and the Netherlands for enjoyable moments and reminding me that there is life outside science, and for listening and understanding the triumphs and troubles of this PhD study.

I thank my parents, Werner and Birgit Knoch, and brother Kevin Knoch for their moral and emotional support and love throughout my life. The warmest thanks to my husband, Thomas Suesse, for his love, dedication and patience during the years.
To them I dedicate this thesis.

Palmerston North, October 2009

TABLE OF CONTENTS

LIST OF TABLES

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 6:

Chapter 7:

Chapter 8:

LIST OF FIGURES

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 6:

Chapter 7:

Chapter 8:

LIST OF ABBREVIATIONS

AA	arachidonic acid
ABC	ATP-binding cassette
ACSL	long chain acyl-CoA synthase
ALA	α-linolenic acid
ALDH	aldehyde dehydrogenase
APC	antigen presenting cells
APOA1	apolipoprotein (apo) AI
B. vulgatus	*Bacteroides vulgatus*
CARD15	caspase activation recruitment domain family member15
CD	Crohn's disease
cDNA	complementary DNA
CES	carboxylesterase
cfu	colony forming units
CLA	conjugated linoleic acid
C. perfringens	*Clostridium perfringens*
CoA	CoenzymeA
COX2	cyclooxygenase2
CYP	cytochrome P450
DGGE	denaturing gradient gel electrophoresis
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DNBS	dinitrobenzene sulfonic acid
DSS	dextran sulfate sodium
GC-clamp	GC-rich sequence
EDTA	ethylenediamine tetraacetic acid
EPA	eicosapentaenoic acid
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
ER	endoplasmic reticulum
FABP	fatty acid binding protein
FATP	fatty acid transport protein
FC	fold change
FDR	false discovery rate
FXR	farnesoid X receptor
GO	gene ontology
H. pullorum	*Helicobacter pullorum*
H&E	haematoxylin and eosin
HDL	high-density lipoprotein
HNF4	hepatocyte nuclear factor 4
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IFNγ	interferon gamma
IκB	inhibitor of κB
IL	interleukin
IL1ß	interleukin1 beta
Il10$^{-/-}$	interleukin-10 gene-deficient
IPA	Ingenuity Pathway Analaysis
JAK1	Janus kinase1
LA	linoleic acid
L. reuteri	*Lactobacillus reuteri*
limma	linear models for microarray data
LOX	lipoxygenase

lsd	least significant difference
LPS	lipopolysaccharide
LT	leukotriene
LX	lipoxin
LXR	liver X receptor
MAPK	mitogen-activated proteine kinase
MHC	major histocompatibility class
MMP	matrix metallopeptidase
mRNA	messenger RNA
MUFA	monounsaturated fatty acid
NCBI	National Centre for Biotechnology Information
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOD2	nucleotide-binding oligomerisation domain containing2
OA	oleic acid
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PPARα, β/δ, γ	peroxisome proliferator-activated receptor alpha, beta/delta or gamma
PPRE	PPAR response element
PUFA	polyunsaturated fatty acids
qRT-PCR	quantitative real-time reverse transcription PCR
Rho GDIβ	Rho GDP dissociation inhibitor (GDI) beta
RNA	ribonucleic acid
rRNA	ribosomal RNA
ROS	reactive oxygen species
RXR	retinoid X receptor
SAA	serum amyloid A
SEM	standard error of mean
SCID	severe combined immune deficiency
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signalling
spp.	species
SREBP	sterol regulatory element binding protein
STAT	signal transducer and activator of transcription
SULT	sulfotransferase
TAE, TBE	tris-acetate-EDTA, Tris-borate-EDTA buffer
TG	triglyceride
Th	T helper
TLR	toll-like receptor
TNBS	trinitrobenzene sulfonic acid
TNFα	tumour necrosis factor alpha
TSS	transcription start site
TX	thromboxane
UC	ulcerative colitis
VNN1	vanin1

Chapter 1

General Introduction

Part of the material presented in this chapter was published as a mini-review:

Knoch B, Barnett MPG, Roy NC & McNabb WC (2009) Study of the effects of dietary polyunsaturated fatty acids: Molecular mechanisms involved in intestinal inflammation. *Grasas y Aceites* **60(1)**, 8-21.

1.1 Background

Traditional nutrition research has dealt with metabolic and physiological responses of the body to nutrients and has focussed on nutrient deficiencies in health and disease. Driven by the advances in molecular biology, biochemistry and genetics, nutrition research has more recently shifted its focus to genotype-based, personalised nutrition to maintain an individual's health and prevent diet-related diseases (Kussmann *et al*., 2006). The research field of gene-nutrient interactions, called nutritional genomics or nutrigenomics, investigates molecular effects of foods and food components with the use of 'omics' technologies. These have the potential to define how nutrition influences metabolic pathways and homeostasis, how diet regulates genes in diet-related diseases and to what extent an individual's genetic background contributes to these diseases (Muller & Kersten, 2003; Bünger *et al*., 2007) or other phenotypes.

Chronic intestinal inflammation is a hallmark of inflammatory bowel disease (IBD) such as Crohn's disease (CD) and Ulcerative Colitis (UC). IBD affects over 3.5 million people worldwide (Bassaganya-Riera & Hontecillas, 2006). Northern Europe and North America have reported the highest incidence and prevalence (Torres & Rios, 2008). CD is a condition for which genetic and dietary factors are known to predispose individuals (Podolsky, 2002), is immunologically mediated, and is characterised by destruction of the intestinal mucosa (Bassaganya-Riera & Hontecillas, 2006). In New Zealand, 3075 new CD cases (representing the total number in the small and/or large intestine, and cases of unspecified or regional enteritis) have been registered from the last half of 2002 to the beginning of 2006. CD in the large intestine is more common with an increase of 16% (246 cases in 2005/06) compared to the small intestine (180 cases in 2005/06). CD identified in both small and large intestine remained constant from 2002 to 2006, with approximately 140 new cases per year (New Zealand Health Information Service, 2007).

The use of 'omics' technologies, together with a range of simplified model systems and molecular tools, is enabling a greater understanding of the basic molecular mechanisms of how foods and food components may ameliorate or prevent a phenotype, e.g. disease (Fig. 1.1; Muller & Kersten, 2003). Accumulating evidence in humans and in animal models has shown a beneficial effect of long-chain polyunsaturated fatty acids (PUFA),

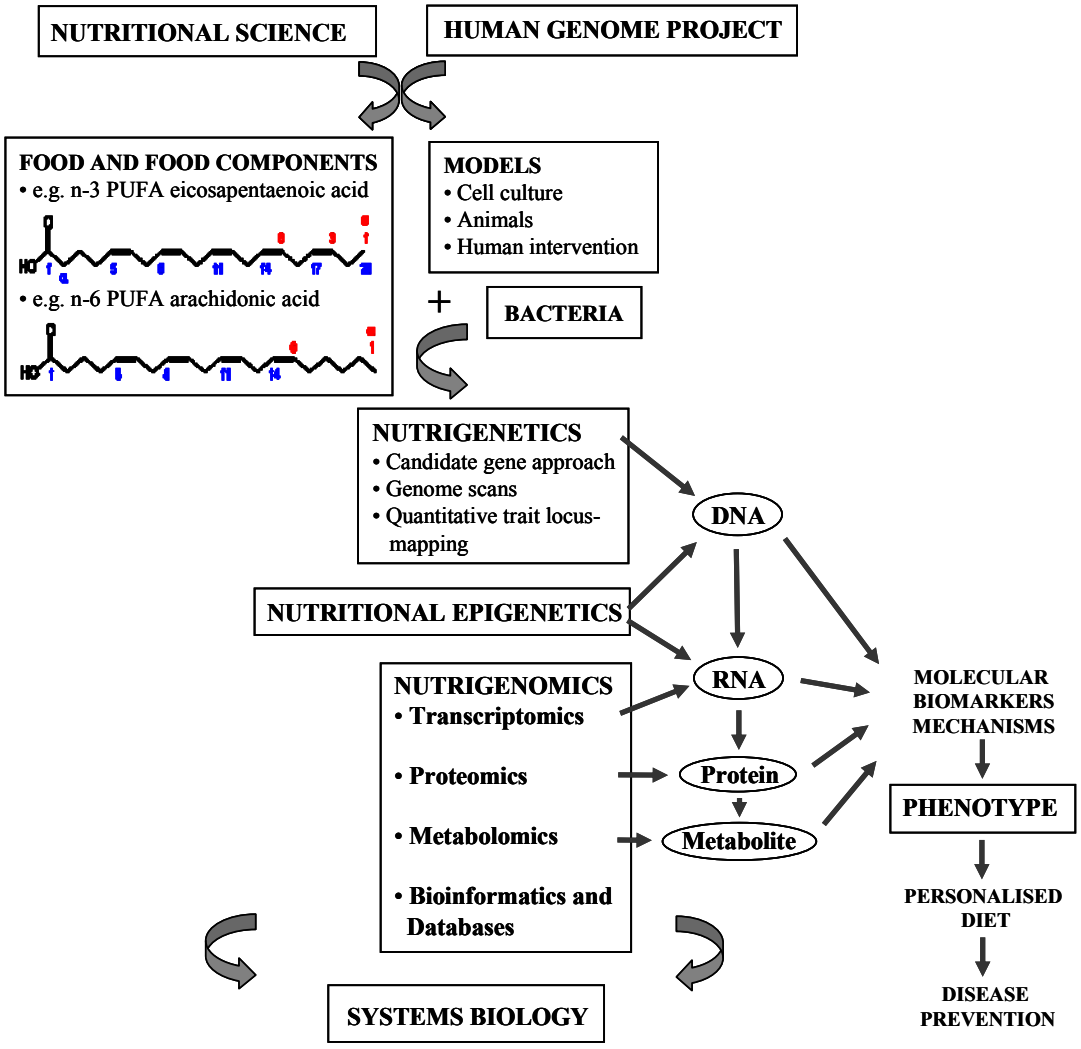


Fig. 1.1 The 'omics' technologies in nutritional science required to establish a phenotype (modified from information in Muller & Kersten, 2003; van Ommen, 2004; Mariman, 2006 and Trujillo *et al*., 2006).

particularly n-3 PUFA, for a variety of inflammatory diseases (Stulnig, 2003), and these molecules may therefore prove to be of benefit for people with either UC or CD. PUFA have been shown to modulate the expression of the genes that code for proteins involved in inflammation, lipid metabolism and energy utilisation (Deckelbaum *et al*., 2006).

1.2 Nutrigenomics

The association between diet and chronic diseases has been identified through epidemiological studies. The emphasis in nutrition research has shifted from epidemiology- and physiology-based to incorporate molecular biology and genetics (Muller & Kersten, 2003; Afman & Muller, 2006). While traditional nutrition research has dealt with nutrition as providing nutrients to nourish populations, modern nutritional research focuses on health promotion, reduction of disease risk and performance improvement through nutrition and lifestyle (Trujillo *et al*., 2006). There has been increasing interest in the molecular mechanisms underlying the beneficial or adverse effects of nutrition and nutrients. Nutrigenomics as a new research field has emerged comprising the fields of human nutrition, genomics, molecular medicine and biotechnology. It studies the genome-wide effects of nutrition at the molecular level (Muller & Kersten, 2003; Kaput & Rodriquez, 2004; Afman & Muller, 2006).

Nutrigenomics promises that specific foods can be developed to promote health and ameliorate disease in individuals with different genotypes and differing genetic susceptibilities to health and disease. There is literature evidence showing the effects of specific nutrients on gene expression changes linked to certain health outcomes such as cardiovascular disease (Sacks et al., 2002), age-related gene expression (Lee *et al*., 1999), DNA damage, oxidative stress and cell-cycle control (Rao *et al*., 2001), zinc metabolism (Blanchard *et al*., 2001) and ulcerative colitis (Ogawa *et al*., 2003).

The long-term goal for nutrigenomics is to deliver personalised nutrition to maintain an individual's health and prevent disease (Kaput, 2008). Nutrigenomics has been widely recognised to have the potential to increase our understanding of how dietary compounds affect metabolic pathways and homeostasis, how this regulation is disturbed in diet-related diseases, and how the individual's genotype contributes to

those diseases (Muller & Kersten, 2003, Kaput & Rodriquez, 2004; Afman & Muller, 2006).

1.2.1 Aspects of nutrition and nutrients

The food pyramid attempts to illustrate how much and what foods to eat to maintain health. These dietary recommendations assume that we are all the same, but we are genetically, culturally, ethnically and socio-economically different and will respond differently to food or food components. Food pyramids are unable to reflect such diversity. There is variation in quantity and type of nutrients consumed among populations and cultures, or in plants grown in different environments and also differences in cooking methods which change the chemical composition of foods and their activity (Netting, 2002; Malfatti & Felton, 2006). The inter-individual differences and preferences and the complexity of food and its preparation further complicates the quantitative assessment of nutrient intake of individuals. Nutrient assessment analyses can be coupled with metabolite profiling (metabolomics), but metabolites in response to the same nutrition could differ because of the genetic variation (Kaput, 2007).

Food is nowadays more than just nutrition, some components might have effects beyond nutrition. Foods are complex mixtures of bioactive food components or chemicals or nutrients (Kaput, 2007). At the cellular level, nutrients can be metabolised for energy production, act as ligands for transcription factor receptors, or directly or indirectly affect signalling processes (Kussmann *et al*., 2006; Kaput, 2007). Metabolism of nutrients leads to alteration of substrate and intermediate concentrations which can also serve as a control mechanism of gene regulation. Fatty acids can serve as ligands for peroxisome proliferator-activated receptor (PPAR), a nuclear receptor that regulates many genes involved in fatty acid metabolism and thus acts as a fatty acid sensor (Auwerx, 1992). Other nuclear receptors such as the sterol regulatory element binding protein (SREBP) and the carbohydrate-responsive element-binding protein (ChREBP) are indirectly regulated by nutrients. Specific nutrients affect signal transduction pathways such as green tea-derived polyphenol which inhibits tyrosine phosphorylation of epigallocatechin-3-gallate and reduces signalling through the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway (Pianetti *et al*., 2002).

Dietary lipid is an important macronutrient and required for organism development. The bioactive components in lipids are fatty acids and/or fatty acid-derived mediator molecules such as ceramides, cholesterol, diacylglycerols, eicosanoids and coenzymeA (CoA) thioesters. Major (glucose, fatty acids, amino acids) and minor (e.g. iron, vitamins) nutrients regulate gene expression in a hormone-independent manner (Mine *et al*., 2009). The molecular mechanism by which fatty acids regulate gene expression involved in their metabolism is described in 1.4.2. An imbalance in levels and composition of dietary lipids has been associated with the development of various chronic diseases such as coronary artery disease, obesity, type 2 diabetes and some forms of cancer (Mine *et al*., 2009). Also an imbalanced intake of the other two macronutrients (carbohydrates and proteins) contributes to the initiation, development and progression of chronic diseases (Kaput & Rodriquez, 2004).

1.2.2 Nutrigenomics approach

The interaction of genes and nutrition can be examined at the genome, transcriptome, proteome and metabolome levels. Genomics refers to the entire genome of an organism, transcriptomics analyses the mRNA expression levels in a cell population or an organ, proteomics is the large scale analysis of proteins and their interactions with other molecules and metabolomics profiles all metabolites in a biological organism (Muller & Kersten, 2003). Integrating genomics (gene analysis), transcriptomics (gene expression analysis), proteomics (protein expression analysis) and metabolomics (metabolite profiling) to define a 'healthy' or a 'disease' phenotype is a major challenge for nutrigenomics (Kussmann *et al*., 2006).

The nutrigenomics approach for tailoring foods or nutrition to match people's genes has to consider the nutrition and its nutrients and the genetic make-up of individuals. Nutrigenomics requires multidisciplinary research teams with expertise in the different areas of nutrigenomics research and large infrastructure support to efficiently deal with the huge amount of data. This has led to the emergence of several nutrigenomcis research collaborations in New Zealand (Nutrigenomics New Zealand, NuNZ), The Netherlands (European Nutrigenomics Organisation, NuGO), the USA, Canada and Brazil. The NuGO research collaboration is the largest with over 20 universities, research institutes and companies all over Europe and focuses mainly on nutrition and

metabolic syndrome. The NuNZ research collaboration is focusing on nutrition and intestinal function. CD has been chosen as an initial proof-of-concept for Nutrigenomics New Zealand (CD has been shown to respond to dietary intervention) for establishing the nutrigenomics approach to dietary disease management. Polymorphisms in the NOD2/CARD15 gene have been associated with CD susceptibility (Ogura *et al*., 2001; Inohara *et al*., 2002) and since then, several other genes linked to CD susceptibility have been reported (Wellcome Trust Case Control Consortium, 2007).

Genetic alterations that can occur to DNA include changes in a single base of the DNA sequence, called single nucleotide polymorphisms (SNP), deletions, gene copy number variants or random repeats some of which can potentially result in a functional change for gene products leading to variation in phenotype and disease susceptibility (Ferguson, 2006). The identification of diet-gene interactions in a population with a CD phenotype is important in order to determine the food components that may prevent, reduce or alleviate the disease symptoms. Dietary intervention is often used by CD patients, as certain foods may help reduce symptoms, where common fruit and vegetables, milk and meat fractions, herbs and spices and plant medicinal products are evaluated. Such food components cover lipids, proteins/peptides/amino acids, carotenoids, phytochemicals and probiotics. In a pilot study it was found that not a very wide range of food or food components could be identified by CD subjects as possibly causing some effects or perceived as beneficial. A study was conducted within the New Zealand CD cohort (446 individuals with the disease) using a food tolerance questionnaire. Subjects who already had the disease provided information about dietary tolerances which were useful when comparing foods in terms of symptom generation in diagnosed patients (Triggs *et al*., 2010). This method enabled more foods and food components to be associated with CD and also showed the variability among subjects in their perception to certain dietary foods and food components.

Results from comparing beneficial and adverse effects of individual foods with special emphasis on fish showed that fresh white fish reduced symptoms in around 25% of subjects while causing few adverse effects, while fried fish appears to lead to symptom enhancement (Triggs *et al*., 2010). Shellfish including mussels, oysters, scallops and paua may enhance symptoms. They also reported that tinned or fresh tuna and salmon

were beneficial to about 15% of subjects, with only a few subjects reporting symptom enhancement. Oily fish such as canned sardines have been reported to be tolerated by around 13% and 25% reported that they enhanced symptoms.

Information from genetic screening combined with information from food components or extracts used in dietary intervention and also literature evidence of a beneficial effect for a particular phenotype, e.g. CD patients, provide potential targets to test in the *in vitro* cell-based high throughput screens. Cell culture systems where human cells are exposed to purified phytochemicals or micronutrients enable the identification of molecular pathways that are affected by the food components and allow their analyses in animal and human studies. Cell-based screening assays have been developed to test food extracts or components for their potential to overcome functional effects of some common SNPs associated with increased risk of CD (Philpott *et al*., 2007). These cell-based screens are classified into inflammatory and genetic screens. Inflammatory screens (such as the tumour necrosis factor alpha (TNFα) screen) test whether a food component or extract has anti- or pro-inflammatory activity. The genetic screen (such as the NOD2 genotype-specific screen) utilises the genetic basis of the variation that has been implicated in CD development, e.g. the association with NOD2/CARD15 gene. It tests whether a food component or extract has a positive effect on an altered gene product.

Cell culture systems show limitations in their complexity compared to *in vivo* systems as they do not have organs or microflora nor that complex metabolic repertoire that affects the metabolism and regulation of nutrients or food components. Animal models are essential in biomedical research. Several mouse models which develop CD-like symptoms are commercially available.

Gene-diet interactions can be studied more in depth using a multi-omics approach (e.g. transcriptomics, proteomics) in animal models by investigating the influence of a diet enriched with a purified dietary component or a single food on the expression of certain genes and proteins in biochemical pathways (Spielbauer & Stahl, 2005). Through such studies, valuable information on the pathways involved in diet-related diseases and on the potential therapeutic mechanisms of the food or dietary component under investigation can be obtained (Philpott *et al*., 2007).

1.2.3 Gene-diet interactions and disease associations

The same food or food component that is beneficial in one individual with CD, may aggravate the condition in another (Riordan *et al*., 1993). Based on dietary questionnaire data, a novel diet-gene interaction in CD has been suggested for mushroom intolerance (Petermann *et al*., 2009). From the 499 New Zealand Caucasian subjects with CD and 370 controls, 39% reported adverse effects and only 2% beneficial effects. Mushrooms contain higher levels of L-ergothionine which is a substrate for the Na-dependent organic cation transporter (OCTN1). It was shown that individuals carrying a functional L503F SNP in the *OCTN1* gene increases the CD risk in some (Peltekova *et al*., 2004), but not all populations (Silverberg, 2006; Ferguson *et al*., 2008). Although case-control data on the New Zealand Caucasians showed no significant differences in CD risk between individuals with the L503F OCTN1 C-allele and those with the variant T-allele, mushroom intolerance was significantly associated with the variant OCTN1 genotype (Petermann *et al*., 2009). Individuals, who reported adverse effects from mushrooms were among those with a higher frequency of the variant T-allele, thus may have an enhanced risk of adverse effects when consuming mushrooms (Petermann *et al*., 2009). Mushroom intolerance was the first gene-diet interaction described for CD.

Coffee is another example for a gene-diet interaction associated with a disease. Coffee intake was associated with an increased risk of myocardial infarction among those with impaired caffeine metabolism (El-Sohemy *et al*., 2007). Caffeine is the major bioactive metabolite in coffee and is metabolised by the polymorphic CYP1A2 enzyme. An A to C substitution at position 734 (*CYP1A2*1F*) in the CYP1A2 gene leads to decreased enzyme inducibility. Individuals who are homozygous for the CYP1A2*1A allele are 'rapid' caffeine metabolisers and carriers of the variant CYP1A2*1F show 'slow' caffeine metabolism. A food frequency questionnaire was used and subjects questioned for their coffee intake. There were protective effects among rapid metabolisers. A fast elimination of caffeine suggested that caffeine might have unmasked the protective effects of other chemicals in coffee such as caffeic acid and chlorogenic acid which have antioxidant properties that might protect against heart disease.

It is important to identify such polymorphic forms of genes and their specific function in order to explain individual's susceptibility to certain dietary factors. "Individual" variation as a consequence of genetic polymorphisms like SNPs can help explain some of the contradiction and limitations of epidemiological studies. The apolipoprotein (apo) AI G-A polymorphism (located 75 base pairs upstream of the transcription start site in the *APOA1* gene promoter) is an example. This G-to-A substitution in the *APOA1* gene has been associated with increased high-density lipoprotein (HDL)-cholesterol concentration in some studies but not in others. The APOA1 protein, which plays a role in lipid metabolism, is linked to coronary heart disease risk (Rader *et al*., 1994), and apo AI and HDL-cholesterol concentrations were higher in individuals carrying the A-allele (G/A or A/A genotype) than in those with the G/G genotype (Jeenah *et al*., 1990). Studies examining this association either partially agreed with this initial finding (Saha *et al*., 1994), found no significant association (Akita *et al*., 1995) or the opposite association (Matsunaga *et al*., 1995). It was suggested that these inconsistencies might be due to interactions with environmental factors (e.g. diet, tobacco smoking) and sample size. Ordovas *et al*. (2002) found no significant difference between G/G subjects and A-allele carriers for any lipid variables, but demonstrated a significant interaction between dietary PUFA intake and the G-A polymorphism on plasma HDL-cholesterol and apo AI concentrations. Generally, their regression model associated the A-allele carriers with lower HDL-cholesterol and apo AI concentrations. In subjects carrying the A-allele though, increased PUFA intake reversed this genetic effect, especially in women, and was associated with higher HDL-cholesterol and apo AI concentrations, whereas the opposite was the case in G/G women. In men, the PUFA intake effect showed only significance when interactions with alcohol consumption and tobacco smoking were considered in the regression model. This example illustrates the complexity of polymorphism-phenotype associations and underlines the importance of considering gene-environment interactions in epidemiological studies.

1.3 Intestinal inflammation in inflammatory bowel disease

CD and UC are the two main forms of IBD and are defined as inflammation of the gastrointestinal tract. CD is characterised by discontinuous (patchy), transmural inflammation with large ulcerations and occasional granuloma and may affect the whole

gastrointestinal tract, from oral cavity to rectum. It most often involves the terminal ileum. Symptoms of CD include abdominal pain and diarrhoea and sometimes also rectal bleeding, weight loss and fever. UC is characterised by a diffuse mucosal inflammation limited only to the large bowel. It evolves from the distal rectum and extends proximally to the colon. The most specific clinical symptom is bloody diarrhoea (Gassull, 2001; Sands, 2002; Loftus, 2004; Macfarlane *et al*., 2009).

Advances have been made in key areas associated with the aetiology of IBD and the underlying inflammatory and mucosal immune responses in CD and UC. There is a better understanding of the complex interactions among genetic, immunological, and environmental (e.g. diet, bacteria) components that are important in an individual's predisposition to and expression of the disease (Macfarlane *et al*., 2009), but most aspects of the exact mechanism of IBD pathogenesis are still unclear.

1.3.1 Genetics of inflammatory bowel disease

The genetic approach towards understanding IBD pathophysiology are family-based genetic linkage and population (case-control)-based association studies. Linkage studies look at chromosomal segments that are shared between family members with the disease, whereas association studies test whether a specific marker allele of a gene is found with higher frequency in individuals with disease compared to the frequency of the marker in healthy individuals (Taylor *et al*., 2003). Linkage studies are particularly challenging with multigene diseases such as IBD because the magnitude of the linkage is often modest, are not universally replicated between studies and the linkage signals occur over wide genomic regions containing hundreds of genes. Genome-wide association studies involve genotyping of a large number of SNPs that test genetic variation throughout the human genome (Cho, 2008). The first susceptibility gene in IBD identified was the nucleotide-binding oligomerisation domain containing 2 or caspase activation recruitment domain family member 15 (*NOD2/CARD15*) at the IBD1 locus on chromosome 16 (Ogura *et al*., 2001). Other established disease associations for IBD that were indicated through genetic linkage studies include the association of CD and UC with the chromosome 5q31cytokine cluster at the IBD5 locus and the major histocompatibility class (*MHC*) and *TNFα* gene studies for the IBD3 locus at chromosome 6p (Cho, 2006). Comparative analyses of gene associations between CD

and UC have successfully identified a number of susceptibility loci common and unique to mechanisms of their immunopathogenesis (Cho, 2008). CD shows strong associations with autophagy related 16-like protein1 (*ATG16L1*), *NOD2* and immunity-related GTPase family M (*IRGM*) which are involved in innate immune response. Polymorphisms of genes of the IL23 pathway including interleukin23 receptor (*IL23R*), p40 subunit of IL12 and IL23 (*IL12B*), signal transducer and activator of transcription3 (*STAT3*) and macrophage stimulating1 (*MST1*) involved in adaptive immune response, have been related to CD and UC (Cho, 2008; Lee & Buchman, 2009).

Once initial disease associations have been established, the approach is to identify additional disease genes by examining close homologs and other members of affected signalling pathways (Cho, 2006). One goal of IBD genetics is to identify multigene associations which increase the disease risk and to reproduce those in murine models for studying the pathogenesis and new therapies (Elson & Weaver, 2003; Cho, 2006).

1.3.1.1 Murine models of intestinal inflammation

Experimental mouse models of intestinal inflammation have proven useful in defining the pathogenesis and candidate genes for human IBD. No animal model can precisely reproduce human IBD which itself has not yet been exactly defined (Elson & Weaver, 2003). Experimental models offer the advantage that they can be used to study the early inductive phases of IBD and the complex interaction amongst genes in a defined genetic background and in controlled environmental conditions (Elson & Weaver, 2003; de Buhr *et al*., 2006).

Numerous mouse models of IBD have been described that show clinical and histopathological analogies for CD or UC. These include gene knockout, defect or transgenic models of spontaneous colitis (Matsumoto *et al*., 1998), models of chemically-induced colitis by chemicals such as dextran sulfate sodium (DSS; Okayasu *et al*., 1990), trinitrobenzene sulfonic acid (TNBS; Elson *et al*., 1996) or oxazolone (Boirivant *et al*., 1998), models of pathogen-induced intestinal lesions by enteroinvasive *E. coli* or *Salmonella typhimurium* (Gill *et al*., 2001), and adoptive transfer models by $CD4^{+}$/$CD45RB^{high}$ cell transfer to $Prkdc^{scid}$ mice (SCID, severe combined immune

deficiency; Powrie *et al*., 1993) as well as by bone marrow cell transfer to CD3ε transgenic mice (Hollander *et al*., 1995).

Genetically engineered mouse models of intestinal inflammation with either insertion (transgenic) or selective deletion (knockout) of a gene are listed in Table 1.1. Animal models also demonstrate that IBD development is influenced by complex genetic interactions. Mouse models for which the genetic background influences the intestinal inflammation include TCRα-deficient mice (Mombaerts *et al*., 1993), IL2-deficient mice (Sadlack *et al*., 1995), *Il10*$^{-/-}$ mice (Berg *et al*., 1996; Bristol *et al*., 2000) and $G\alpha_{i2}$-deficient mice (Hörnquist *et al*., 1997). These background strain differences also apply to DSS- (Mähler *et al*., 1998) and TNBS- (Elson *et al*., 1996) induced colitis. One of these genetically manipulated mouse models is the *Il10*$^{-/-}$ mouse (Kühn *et al*., 1993) which is commonly used to study intestinal inflammation, in particular IBD.

Despite the IBD-like symptoms that develop in *Il10*$^{-/-}$ mice, IL10 gene variants have not consistently been found in IBD patients, whereas variants of the IL23 receptor have been, e.g., in the New Zealand population (Roberts *et al*., 2007). IL23 has been shown to regulate T cell-mediated colitis in the *Il10*$^{-/-}$ mice (Yen *et al*., 2006). The most common human IBD susceptibility gene is NOD2 (Ferguson *et al*., 2007, Torres & Ríos, 2008), and one common variant in this gene is a C insertion at position 3020, which leads to a truncated gene product (NOD2Δ33). The NOD2Δ33 mouse develops colon inflammation and ulceration in response to bacterial invasion (Maeda *et al*., 2005), because of a failure to differentiate between existing intestinal flora and invading bacteria.

1.3.1.1.1 The interleukin-10 gene-deficient mouse model

The *Il10*$^{-/-}$ mouse (formal designation *Il10*^{tm1Cgn}) was generated at the Institute for Genetics at the University of Cologne in 1993 by targeted mutation (Kühn *et al*., 1993). Part of the first exon of the *Il10* gene (codons 5-55) was replaced by a termination codon and a *neo* gene as well as by a termination codon in exon 3 of the gene by homologous recombination. Mice with this null mutation develop a spontaneous form of IBD with some characteristics of CD. The chronic enterocolitis involves the entire intestinal tract, but is most severe in the proximal colon. The mucosal inflammation is associated with segmental and also transmural lesions of the intestinal epithelium.

Table 1.1 Genetically manipulated mouse models of intestinal inflammation

Mouse model	Area involved	Reference
A20-deficient mouse	Multifocal	(Beg *et al*., 1995)
alpha1,2-fucosyltransferase transgenic mouse	Colon, caecum	(Miller *et al*., 2000)
G-protein subunit α_{i2} ($G\alpha_{i2}$)-deficient mouse	Entire colon	(Rudolph *et al*., 1995)
Interleukin-10 (*Il10*)-deficient mouse	Colon, caecum	(Kühn *et al*., 1993)
IL10 receptor beta (*Il10rb*)-deficient mouse	Colon, caecum	(Spencer *et al*., 1998)
IL2 (*Il2*)-deficient mouse	Colon, caecum	(Schorle *et al*., 1991)
IL2 receptor alpha and beta (*Il2ra, b*) chain-deficient mouse	Small and large bowel	(Poussier *et al*., 2000)
IL7 transgenic mouse	Colon	(Watanabe *et al*., 1998)
Keratin-8 (*K8*)-deficient mouse (FVB/N background)	Colon, caecum	(Baribault *et al*., 1994)
Macrophage- and neutrophilic granulocyte-specific *Stat3*-deficient mouse	Colon, caecum	(Takeda *et al*., 1999)
Multiple drug resistence 1 alpha (*mdr1*a)-deficient mouse	Colon	(Panwala *et al*., 1998)
N-cadherin ($^{\Delta NCAD}$) transgenic mouse	Small bowel, caecum	(Hermiston & Gordon, 1995)
Nitric oxide synthase 2 (*Nos2*)-deficient mouse	Multifocal	(Laubach *et al*., 1995)
Nuclear factor of kappa light chain gene enhancer in B-cells 1 (*Nfkb1*)-deficient mouse	Multifocal	(Sha *et al*., 1995)
Nucleotide-binding oligomerisation domain protein 2 (*Nod2*)-deficient mouse	Colon	(Maeda *et al*., 2005)
Signal transducer and activation of transcription 4 (*Stat4*) transgenic mouse	Ileum, colon	(Wirtz *et al*., 1999)
Sma and Mad-related protein 3 (*Smad3*)-deficient mouse	Multifocal	(Yang *et al*., 1999)
T cell receptor alpha (*Tcra*) chain-deficient mouse, also *Tcrb* and *Tcrd*	Colon, caecum	(Mombaerts *et al*., 1993)
Transforming growth factor beta (*Tgfb*) dominant negative mutant type II (dnRII) transgenic mouse	Multifocal	(Hahm *et al*., 2001)
Tumor necrosis factor AU-rich elements ($Tnf^{\Delta ARE}$)-deficient mouse	Terminal ileum > proximal colon	(Kontoyiannis *et al*., 1999)
Wiskott-Aldrich syndrome protein (*Wasp*)-deficient mouse	Colon	(Snapper *et al*., 1998)

Histological characteristics include inflammatory cell infiltration into lamina propria and submucosa, erosions, ulcerations and hyperplasia of the mucosa, abnormal crypt and villus structures as well as crypt abscesses (Kühn *et al*., 1993; Löhler *et al*., 1995; Berg *et al*., 1996). The disease in *Il10*$^{-/-}$ mice is progressive and non-remittent (Sellon *et al*., 1998). Mice surviving six months or more can develop colorectal cancer (Berg *et al*., 1996). Besides the intestinal disruptions, the mice develop a microcytic and hypochromic anaemia which has been linked with the intestinal inflammation, on the one hand by blood loss through the intestine and on the other hand by disruption of the absorption of iron and vitamin B-complexes (Löhler *et al*., 1995; Davidson *et al*., 2000). Except for the intestinal tract, no other organs are affected by inflammation in the *Il10*$^{-/-}$ mouse, therefore this model is well suited for the study of IBD.

The *Il10*$^{-/-}$ mouse is a particularly well-characterised colitis model that develops intestinal inflammation when exposed to non-pathogenic (commensal) bacteria, unlike its wildtype counterpart, suggesting the importance of IL10 in preventing colitis (Kim *et al*., 2007). *Il10*$^{-/-}$ mice that are maintained in germ-free conditions do not show intestinal inflammation and immune activation (Sellon *et al*., 1998). This underlines the importance of resident enteric bacteria in the development of the spontaneous colitis in this model.

The effector cell mediating colitis in *Il10*$^{-/-}$ mice is the CD4^{+} T cell of the T helper (Th) cell type 1 subset in that interferon gamma (IFNγ) is the main pro-inflammatory cytokine (Elson *et al*., 2005). Recent data indicated that production of the pro-inflammatory cytokine IL17 in germ-free *Il10*$^{-/-}$ mice co-colonised with *Enterococcus faecalis* (*E. faecalis*) and *Escherichia coli* (*E. coli*, commensal bacteria) accompanied the subsequent inflammation that developed (Kim *et al*., 2005; Kim *et al*., 2007). This showed the involvement of both Th1 and Th17 immune responses in the bacteria-driven colonic inflammation in the *Il10*$^{-/-}$ mouse model. IL17 is produced by CD4^{+} and CD4^{+}CD8^{-} T cells of the Th17 subset and involved in the protective immunity against extracellular and intracellular pathogens (Matsuzaki & Umemura, 2007).

The impact of the IL10 mutation on colitis development varies markedly as a function of the inbred strain background. The severity and accordingly, the time-course of the colitis are influenced by the genetic background strain when maintained under the same

conditions. The colitis in *Il10*$^{-/-}$ mice on the C57BL/6J background is mild (Berg *et al*., 1996), whereas colitis on the BALB/c (Takeda *et al*., 1999), NOD/Lt and NOD.NON-*H2*^{nbl} (Mähler & Leiter, 2002) background is intermediate and progressive. In contrast, 129/SvEv (Takeda *et al*., 1999), C3H/HeJBir (Bristol *et al*., 2000) and C3H.SW (Mähler & Leiter, 2002) background strains develop severe and progressive colitis to IL10 deficiency, starting early in the C3H strains. This suggests that other genes of the particular background strain modify the development of colitis.

1.3.1.1.2 Interleukin-10 signalling

IL10 is a pleiotropic cytokine and produced by lymphoid (T and B cells, natural killer cells) and myeloid cells (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells) (Lee & Chau, 2002; Murray, 2006). IL10 derived from T cells of either the Th1 or Th2 subset are important in the regulation of inflammation in Th1- or Th2-driven immune responses (Murray, 2006). IL10 has been shown to signal through Janus kinase1 (JAK1)/STAT3 and p38 mitogen-activated protein kinase (MAPK)-dependent pathways to induce suppressor of cytokine signalling (SOCS)-mediated (Alexander & Hilton, 2004) or heme oxygenase-1-dependent (Lee & Chau, 2002) anti-inflammatory mechanisms (Fig.1.2). IL10 binds to a specific IL10 receptor (IL10R) on its target cells such as macrophages and neutrophils. After ligand binding, the IL10 receptor activates JAK1, which leads to recruitment of STAT3 and activation of STAT3-responsive genes (e.g. SOCS3) that inhibit pro-inflammatory cytokines. STAT3 is a key mediator in the IL10-IL10R signalling in macrophages and neutrophils and the only STAT protein required for the anti-inflammatory effects of IL10 (Murray, 2006). IL10 can inhibit the expression of genes induced by LPS-stimulated toll-like receptor (TLR) signalling. These IL10-regulated genes code for cytokines, chemokines and cell-surface receptors (Lang *et al*., 2002).

A novel anti-inflammatory mechanism has been identified for IL10 in blocking endoplasmic reticulum (ER) stress in intestinal epithelial cells (Fig.1.2) (Shkoda *et al*., 2007). IL10-mediated p38 MAPK signalling inhibited the recruitment of activating transcription factor6 to the glucose-regulated protein78 (GRP78) promoter and its function on TNF-induced NFκB signalling. It has been proposed that host-derived immune signals such as IL10 signalling cascades are critical in maintaining epithelial

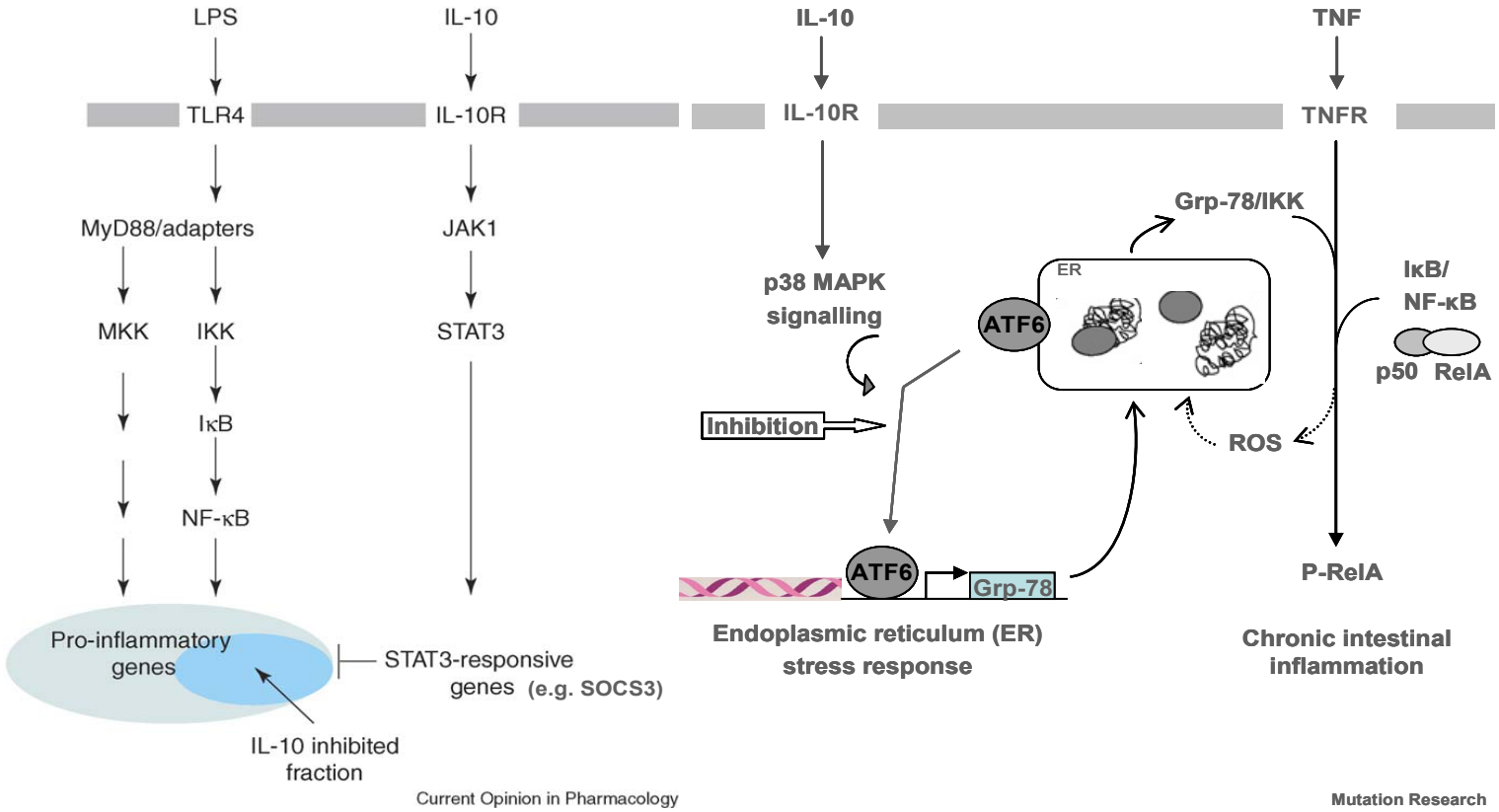


Fig. 1.2 Overview of IL10 signalling. IL10 signals though JAK1/STAT3- and p38 MAPK-dependent pathways to induce anti-inflammatory mechanisms (e.g. mediated through SOCS3). The IL10-mediated p38 MAPK cascade can block endoplasmic reticulum (ER) stress protein (Grp-78) response by inhibition of ATF-6 transcription factor recruitment. Abbreviations are discussed in the text. The left-hand pathway has been adopted from Murray, 2006 and the right-hand pathways from Werner & Haller, 2007.

cell homeostasis and therefore in controlling the development of chronic intestinal inflammation (Werner & Haller, 2007).

1.3.2 Immunology in inflammatory bowel disease

The immune system is heavily involved in the inflammatory processes that cause IBD. In a healthy intestine, the immune system is self-tolerant and can recognise and eliminate infectious agents (including bacteria, viruses, fungi) by tightly controlled overlapping mechanisms (Nagler-Anderson, 2006). It is the interaction of commensal bacterial antigens with host genetic factors that determines the individual's immune response and mucosal barrier function (Sartor, 1997).

1.3.2.1 Mucosal surface and defence system

The structure of the small and large intestine consists of four layers: mucosa, submucosa, muscularis (muscle layers) and serosa (Fig. 1.3). The mucosa represents the inner lining of the intestinal tract and is made up of a single epithelial cell layer attached to a basement membrane which overlays the lamina propria. The lamina propria consists of subepithelial connective tissue and lymph nodes. The muscularis mucosae, which is a thin muscle layer, follows underneath, and below this is the submucosa. There are four major intestinal cell types: columnar absorptive cells, mucus secreting goblet cells, endocrine cells and Paneth cells (at the base of the crypt) (Pearson & Brownlee, 2005). There are lymphoid nodules present in the mucosa and the larger constructs of lymphoid tissue are known as Peyer's Patches (Burgess, 1998).

An intact intestinal mucosa, with normal intestinal permeability and barrier integrity, has an essential role in digestion, absorption and secretion of fluids, transport of oxygen, electrolytes and nutrients. It represents a secretory physical barrier that is critical in the elimination of potential harmful antigens and bacteria by producing secretory immunoglobulin A and mucus. The secretory immunoglobulin A can inhibit the adherence of antigens to the mucosal surface and the mucus can prevent bacterial infestation (Pessi *et al*., 1998). Intestinal epithelial cells lining the intestinal mucosa form an initial barrier and are regarded as the outer defence system protecting systemic organs and tissues against bacteria including commensals, and endotoxins (Kagnoff & Eckmann, 1997; Foitzik *et al*., 1999; Fukushima *et al*., 1999).

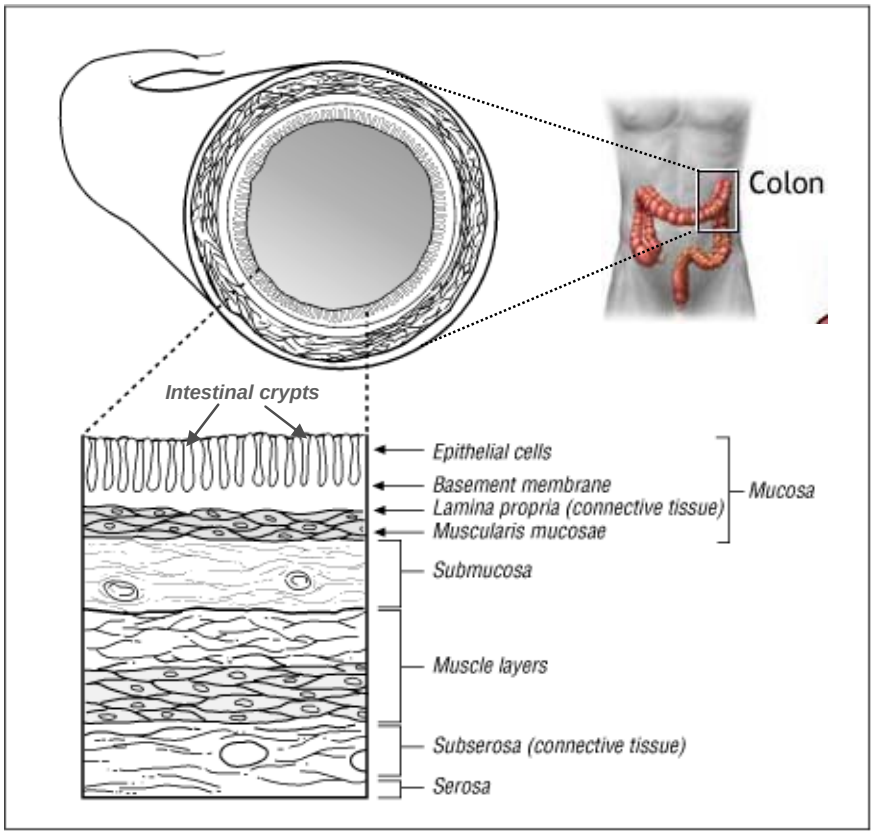


Fig. 1.3 Structure of the large intestine (colon) with enlarged inset to show details of its layers mucosa, submucosa, muscularis (muscle layers) and serosa.

1.3.2.2 Mucosal immunity

The gastrointestinal tract is an important part of the mucosal immune system (Coico *et al*., 2003). The mucosal immune system which is harboured by the lamina propria, can be divided into components that involve the epithelial barrier, innate and adaptive immune responses. The innate and adaptive immune system consists of a complex network of cells and factors responsible for detection and elimination of pathogens (Kufer *et al*., 2006). IBD patients have shown activated innate (macrophages and neutrophils) and adaptive (T and B cells) immune responses and a loss of tolerance to commensal bacteria (Duchmann *et al.*, 1995; Sartor, 2006).

1.3.2.2.1 Epithelial barrier

Intestinal immunity involves an interaction of local immune cells with non-immune cells, such as epithelial cells which together with other cells form the epithelium (Janeway *et al*., 2001). Epithelial cells of the small and large intestine express MHC class II proteins, thus can function as antigen presenting cells (APC), and $CD4^+$ Th cells react to the antigens displayed on their surfaces (Janeway *et al*., 2001). Epithelial cells are important in the prevention of TLR activation by commensal bacteria. The epithelial barrier consists of the cells themselves and tight junctions in the paracellular space joining epithelial cells. Tight junctions are multiprotein structures that control permeation of fluids, nutrients, and selected bacteria (Madara, 2000; Cobrin & Abreu, 2005).

The intestinal epithelium has to adapt to constant changes in its environment by processing bacteria- and host-derived immune signals of the luminal content in order to maintain epithelial cell homeostasis (Werner & Haller, 2007). A defect in barrier function increases the paracellular- (bacteria penetration through opening tight junctions) or transcellular (bacteria uptake by endocytosis) migration of bacteria. The mucosal immune system is then exposed to potentially harmful luminal contents which may activate the production of pro-inflammatory cytokines (Cobrin & Abreu, 2005). A defect in epithelial barrier function with increased intestinal permeability has been found in CD patients (Katz *et al*., 1989; Irvine & Marshall, 2000) and also in *Il10*$^{-/-}$ mice (Madsen *et al*., 1999) that precedes the development of mucosal inflammation. Thus, a primary barrier defect and loss of epithelial cell homeostasis plays a critical role in mucosal inflammation (Madsen *et al*., 1999; Werner & Haller, 2007).

1.3.2.2.2 Innate immune response

The innate immune response represents the first line (early phase) of defence against pathogenic bacteria and antigens (Kufer *et al*., 2006). The innate immune response relies on the recognition of highly conserved structures of bacterial pathogens by pattern recognition receptors such as TLR and CARD receptors (Fig.1.4). These conserved structures include extracellular and intracellular bacterial patterns (e.g. bacterial LPS and peptidoglycan from the cell walls) of pathogenic and commensal bacteria (Cobrin & Abreu, 2005; Werner & Haller, 2007). These receptors are transmembrane proteins expressed on the surface of various effector cells including macrophages, dendritic cells and B cells within the mucosa (Cobrin & Abreu, 2005). In response to their ligands, they initiate various signal transduction cascades that activate MAPK and inhibitor of κB (IκB)/NFκB (Werner & Haller, 2007). These transcription factors regulate the expression of pro- (*IL1B*, *TNF*, *IL6*, *IL8* and adhesion molecules) and anti-inflammatory (TNF-induced protein 3, *CARD15*, cyclooxygenase 2 (COX2), β-defensins, peroxisome proliferator-activated receptor gamma (*PPARγ*) and *IκBα*) genes (Sartor, 2006). TLR-associated recognition of bacterial patterns, can lead to antibacterial peptide expression, epithelial barrier fortification and epithelial cell proliferation in aid of barrier integrity (Cobrin & Abreu, 2005). In a healthy intestine, epithelial cells express low levels of TLRs, which allows the cells to reside in the high bacterial concentration of the distal ileum and colon (Sartor, 2006). TLR expression is markedly up-regulated in IBD (Eckburg & Relman, (2007). Each type of TLR binds specific bacterial components (e.g. TLR4 binds LPS, TLR2 binds peptidoglycan, TLR5 binds monomeric flagellin, and TLR9 binds to oligodeoxynucleotides), but all these signals lead into activation of inflammatory pathways (Sartor, 2006).

The other set of recognition receptors, CARD4 (formerly NOD1) and CARD15 (formerly NOD2), are cytoplasmic sensors of bacterial components and bind to diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively, to induce NFκB activation and the production of pro-inflammatory mediators. CARD proteins regulate host innate immune response to pathogens through a protective antibacterial function and prevent pathogenic invasion (Cobrin & Abreu, 2005; Sartor, 2006). There have been more than 20 CARD proteins identified, but CARD4 and CARD15 are the focus of current CD research. CARD4 protein is expressed by intestinal epithelial cells and CARD15 protein in monocytes, macrophages, T and B cells, dendritic and Paneth

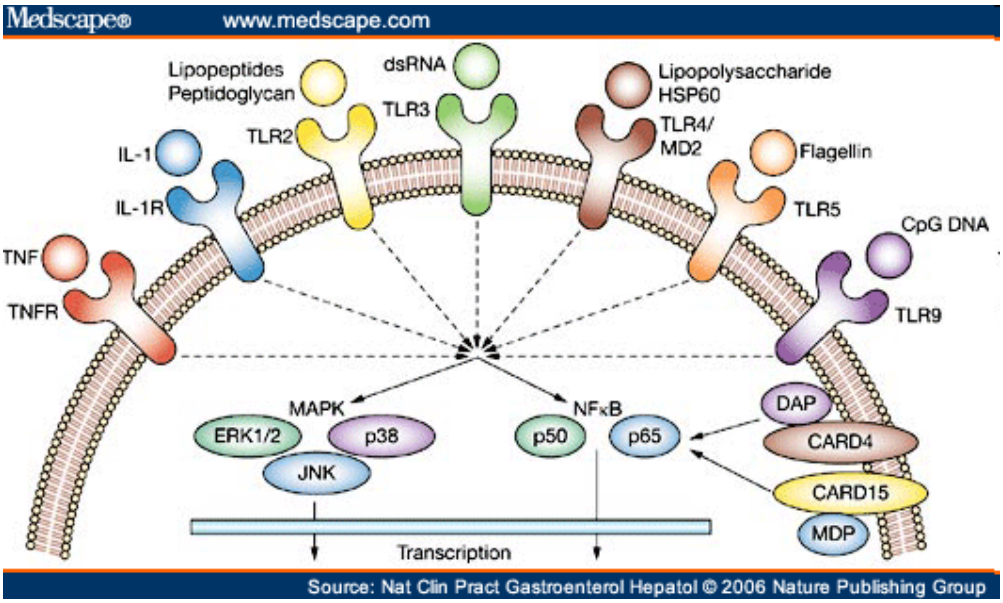


Fig. 1.4 Extracellular and intracellular pattern recognition receptors (TLR and CARD) of the innate immune response. TLR on the membrane can bind to bacterial lipopolysaccharide and peptidoglycan to induce signalling pathways that activate NFκB and MAPK pathways. These transcription factors stimulate the expression of various pro-inflammatory and anti-inflammatory genes. CARD4 (formerly NOD1) and CARD15 (formerly NOD2) bind to DAP and MDP to activate NFκB. Abbreviations are discussed in the text. This figure has been adapted from Sartor, 2006.

cells, and intestinal epithelial cells (Cobrin & Abreu, 2005).

Pattern recognition receptor signalling alerts and protects the host against deregulated inflammation in the absence of pathogens in the intestine. Changes in these innate signalling pathways due to a genetically predisposed host (e.g. TLR4 (Franchimont *et al*., 2004) and NOD2/CARD15 (Ogura *et al*., 2001) mutations identified in IBD patients), may underlie chronic intestinal inflammation (Werner & Haller, 2007).

1.3.2.2.3 Adaptive immune response

The adaptive immune responses at the mucosal surface are based on complex interactions between epithelial cells, mucosa-associated lymphoid tissue and systemic immune cells (Fig. 1.5). Antigens are sampled from the intestinal lumen across the epithelial barrier by microfold-epithelial (M) cells. M cells form an intraepithelial pocket-like structure containing APCs (dendritic cells and macrophages) and T and B cells which play an important role in generating an antigen-specific response (Pasetti *et al*., 2005; Fiocchi, 2003). Most T cells in the intraepithelial pocket are $CD4^+$ T cells, whereas $CD8^+$ T cells reside within the epithelial layer. In contrast to innate immune responses that are similarly activated in CD and UC, T cell profiles of the adaptive immune response can be distinguished in CD and UC (Sartor, 2006). Th cell types produce cytokines and chemokines and are classified into Th1 and Th2 subsets. Th1 cells regulate the cellular immunity and macrophage activation and result in increased expression of IL2, IFNγ and IL12 cytokines with subsequent induction of TNFα and IL18 and a compensatory increase in IL10 and tumour growth factor beta (TGFβ). Th2 cells regulate the humoral immunity and generate IL4, IL5, IL6, IL10 and IL13. IFNγ production can inhibit the differentiation of Th2 cells, and IL10 can decrease Th1 responses (O'Neil & Steidler, 2003; Madsen, 2002). The imbalance of cellular and humoral immune responses seems to play an important role in IBD pathogenesis, because the immunological response in CD is Th1 cell-mediated, whereas UC is based on Th2 cell activation. Also a Th17 cell-mediated response has been implicated in CD (Sartor, 2006).

Activated B cells, called plasma cells, produce antibodies or immunoglobulins (Ig), of which there are five classes (IgA, IgG, IgM, IgD and IgE) at the mucosal surface, binding to the antigens and eventually leading to destruction (Kett *et al*., 1987).

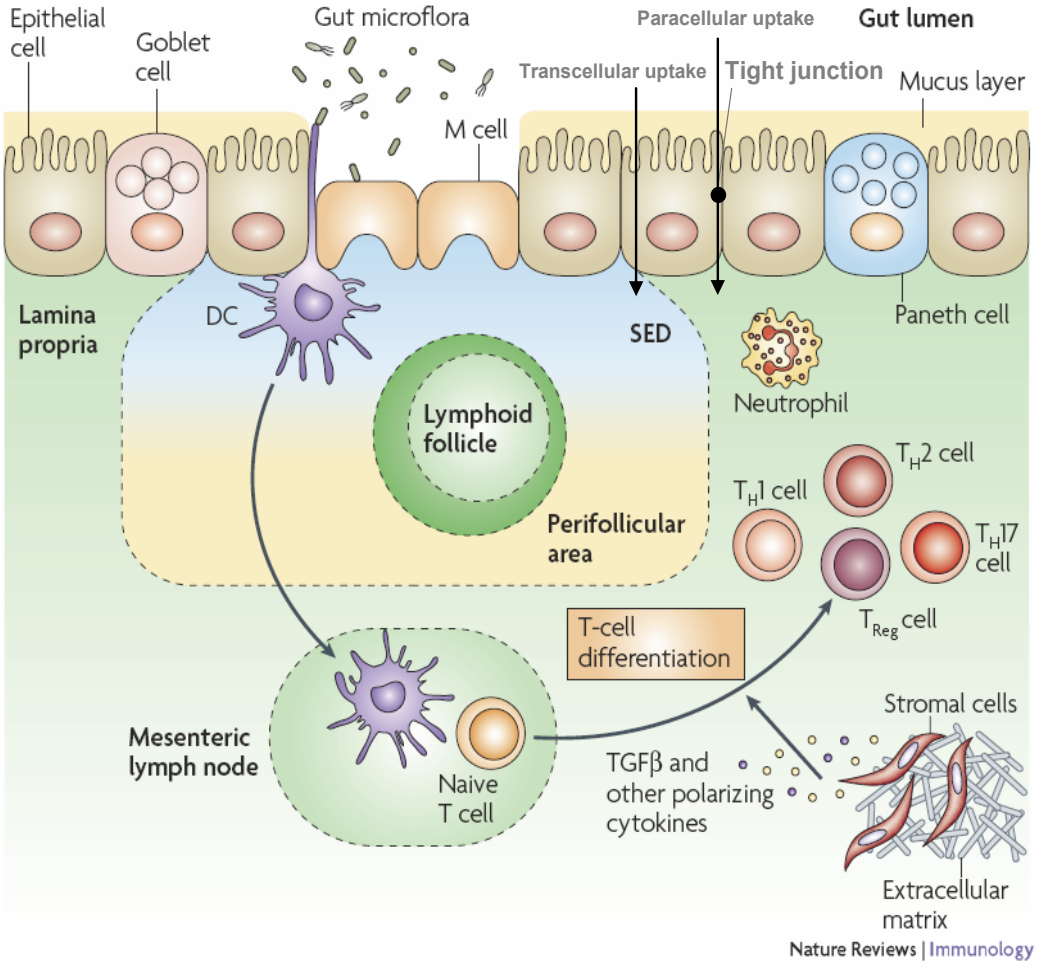


Fig. 1.5 Intestinal immune system showing features of the adaptive immune response. Antigens are sampled from the intestinal lumen across the epithelial barrier by M cells which form an intraepithelial pocket-like structure containing dendritic cells (DC), macrophages, T and B cells. The presence of bacterial antigens leads to activation and migration of DC that induce naïve T cells to undergo differentiation. SED, subepithelial dome; TGFβ, transforming growth factor beta; Th, T helper; T_{Reg}, T regulatory. Other abbreviations are discussed in the text. This figure has been adapted from Cho, 2008.

B cells in the intraepithelial pocket that produce secretory and memory markers serve as antigen-presenting B cells and interact with T cells to promote immunological memory (Pasetti *et al*., 2005). High numbers of IgG, followed by IgM and IgA, producing plasma cells are found in IBD patients with more IgG1 in UC and IgG2 in CD. This difference may be due to the distinct immunoregulatory mechanisms that lead a Th1 (CD) or Th2 (UC) response (Kett *et al*., 1987).

1.3.3 Bacteria in inflammatory bowel disease

Bacteria are an environmental factor that can interact with a genetically susceptible host and be a risk factor for the development of intestinal inflammation. The influence of diet, especially dietary fat, as another environmental factor in intestinal inflammation will be described in section 1.4.

1.3.3.1 Bacteria of the gastrointestinal tract and their role in intestinal inflammation

The gastrointestinal tract, especially the terminal ileum and the large bowel (caecum and colon), harbour complex and numerous bacterial communities (Tannock, 2005). Fig. 1.6 illustrates the different compartments of the human gastrointestinal tract, physiological processes and conditions and the major bacterial genera found herein (Manson *et al*., 2008). The intestinal bacteria in humans comprises 500-1000 bacterial species (laboratory mouse limited to 6-10 species) and complex metabolic relationships exist among bacterial communities and their host (MacDonald, 2003). Very little is known about these inter-bacterial relationships and how they relate to host health, but it is known that increasing diversity of the large bowel bacteria promotes metabolic homeostasis and ability to resist invading pathogens (Macfarlane *et al*., 2009). Colonisation of the large bowel by bacteria can be influenced by diet, environment and host metabolism (Macfarlane *et al*., 2009).

Colonic bacteria metabolise mainly carbohydrates and proteins and host-produced substances (e.g. mucin) to salvage energy by anaerobic fermentation. Protein fermentation metabolites such as phenolic and indolic compounds, branched-chain fatty acids, sulphur-containing compounds and ammonia, seem to affect IBD by damaging epithelial cells in genetically susceptible hosts, thereby resulting in inflammation

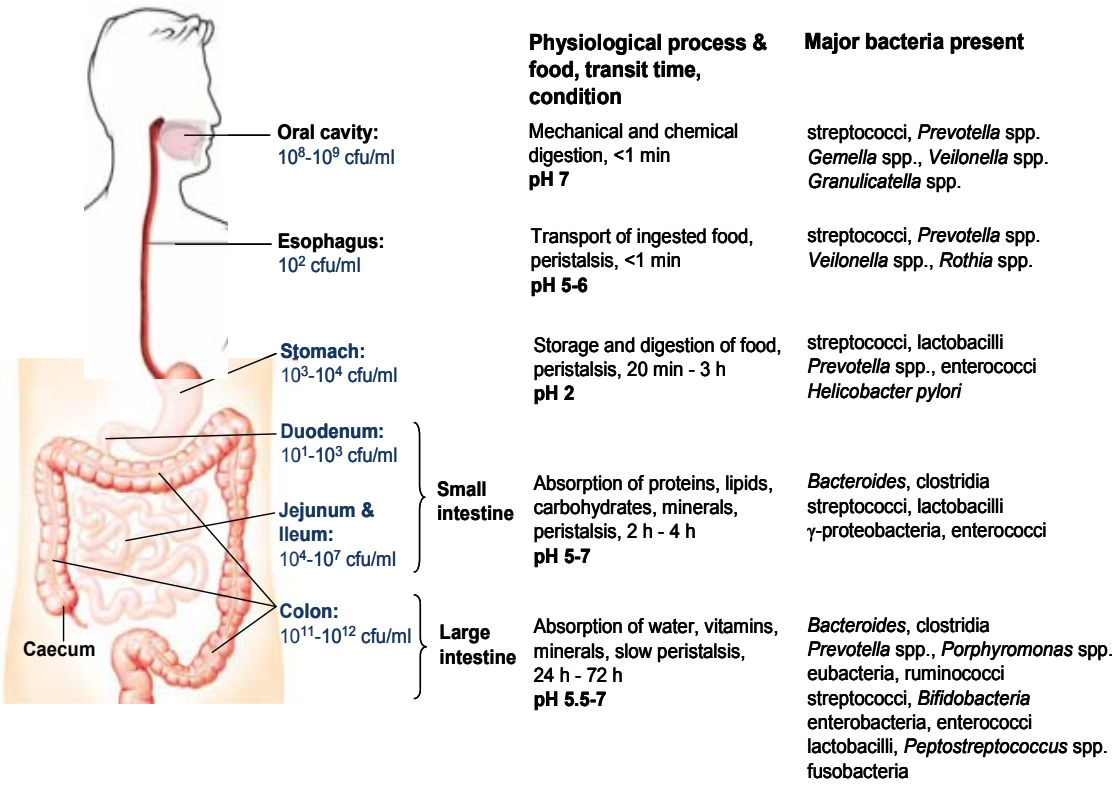


Fig. 1.6 Compartments of the human gastrointestinal tract, physiological processes and conditions and the major bacterial genera found herein. cfu, colony forming units. This figure has been adapted from Manson *et al*. (2008).

(Gottschalk, 1986; MacFarlane *et al*., 1992). Butyrate, a short-chain fatty acid produced by bacteria and a major fermentation product in the large bowel, is an important energy source for colonocytes. Inhibition of butyrate metabolism by reduced sulfide-containing compounds from luminal bacteria, can lead to loss of epithelial barrier integrity and induces inflammatory lesions similar to those seen in UC (Roedinger & Duncan, 1993; Mariadason *et al*., 1997; Wachtershauser & Stein, 2000; Macfarlane *et al*., 2009).

Bacteria provide a continuous stimulus for the host immune system. An immunological tolerance towards commensals normally prevents intestinal inflammation (Duchmann *et al*., 1995). Studies with IBD patients demonstrated that commensal bacteria are a critical factor in the development of intestinal inflammation (Duchmann *et al*., 1999; Kraus *et al*., 2004). Mouse models showed that the interaction between genetic susceptibility and bacteria is important in the aetiology of IBD (Table 1.2). Some mice did not develop IBD when maintained in germ-free conditions and in others the disease development was reduced in specific pathogen-free conditions or by antibiotic administration. On the other hand it has been shown that colonisation of the gastrointestinal tract with specific bacteria, for instance *Lactobacillus* spp., can have anti-inflammatory effects. These beneficial bacteria were host-specific. Bacteria with pro-inflammatory effects include *E. faecalis* which can induce a more consistent intestinal inflammation when inoculated into *Il10*$^{-/-}$ mice (Balish & Warner, 2002) or when pure Enterococcus isolates (*E. faecalis* and *E. faecium*) are combined with a mixed "complex intestinal flora" derived from healthy C57BL/6J mice raised under conventional conditions (Roy *et al*., 2007).

Intestinal inflammation influences the gastrointestinal environment, where some bacterial species flourish and others decrease in numbers (McBurney *et al*., 2006). In contrast to healthy individuals, colon biopsy samples from CD patients are characterised by a higher number of bacteria and a thicker mucosal layer of bacteria associated with the intestinal surface. However, the overall bacterial diversity appears to be reduced in individuals with CD with predominance of members of the phyla *Proteobacteria* and *Bacteroidetes* as compared to UC patients and healthy subjects. It remains to be elucidated which bacterial populations are most important in the aetiology of CD or UC and whether a certain degree of variation in the community profile reflects inter-individual differences among intestinal bacteria. In order to address these important

Table 1.2 Interaction of the genetic susceptible host with environmental factors such as bacteria

Model	Reference
No colitis in germ-free condition	
IL2-deficient mouse	(Sadlack *et al*., 1993)
IL10-deficient mouse	(Sellon *et al*., 1998)
TCRα-deficient mouse	(Dianda *et al*., 1997)
Reduced colitis in specific pathogen-free condition or after antibiotic treatment	
IL2-deficient mouse	(Sadlack *et al*., 1993)
IL10-deficient mouse	(Kühn *et al*., 1993; Madsen *et al*., 2000)
Mdr1α-deficient mouse	(Panwala *et al*., 1998)
TGFβ dnRII transgenic mouse	(Hahm *et al*., 2001)
Bacteria with anti-inflammatory effects	
Lactobacillus sp. in IL10-deficient mouse	(Madsen *et al*., 1999; Schultz & Sartor, 2000)
Bacteria with pro-inflammatory effects	
Bacteroides vulgatus in HLA-B27/β2m transgenic rats	(Rath *et al*., 1999)
Enterococcus faecalis in IL10-deficient mouse	(Balish & Warner, 2002; Kim *et al*., 2005)
Escherichia coli in IL10-deficient mouse	(Kim *et al*., 2005)
Helicobacter hepaticus in IL10-deficient and TGFαβ dnRII transgenic mouse	(Kullberg *et al*., 1998; Chin *et al*., 2000))

questions in more detail, robust statistical methods will be required to analyse large clone libraries (Eckburg & Relman, 2007) or pyrosequencing (a high throughput, next-generation DNA sequencing technique; Margulies *et al*., 2005) data sets. Furthermore, a consistent use of the phylotype definition would facilitate more productive comparisons of results from different studies.

1.3.3.2 Qualitative and quantitative analysis of bacteria involved in intestinal inflammation

A variety of bacteria, including *Mycobacterium avium* subspecies *paratuberculosis,* adherent-invasive *E. coli, Pseudomonas aeruginosa, Listeria monocytogenes*, *Mycoplasma* spp., *Chlamydia* spp., *Coxiella* spp., *Streptococci, Yersinia pseudotuberculosis, Saccharomyces cerevisiae, Measles* virus*, Helicobacter pylori, Bacteroides vulgatus (B. vulgatus), Clostridium difficile* (*C. difficile*)*, Campylobacter jejuni, Salmonella typhimurium* and *Klebsiella pneumoniae*, have been linked to the pathogenesis of IBD (Chadwick & Anderson, 1992; Darfeuille-Michaud *et al*., 1998; Linskens *et al*., 2001; Eckburg & Relman, 2007). Active intestinal inflammation in IBD patients is accompanied by accumulation of *Bacteroides* and *E. coli* spp. (Swidinski *et al*., 2005). *Bacteroides* spp., *Bifidobacterium animalis*, *C. cocleatum* and *Enterococcus* spp. were more apparent in the large bowel of colitic *Il10*$^{-/-}$ mice (Bibiloni *et al*., 2005). Each individual bacterial profile is unique and its composition and metabolic activity depends for example on the individual's geographic location, diet, grade of illness, age or host genotype (Eckburg & Relman, 2007).

The human gastrointestinal tract is a habitat for diverse bacterial communities. Most of them are anaerobes, and only about 20-40% are cultivable in the laboratory using classical plating and speciation techniques (Tannock, 2005). Traditional culture methods based on phenotypic characterisation are insufficient to detect the whole bacterial community in the intestine, due to low coverage, low reproducibility, low sensitivity, and being time-consuming and laborious (Furham *et al*., 1992; Dutta *et al*., 2001). Nevertheless, traditional cultivation techniques provided early insights into the physiology of cultivable bacteria in the intestine and are still applied in clinical microbiology.

Culture-independent and higher resolution molecular methods have become an important tool in studying the diversity, abundance and population dynamics of complex bacterial communities (Amor & Vaughan, 2006). These molecular methods are based mainly on the detection of 16S ribosomal RNA (rRNA) or ribosomal RNA genes (rRNA genes; DNA encoding the rRNA) (Franks *et al.*, 1998). The use of RNA isolated from bacterial cells indicates the metabolic activity, because the ribosome-per-cell is approximately proportional to bacterial growth rate. The use of DNA provides a phylogenetic overview of the community comprising DNA from living active or dormant cells, lysed or dead cells (Tannock, 2005). The 16S rRNA gene has become a key bacterial marker due to its unique composition of highly conserved as well as variable genomic regions, its evolutionary stability and its high copy number within bacterial cells (Amann *et al.*, 1990). Differences in base pair sequence within the variable regions are used to distinguish among bacterial genera and species. The highly conserved regions of the ribosomal RNA or DNA can be used for design of universal probes targeting the entirety of the bacteria, whereas variable regions are used for targeting specific groups of bacteria. Numerous 16S rRNA gene sequences including uncultured bacterial clones are available in the database of the National Centre for Biotechnology Information (NCBI), which allows for sequence comparisons across the world.

The bacterial community in a sample can be analysed using polymerase chain reaction (PCR) combined with temperature or denaturing gradient gel electrophoresis (TGGE or DGGE), or dot blot hybridisation with specific synthetic oligonucleotide probes, or having fixed bacterial cells by fluorescent *in situ* hybridisation (FISH) combined with flow cytometry or microscopic analysis, or DNA-arrays (DNA chips) (Amann *et al.*, 1995; Wang *et al.*, 1996; Wilson & Blitchington, 1996; Yu & Morrison, 2004; Tannock, 2005). Quantitative PCR enables the quantification of all DNA fragments (Rinttilä *et al*., 2004). Multiplex PCR even allows the amplification of several target regions of the 16S rRNA gene in a single reaction with the respective primers (García *et al*., 1998). These methods have found wide application, especially in the field of environmental microbiology (e.g. in marine, soil, wastewater habitats etc.). However, in recent years these techniques have also been used to study the ecology of intestinal bacteria of humans and animals. Hereby, the detection of bacterial shifts in the gastrointestinal tract related to factors such as diet, disease or age are of particular interest. Because of the

difficulty in collecting samples from different parts of the gastrointestinal tract, faeces is often collected in studies of gastrointestinal bacteria. However, faecal bacteria merely represent the community in the distal colon, not that of the proximal colon or terminal ileum. It most likely does not accurately represent the upper intestinal bacteria nor its metabolic activity.

The following paragraphs describe some of the qualitative and quantitative molecular methods that are commonly applied in gastrointestinal microbiology, paying special attention to fingerprinting (e.g. DGGE) and quantitative (e.g. real-time PCR) techniques.

1.3.3.2.1 Community fingerprinting techniques

Molecular fingerprinting techniques such as terminal restriction fragment length polymorphism (T-RFLP) and DGGE allow the rapid assessment of predominant bacterial species of complex and dynamic bacterial communities in multiple samples simultaneously (Liu *et al.*, 1997; Bibiloni *et al*., 2006; McBurney *et al*., 2006). They are based on differences in the 16S rRNA marker genes of different bacterial species. The profiles obtained reflect the diversity of the bacterial community in the analysed sample. In T-RFLP, a restriction endonuclease is used to obtain a species-specific terminal restriction fragment from each species within the community (Hozapfel *et al*., 2001).

DGGE was first introduced in bacterial ecology by Muyzer *et al*. (1993). The DGGE technique is based on 16S rRNA gene sequence-specific melting behaviour because DGGE is carried out with double-stranded DNA. A single hypervariable (V) region of the 16S rRNA gene is usually used in DGGE. The amplification of either the V1 and V3 region provided best results for short amplicons, whereas the V3 to V5 or V6 to V8 region may be used for longer amplicons (Yu & Morrison, 2004). The PCR amplicons are generated with primers of which one has a GC-rich sequence (GC-clamp) attached (Fig. 1.7). A temperature (TGGE) or denaturing (DGGE) gradient is generated in a polyacrylamide gel in parallel with an electric field. The 16S rRNA genes migrate through the gel and become exposed to an increasing temperature or chemical gradient. The migration of the double-stranded DNA stops at the point in the gradient where the amplicon (except for the GC-clamp) fully denatures. Thus, sequences of the same size but of different thermal or chemical (based on G+C content) stability can be separated.

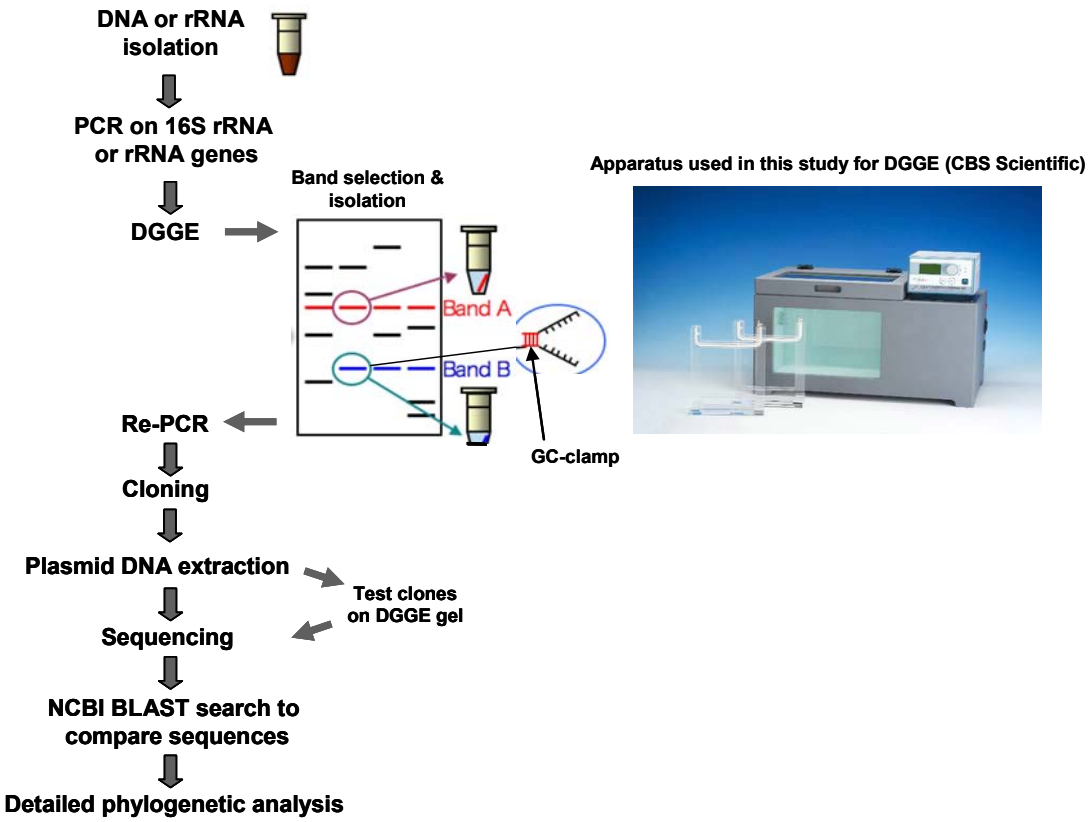


Fig. 1.7 Workflow for denaturing gradient gel electrophoresis (DGGE), a fingerprinting technique to assess complex bacterial communities. The 16S rRNA genes migrate through the gel in an increasing denaturing gradient of urea and formamide. The migration of the double-stranded DNA stops at the point in the gradient where the DNA almost fully denatures and sequences of the same size but different chemical (based on G+C content) stability are separated. The GC-clamp attached to one of the primers ensures that the DNA remains partially double-stranded. After visualisation of the DNA fragments, they can be excised from the gel, cloned into *E. coli* and sequenced for identification. Sequences are compared against numerous 16S rRNA gene sequences available from the online database of the National Centre for Biotechnology Information (NCBI).

The DNA fragments with different gene sequences migrate to their respective positions in the gel to form an operational taxonomic unit. The bacterial community profiles can be visualised using several staining techniques such as ethidium bromide, silver stain, SYBR Green or SYBR Gold. In order to link the position of a certain operational taxonomic unit to the bacterial species, DNA bands can be excised from the gel, cloned into *E. coli* and sequenced (Muyzer & Smalla, 1998; Simpson *et al*., 1999).

In order to compare DGGE profiles from different samples statistical methods such as Dice (band-based) or Pearson (curve-based) similarity coefficients can be used. The Shannon-Wiener diversity index takes into account the richness and proportion of subspecies and is therefore a means to describe the diversity of a sample (Yu & Morrison, 2004). The similarities between samples can be visualised in the form of a dendrogram. Limitations of DGGE are that a single isolate can show multiple bands by DGGE or a single band in a DGGE gel can represent multiple bacterial species (Fromin *et al*., 2002). Since the length of the PCR amplicons is limited to not more than 600 bp which represents only a fraction of the whole 16S rRNA gene, unambiguous phylogenetic identification of bacterial species can sometimes prove challenging (Nubel *et al*., 1996).

1.3.3.2.2 Quantitative techniques

Several methods can be used to quantify bacteria in the gastrointestinal tract. There are quantitative PCR-based methods such as dilution or real-time PCR and methods that are not dependent on previous amplification but based on oligonucleotide probes labelled with fluorescent dyes, e.g. FISH, dot blot and microarrays (Tannock, 2005). The following paragraphs will focus on real-time PCR because this technique is the most frequently used in gastrointestinal microbiology.

In real-time PCR, the amplified target gene fragment is quantified as it accumulates in real time after each amplification cycle. This is done by quantification of a fluorescent reporter that increases its signal proportionally to the amount of PCR product that it binds to in the exponential phase. The data are collected throughout the entire PCR process rather than at the end as it is for conventional PCR reactions. Gene expression in real-time PCR can be measured by absolute or relative quantification. Absolute quantification uses calibration curves based on standards of known DNA concentrations

to estimate the number of target DNA in the sample. Relative quantification calculates the relative expression of a target gene versus a reference gene and does not need a calibration curve (Pfaffl, 2004).

Common methods for real-time PCR include the TaqMan assay (Holland *et al*., 1991) and the SYBR Green dye method (Morrison *et al*., 1998). The TaqMan assay uses fluorogenic labelled oligonucleotide probes together with the primers in the PCR reaction. The SYBR Green dye method uses a non-sequence specific fluorogenic dye (SYBR Green) that emits a strong signal when bound to double-stranded DNA. The target sequence is amplified by a polymerase during the PCR. The SYBR Green dye binds to each new double-stranded DNA copy which increases the fluorogenic signal as more amplicons are produced. A disadvantage for the SYBR Green dye method is that it binds to all double-stranded DNA, including non-specific binding and primer dimmers, thus, requires well-optimised PCR conditions. On the other hand, for the TaqMan assay the different probes for the target sequences have to be synthesised and requires more assay planning and costs.

In summary, despite a few limitations, the fingerprinting and quantitative techniques described above are powerful and reproducible methods that have been used by several groups in recent years to study intestinal bacteria in rodent models of IBD or in biopsy samples from IBD patients. PCR-DGGE has been applied to monitor changes in the small and large bowel bacteria during rodent colitis (Schultz *et al*., 2004; McBurney *et al*., 2006) combined with quantification analysis (Heimesaat *et al*., 2006; Heimesaat *et al*., 2007) by FISH (Hoentjen *et al*., 2005; Heimesaat *et al*., 2006), or by dot blot hydridisation (Bibiloni *et al*., 2005). Conte *et al*. (2006) investigated changes in large bowel bacteria of IBD patients using culture analysis and 16S rRNA gene based amplification and real-time PCR. PCR-DGGE and real-time PCR (Bibiloni *et al*., 2006) in addition to cluster analysis and FISH (Ahmed *et al*., 2007) were used to study the bacterial diversity in small and large bowel biopsies of IBD patients.

1.4 Study of the effects of dietary polyunsaturated fatty acids: Molecular mechanisms involved in intestinal inflammation

The use of 'omics' techniques in combination with model systems and molecular tools helps to understand how foods and food components act on metabolic and signalling processes. PUFA have distinctive nutritional and metabolic effects because they give rise to lipid mediator products and affect the expression of various genes involved in intestinal inflammation. The mechanisms whereby n-3 and n-6 PUFA modulate gene expression in intestinal inflammation involving multiple metabolic pathways and cross-talk between genes controlling lipid homeostasis, and the existing knowledge will be presented in the following sections.

1.4.1 Polyunsaturated fatty acids, inflammation and immune response

PUFA modulate multiple processes including innate and adaptive immunity, infectious pathologies due to bacteria, and the course of chronic diseases such as inflammatory diseases (Calder, 2003; Mutch *et al*., 2005). Two major classes of PUFA, n-3 and n-6, are named after the first unsaturated carbon counting from the methyl end of the molecule. Their precursors α-linolenic acid (ALA) and linoleic acid (LA), are nutritionally essential and must be derived from the diet. LA is the major PUFA in Western-type diets, followed by ALA (Schmitz & Ecker, 2008). The major sources of LA are vegetable oils such as corn, safflower, soybean and sunflower oil, whereas ALA can be mostly found in linseed oil, rapeseed, walnuts and blackcurrant oil, but also in dark green leafy plants (Bartsch *et al*., 1999).

LA and ALA are metabolised in mammalian cells to produce AA and EPA, respectively, by a series of competitive desaturation and elongation steps, initiated by Δ6-desaturation, followed by an elongation and Δ5-desaturation (Grammatikos *et al*., 1994). The processes are shown in Fig. 1.8. These steps are limited by the activity of Δ6-desaturase which is bound with greater affinities by n-3 PUFA. Thus, increased intake of n-3 PUFA can reduce the desaturation of LA and subsequently the production of AA (Rose & Connolly, 1999). LA is converted to γ-linolenic acid and dihomo-γ-linolenic acid to form AA (Schmitz & Ecker, 2008). Dietary LA is the main source of tissue n-6 AA, although dietary AA is also found in lean meats and meat fat (Rose & Connolly, 1999). The n-3 fatty acid ALA is converted to stearidonic acid and

eicosatetraenoic acid, then further converted to EPA and docosahexaenoic acid (DHA). EPA and DHA are found in marine fish such as salmon, tuna, herring, mackerel and anchovy (Rose & Connolly, 1999). AA and EPA can be further metabolised to more highly unsaturated fatty acids or to a series of lipid mediators as shown in Fig. 1.8 (Schmitz & Ecker, 2008).

1.4.1.1 PUFA-derived mediators

Inflammation and inflammatory responses are part of the normal, innate immune response as long as they occur in a controlled manner. Inappropriate inflammatory responses are characterised by up-regulation of adhesion molecules and subsequent induction of leukocyte movement into sites of inflammation, production of inflammatory mediators and host tissue damage (Calder, 2006). Eicosanoids generated from n-6 20-carbon PUFA are the key link between PUFA and inflammation. Inflammatory cells usually contain large amounts of AA and small amounts of other 20-carbon PUFA such as EPA. Large amounts of interleukins and the pro-inflammatory eicosanoids derived from AA released from membrane phospholipids have an important role in the early phase of inflammatory activation (Simopoulos, 2002; Calder, 2008). AA can be converted to series-2 prostaglandins (PG) and thromboxanes (TX), series-4 leukotrienes (LT), to several hydroperoxy- and hydroxy-eicosatetraenoic derivatives, and to lipoxins (LX). COX and lipoxygenases (LOX) are enzymes that catalyse these conversions (Fig. 1.8). EPA is converted to series-3 PG and TX eicosanoids, series-5 LT, and to several hydroperoxy- and hydroxy-eicosapentaenoic derivatives and resolvins. Thus EPA supplementation can lead to competitive inhibition of AA metabolism and to the production of the less active metabolites (Fisher, 1997). Mediators derived from EPA and DHA typically have anti-inflammatory properties while those formed from AA exert mostly pro-inflammatory or other metabolic effects (Table 1.3). Study of their pro- or anti-inflammatory action continues.

The theory that dietary n-3 PUFA exert anti-inflammatory effects by substituting for AA incorporation into membrane phospholipids, and thereby block the production of pro-inflammatory eicosanoids (especially PGE_2 and LTB_4), has long been known (Lands, 1987). The AA-derived PGE_2 has anti- and pro-inflammatory modulation effects. It can induce COX2, and in this way induces its own synthesis and the production of the pro-inflammatory cytokine IL6 in macrophages. In contrast, PGE_2

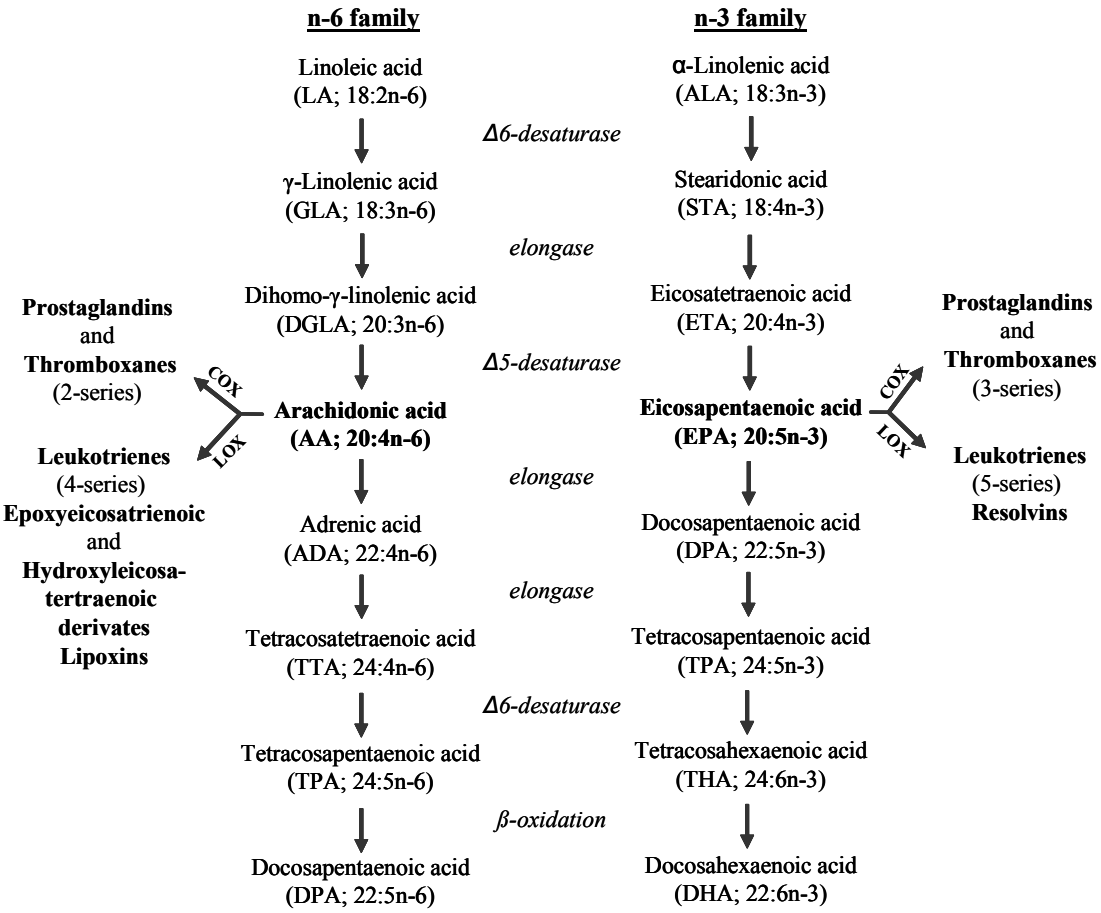


Fig. 1.8 Metabolism of n-3 and n-6 PUFA (created from information in Mills, 2005 and Schmitz & Ecker, 2008). COX, cyclooxygenase; LOX, lipoxygenase.

Table 1.3 n-3 and n-6 PUFA-derived lipid mediators and their metabolic effects

Mediator effects	**AA (n-6)-derived lipid mediators**		**EPA (n-3)-derived lipid mediators**	**Mediator effects**
Anti-inflammatory	PGD_2	**Prostaglandins**	PGD_3	Less inflammatory
Pro-inflammatory	PGE_2		PGE_3	Anti-inflammatory
Vasodilatation	PGF_2		PGF_3	
	PGI_2		PGI_3	
Pro-inflammatory	TXA_2	**Thromboxanes**	TXA_3	Less inflammatory
Vasoconstriction	TXB_2		TXB_3	Vasodilatation
Platelet aggregation				Weaker platelet aggregation
Pro-inflammatory	LTB_4	**Leukotrienes**	LTB_5	Less inflammatory
Chemotaxis of	LTC_4		LTC_5	
phagocytes	LTD_4		LTD_5	
	LTE_4		LTE_5	
Anti-inflammatory	LXA_4	**Lipoxins**		
Pro-resolving	LXB_4			
		Resolvins	RvE1	Anti-inflammatory
			RvE2	

inhibits 5-LOX to decrease the production of pro-inflammatory 4-series LT and can induce 15-LOX to form anti-inflammatory LX (Schmitz & Ecker, 2008).

Lipoxins belong to a new family of PUFA-derived lipid mediators, including AA-derived aspirin-triggered LX; EPA-derived resolvin E-series (RvEs); DHA-derived resolvin D-series (RvDs) and protectins, which serve as endogenous agonists controlling inflammation by stimulating resolution (Serhan & Chiang, 2008). Bannenberg *et al*. (2007) defined resolution as an essential part of the acute inflammatory response including the formation of pro- and anti-inflammatory and pro-resolution lipid mediators. These mediators serve as ligands to activate specific receptors and thus play an important role in the progression of the normal inflammatory response. Resolution of inflammation contributes to molecular events that remove inflammatory cells and restore tissue integrity.

LXA_4 exerts potent anti-inflammatory effects mediated through the activation of a specific G-protein coupled LX receptor and activates receptor-mediated molecular pathways to counter-regulate acute inflammation (Takano *et al*., 1997; Fiore *et al*., 1994). Resolvins, first identified during the resolution phase of acute inflammation (Serhan *et al*., 2000), are oxygenated products derived from EPA and DHA (Bannenberg *et al*., 2007). The EPA-derived resolvin E1, shows anti-inflammatory actions by binding to a G-protein coupled receptor to inhibit NFκB activation and thus block the synthesis of pro-inflammatory cytokines and chemokines. RvE1 receptor is also expressed in gastrointestinal tissue (Arita *et al*., 2005).

1.4.1.2. Immunomodulatory mechanisms

From early studies of patients with IBD a link between PUFA, the immune system and pathogenesis of CD was suggested. These studies reported decreased concentrations of n-3 PUFA in plasma phospholipids (Sheehan *et al*., 1992) and in adipose tissue (Geerling *et al*., 1999) and also decreased serum concentrations of total n-3 PUFA (Kuroki *et al*., 1997) in IBD patients compared with control patients. The loss of n-3 PUFA in body fluids and tissues increases the n-6:n-3 ratio, which can lead to the production of more pro-inflammatory eicosanoids.

Several aspects of the immunomodulatory effects of PUFA in intestinal inflammation have recently been investigated, e.g. the role of PUFA in modulation of pro- and anti-inflammatory eicosanoids, membrane fluidity, signal transduction, antigen presentation, gene expression and gastrointestinal flora (Mills *et al*., 2005). They reviewed studies in humans and animals which reported that dietary n-3-rich fish oil supplementation decreased AA levels in the membrane as AA was replaced by n-3 PUFA. As a result of the competitive inhibition of AA metabolism, less pro-inflammatory eicosanoids were produced by AA. Immune cells, such as macrophages, are the main source of eicosanoids. Prostaglandins, for example, can suppress T lymphocyte proliferation, T cell-mediated toxicity, IL2 production and natural killer cell activity *in vitro* (Goodwin & Ceuppens, 1983). Thus eicosanoids act directly on immune cells and are modulated themselves by PUFA (Mills *et al*., 2005).

PUFA have an important role in membrane structure. Incorporation of PUFA into membrane lipids increases membrane fluidity and affects the conformation of membrane protein complexes. Some membrane-bound enzymes and receptors are sensitive to the fatty acids that surround them. There are also membrane regions (the so-called lipid rafts) containing cholesterol, sphingolipids and signalling molecules, where signalling events can take place (Viola *et al*., 1999; Xavier *et al*., 1998). Dietary n-3 PUFA are able to decrease the sphingomyelin content of lipid rafts *in vivo*. Displacement of acylated proteins from membrane lipid rafts is observed with EPA but to a lesser extent with AA and could be due to altered protein acylation after PUFA incorporation or changes in lipid raft composition (Stulnig, 2003).

PUFA are involved in many intracellular signal transduction pathways in immune cells, specifically in T and B lymphocytes. Long chain fatty acids can modulate signalling pathways and target gene expression by reciprocal modulation of the activation of TLR4 and its downstream signalling pathways. They suppress NFκB activation and COX2 expression that are inducible by a TLR2 agonist in macrophages. Different types of fatty acids modulate different TLR molecules, suggesting a modulatory role for dietary fatty acids in immune and inflammatory responses by TLR activation in the intestine. Changes in dietary fat composition could modulate signalling pathways downstream of TLR molecules, as well as targeting gene expression and subsequent cellular responses (Mills *et al*., 2005).

In vitro and *in vivo* experiments suggest that the immunomodulatory mechanism of n-3 PUFA might occur via modulation of antigen-presenting cell function. Fish oil supplementation to healthy volunteers decreased the expression of membrane-bound MHC II antigen-presenting molecules in peripheral blood monocytes. Furthermore, a role for PUFA in modulating APC function is supported by the observation that the expression of adhesion molecules such as intercellular adhesion molecule1 (ICAM1) protein is down-regulated by n-3 PUFA. Adhesion molecule expression enables the antigen bearing cells in the peripheral circulation to reach lymphoid areas to present antigen to T lymphocytes and stimulate an immune response (Mills *et al*., 2005).

PUFA may affect the composition of the intestinal bacteria and thus alter the balance of inflammation in the intestine. It is suggested that dietary PUFA affect mucosal adhesion sites for gastrointestinal bacteria (e.g. lactobacilli) by altering the composition of the intestinal wall (Kankaanpaa *et al*., 2001). This implies that dietary PUFA could enhance the functions of probiotic bacteria in the gastrointestinal tract, e.g. by stimulating adhesion (Mills *et al*., 2005; Torres & Ríos, 2008).

Immune responses in IBD patients are triggered by the presence of commensal intestinal bacteria (Ewaschuk & Dieleman, 2006). Most animal models of IBD (such as *Il10*$^{-/-}$ mice and HLA-B27 transgenic rats) fail to develop chronic intestinal inflammation when raised in germ-free conditions (Sellon *et al*., 1998; Taurog *et al*., 1994). *E. faecalis* is a commensal bacterium and has been used to induce colitis in *Il10*$^{-/-}$ mice, both as a single defined strain (Balish & Warner, 2002) and in a combined inoculum with complex intestinal flora (Roy *et al*., 2007). It would therefore seem that the balance between commensal intestinal bacteria is an important factor which can influence the transition from mucosal homeostasis to chronic inflammation (Ewaschuk & Dieleman, 2006).

1.4.2 Polyunsaturated fatty acid regulation of gene expression in IBD

Long chain fatty acids have been known to contribute to enterocyte function and pathology (Heimerl *et al*., 2006). In particular, long chain n-3 and n-6 PUFA are incorporated into biological membranes and regulate such processes as energy metabolism, eicosanoid production, signal transduction and regulation of gene

expression (Mutch *et al*., 2005; Deckelbaum *et al*., 2006). n-3 PUFA modulate the expression of genes that code for proteins involved in inflammation, lipid metabolism and energy utilisation in several tissues and organs. They reduce inflammation and induce lipid oxidation for enhanced energy utilisation (Deckelbaum *et al*., 2006). Long chain PUFA modulate inflammatory processes by altering fatty acid metabolism in the intestinal epithelial cells (Belluzzi, 2002). There are multiple mechanisms whereby long chain PUFA, especially n-3 PUFA, can affect transcription factor-dependent gene expression, the expression of nuclear hormone receptors and lipid second messengers. The latter activate other genes or other cellular events which affect gene expression, e.g. production of n-3 metabolites and subsequent gene expression (Deckelbaum *et al*., 2006).

1.4.2.1 Fatty acid metabolism

It has been shown that the altered expression of genes involved in fatty acid metabolism in IBD may be a consequence of inflammation, but may also contribute to IBD pathophysiology. Genes involved in uptake and activation of fatty acids include fatty acid transport proteins (FATP, solute carrier family 27), fatty acid binding proteins (FABP) and long chain acyl-CoA synthases (ACSL) (Heimerl *et al*., 2006). The cellular uptake of long chain fatty acids is shown in Fig. 1.9.

The FATP transmembrane proteins in the intestinal membrane increase the uptake of long chain PUFA into cells and FATP gene expression levels directly correlate with long chain PUFA uptake. Six FATP proteins have been found in all fatty acid-metabolising tissues of the human body (Stahl, 2004). High expression levels of FATP2 and FATP4 transporter genes have been found in ileum and colon tissues of healthy subjects. In the colon of CD and UC patients, FATP4 gene expression was down-regulated compared to control tissues (Heimerl *et al*., 2006).

Other proteins (CD36 molecule, FABP and ACSL) can facilitate the uptake of PUFA. It is suggested that long chain PUFA either bind directly to FATP complexes, or bind first to CD36 which then releases the PUFA to FATP. Considerable amounts of CD36 mRNA were observed in healthy intestinal biopsies (Heimerl *et al*., 2006). A study with CD36-deficient mice suggested only a minor role for CD36 in the lipid absorption in enterocytes (Goudriaan *et al*., 2002).

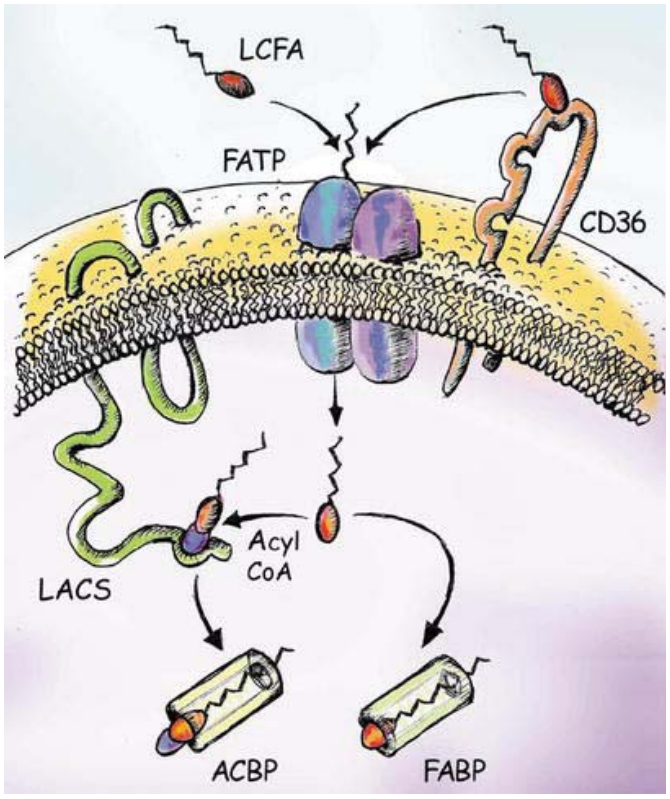


Fig. 1.9 Cellular uptake of long chain PUFA. LCFA, long chain fatty acids; FATP, fatty acid transport protein; CD36 molecule; LACS, long-chain acyl-CoA synthetase; FABP, fatty acid binding protein; ACBP, acyl-CoA-binding protein. This figure has been adapted from Stahl, 2004.

The cytoplasmic protein FABP binds PUFA inside the cell for further fatty acid metabolism. Only three of the seven FABP members described to date (FABP1, FABP2 and FABP6) have major roles in the intestinal tissue. High mRNA levels have been detected in ileum (FABP1, FABP2 and FABP6) and colon (FABP1 and FABP2) biopsies. The expression of FABP2 and FABP6 genes was down-regulated in colon of UC patients (Heimerl *et al*., 2006). FABP has also been considered as a plasma marker of intestinal injury in patients with UC, as the serum concentration of intestinal FABP was elevated in these patients and may indicate ileitis (Wiercinska-Drapalo *et al*., 2008).

For the next step in fatty acid metabolism, acetylation of long chain fatty acids by ACSL is required. ACSL are integral membrane proteins with active sites facing the cytosol. Five human ACSL genes and several isoforms have been identified (Heimerl *et al*., 2006; Soupene & Kuypers, 2008). mRNA levels of ACSL1 and ACSL4 were found to be increased in ileum and colon of IBD patients. The ACSL1 and ACSL4 isoforms provide acyl-CoA for the synthesis of triacylglycerol and phospholipid; the latter of which is incorporated into the intestinal membrane to maintain epithelial barrier function. Therefore, an up-regulation of some ACSL members by the fatty acids present in the enterocyte could support membrane integrity and decelerate the course of inflammation in IBD (Heimerl *et al*., 2006).

Inside the cytoplasm, fatty acids can be elongated and desaturated by specific elongases and desaturases, β-oxidised in mitochondria or peroxisomes for energy production, ω-oxidised in microsomes, peroxidised, involved in membrane phospholipid or eicosanoid (PG, LT and TX) synthesis (Dulpus *et al*., 2000). PUFA are able to down-regulate the expression of genes coding for enzymes involved in fatty acid synthesis, including acetyl-CoA carboxylase, fatty acid synthase and stearoyl-CoA desaturase (Salter & Tarling, 2007).

1.4.2.2 Transcription factors

Dietary PUFA can also directly regulate gene expression by interaction with transcription factors to exert their effects on various metabolic states in health and disease (Salter & Tarling, 2007). Transcription factors play an important role in IBD (Heimerl *et al*., 2006), for example NFκB in regulating production and activation of pro-inflammatory cytokines (Mitsuyama *et al*., 2001). Nuclear receptors, a family of

ligand-activated transcription factors, are most relevant in mediating the effect of nutrients and their metabolites on gene transcription (De Vogel-van den Bosch *et al*., 2008) and can directly or indirectly regulate the expression of genes involved in lipid metabolism and inflammatory signalling (Schmitz & Ecker, 2008). Nuclear receptors that regulate PUFA-induced gene expression include PPAR (NR1C), liver X receptor (LXR, NR1H), retinoid X receptor (RXR, NR2B), hepatocyte nuclear factor 4 (HNF4), farnesoid X receptor (FXR, NR1H) and pregnane X receptor (PXR, NR1I) (Heimerl *et al*., 2006; Salter & Tarling, 2007). Transcriptional regulation by these nuclear receptors requires heterodimerisation with RXR. Receptors that also mediate effects of dietary fatty acids on gene expression include the transcription factors SREBP1 and NFκB (Sanderson *et al*., 2008). The interaction of PUFA with these transcription factors in the regulation of fatty acid metabolism genes is still poorly defined *in vivo*.

PPAR can be activated by a range of natural and synthetic compounds, including fibrates, xenobiotics (Issemann & Green, 1990), and both saturated and unsaturated fatty acids (Kliewer *et al*., 1997). PPAR isoforms, which belong to the steroid hormone nuclear receptor superfamily of ligand-activated transcription factors (which includes retinoic acid receptor (RAR), LXR and RXR), are particularly relevant to dietary fatty acid effects on gene expression. Three different isoforms of PPAR (PPARα (NR1C1), PPARβ (also known as δ; NR1C2), and PPARγ (NR1C3)) have been characterised (Kersten *et al*., 2007).

n-3 and n-6 PUFA serve as endogenous ligands for all three PPAR isoforms with varying affinity for the receptor subtypes (Kliewer *et al*., 1997). Furthermore, transactivation and competitive binding assays have revealed that PUFA metabolites, such as the naturally occurring eicosanoids, can also activate PPAR isoforms with greater affinity than their parent compounds (Sampath & Ntambi, 2005). n-3 PUFA are more potent activators of PPARα than n-6 PUFA. After activation by a ligand, PPAR dimerise with RXR and the PPAR/RXR heterodimer binds to DNA response elements, called the PPAR response element, in the promoter of target genes (Ijpenberg *et al*., 1997).

PPAR*α* is the main PPAR isoform involved in the regulation of genes of lipid and carbohydrate metabolism in hepatocytes (Sampath & Ntambi, 2005). PPARα has been

shown to be highly expressed in the small intestine (Bünger *et al*., 2007). PPARα is also expressed in kidney, whereas PPARγ (subforms γ1, γ2 and γ3 arising from alternative splicing of one gene) is mainly expressed in adipose tissue and macrophages (Salter & Tarling, 2007). PPARβ is ubiquitously expressed (Brown & Plutzky, 2007). PPARα is associated with transcriptional up-regulation of the expression of genes involved in fatty acid oxidation and lipoprotein metabolism, including acyl-CoA oxidase, FATP, carnitine palmitoyl transferase1, lipoprotein lipase and APOA1 and APOC3. PPARα inhibits the expression of genes coding for pro-inflammatory proteins, including vascular cell adhesion molecule1 (a membrane protein that mediates leukocyte extravasation to sites of tissue inflammation) and IL6, in the vascular endothelium (Marx *et al*., 2004). There is some evidence that PUFA inhibit lipogenic gene expression by repression of SREBP mRNA (Sampath & Ntambi, 2005).

SREBP belong to the membrane-bound members of the basic helix–loop–helix leucine zipper family of transcription factors that were first characterised by binding to a sterol response element found on genes involved in cholesterolgenesis. Three isoforms of SREBP have been identified; SREBP1a and 1c, which regulate the expression of genes involved in fatty acid and cholesterol synthesis pathways, and SREBP2, which controls genes important for cholesterol homeostasis (Osborne, 2000). The inactive precursor form of SREBP is located in the ER, linked to SREBP cleavage-activating protein (SCAP). With low sterol concentrations in the cell, SCAP moves with the SREBP to the Golgi apparatus where the active mature form of SREBP is released. The mature SREBP migrates to the nucleus and can bind to the sterol response element in the promoter region of target genes involved in cholesterol, triglyceride (TG) and fatty acid synthesis. When cellular sterol concentrations are high, sterol interacts with the protein Insig, an ER anchor protein, on the SREBP-SCAP complex and SREBP maturation is inhibited. As a consequence the transcription of target genes involved in lipid and lipoprotein metabolism is down-regulated (Salter & Tarling, 2007; Sampath & Ntambi, 2005).

SREBP1 expression is mainly regulated at the level of gene transcription, whereas SREBP2 expression is mainly regulated at a post-translational level, through modulation of cleavage of its membrane-bound precursor to release the active nuclear form (Salter & Tarling, 2007). As reviewed by Salter & Tarling (2007), PUFA regulate

SREBP expression partly through LXR. PUFA can inhibit the interaction of oxysterol ligands with LXR and thus inhibit activation of SREBP1c gene transcription by LXR. Another possible mechanism is the inhibition of LXR transcriptional activity by PUFA activation of PPAR. Increased competition between PPAR and LXR for the connection with its transcriptional partner RXR has been reported. The effect of dietary PUFA on cell lipid homeostasis is a result of complex, controlled interactions between SREBP, PPAR, LXR and RXR transcription factors (Sampath & Ntambi, 2005; Deckelbaum *et al*., 2006; Salter & Tarling, 2007). An overview of PUFA and its influence on transcription factors is presented in Fig. 1.10.

There is also evidence that n-3 PUFA affect HNF4α transcriptional activity and exert hypotriglyceridemic effects. The HNF4α gene is highly expressed in liver, but is also found in kidney, intestine and pancreas. This transcription factor is associated with the expression of genes involved in lipid and lipoprotein metabolism, such as apo AII, AIV, CII and CIII, the microsomal TG transfer protein, and cytochrome P450, family 7, subfamily A, polypeptide 1 (CYP7A1). HNF4α binds with high affinity to unsaturated long-chain fatty acyl CoA which in turn inhibits HNF-4a transcriptional activity. HNF4α binds to direct repeat 1 response elements as a homo-dimer and can compete with PPAR/RXR heterodimers for DNA binding (Salter & Tarling, 2007).

Another mechanism of PUFA-mediated gene expression is the regulation of the transcription factor NFκB. NFκB and IκB are co-located in the cytoplasm of cells as an inactive heterodimer. When IκB is phosphorylated, NFκB separates from IκB and translocates to the nucleus to influence the transcription of a variety of pro-inflammatory genes, such as COX2 and IL6. AA stimulates NFκB translocation and thus induces NFκB target gene transcription, whereas EPA inhibits translocation by PPARα-mediated activation, resulting in inhibition of the transcription of NFκB target genes (Sampath & Ntambi, 2005).

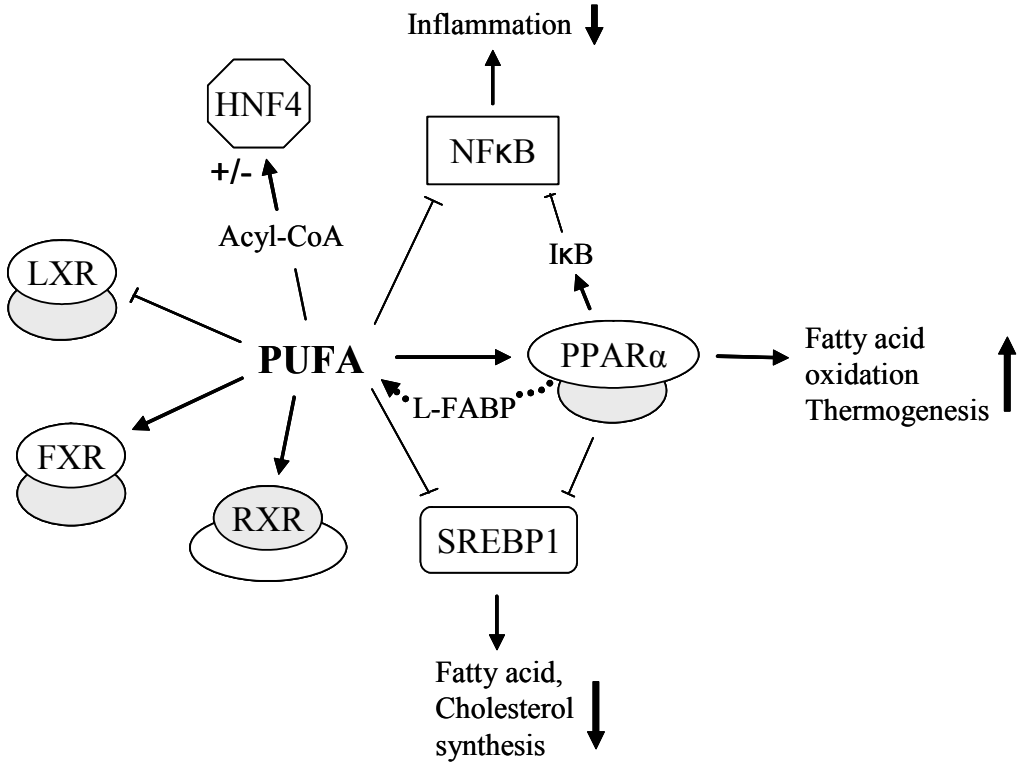


Fig. 1.10 Transcription factors influenced by PUFA. PUFA can activate (PPARα, RXR and FXR) or repress (SREBP1, LXR and NFκB) various transcription factors. L-FABP as PPAR-binding protein can transport and deliver PUFA to PPARα. HNF4 transactivation is repressed by PUFA and activated by PUFA-CoA ester. RXR is the dimerisation partner for PPARα, LXR and FXR and represents a PUFA-binding protein. PPARα, peroxisome proliferator-activated receptor α; RXR, retinoid X receptor; FXR, farnesoid X receptor; SREBP1, sterol regulatory element binding protein 1; LXR, liver X receptor; NFκB, nuclear factor-κB; L-FABP, liver fatty acid binding protein; HNF4, hepatic nuclear factor 4 (created from information in Schmitz & Ecker, 2008 and Dulpus *et al*., 2002).

1.4.3 A molecular approach to study polyunsaturated fatty acid-regulated genes in intestinal inflammation

An approach to define the molecular mechanisms whereby dietary PUFA alter intestinal inflammation is to identify PUFA-regulated genes that are expressed at the onset of intestinal inflammation.

Methods for gene expression analysis that require the pre-selection of single genes, such as Northern blotting and quantitative PCR, are biased. These methods are useful, but they miss important effects on biological processes, such as metabolic and signalling pathways and transcriptional networks across several pathways (Subramanian *et al*., 2005).

DNA and oligonucleotide microarray technologies enable the analysis of genome-wide expression patterns (e.g. for particular diseases or physiological states) of organisms with a sequenced genome, allowing investigators to establish gene networks and identify new genes involved in the investigated trait. Gene expression profiling, or transcriptomics, is widely used in biomedical (Subramanian *et al*., 2005) and nutrition (Muller & Kersten, 2003) research to characterise molecular pathway and network data in a variety of tissues in laboratory animals (Langmann *et al*., 2004; Dommels *et al*., 2007; Rivera *et al*., 2006) and in response to dietary treatments to predict a phenotypic outcome (Nones *et al*., 2008; Sanderson *et al*., 2008; De Vogel-van den Bosch *et al*., 2008).

However, extracting the biological significance of a large amount of gene data is still a challenge. Information about genes is organised in ontologies, such as Gene Ontology (GO) (Prüfer *et al*., 2007). There are several tools for gene ontology enrichment analysis (Ingenuity pathway analysis or IPA, FUNC and many others) available to help interpret the extensive gene lists generated using transcriptomics.

IPA software has a manually created knowledge base and combines GO enrichment with pathway enrichment analysis, network construction and comparison analysis. The biological interaction networks in IPA identify nodes that are central in connections (hub genes) between the differentially expressed genes. FUNC is also a useful tool to

analyse large-scale gene expression data in the context of functional annotations (Prüfer *et al*., 2007). The disadvantage of the FUNC approach compared to that of IPA is that it does not incorporate a function or pathway analysis and lacks a disease focus.

Hub genes are validated by quantitative real-time PCR to confirm their expression as seen in microarrays. More importantly, their function and possible target genes can be further investigated through cell-based transactivation assays.

1.4.3.1 Studies on inflammatory lesions and mediator production

In this section, results from studies using animal models of IBD fed diets supplemented with PUFA are discussed, with particular emphasis on inflammatory lesion development as evaluated by histology, immunohistochemistry, lipid and protein mediator profiles and changes in intestinal gene expression.

Reviews of studies using dietary long chain PUFA (as fish oil) in IBD patients (Belluzzi, 2002; Calder, 2008) have concluded some benefits of fish oil including improvement of clinical score and intestinal mucosal histology and decreased relapse (Calder, 2008). Despite these beneficial effects of fish oil, there are also studies that report no effect on disease activity, higher rate of relapse or no effect on relapse (Calder, 2008). Overall evidence of clinical benefits of n-3 PUFA in IBD is weak.

Several *in vitro* studies have been carried out to investigate the effect of essential fatty acids on various cell cultures (Grammatikos *et al*., 1994). The current *in vitro* models are useful for screening food components, but they are unable to mimic the complexity of the intestinal tract. Thus *in vivo* models of IBD are important for unravelling the complex interactions between food, intestinal bacteria and intestinal cells and thus elucidating the mechanisms of initiation and progression of intestinal inflammation. These animal models display some of the key characteristics of intestinal inflammation (such as morphological changes, inflammation status and onset and progression of disease pathophysiology) and have a well-defined genetic background and immune system. Beside the genetically engineered rodent models developing spontaneous colitis, many animal models of inducible colitis (chemicals, pathogenic bacteria) are used (Gill *et al*., 2001; Jurjus *et al*., 2004). The parameters commonly monitored in

chemically- and pathogenic bacteria-induced colitis models are histological lesions and biochemical parameters such as myeloperoxidase activity, glutathione concentration and cytokine profiles.

Only a few rodent (e.g. Hansen Petrik *et al*., 2000) and human (e.g. Shimizu *et al*., 2003) studies have investigated the effects of purified PUFA in intestinal inflammation. The study of Hansen Petrik *et al*. (2000) evaluated the effect of purified AA and EPA ethyl ester alone and in combination on colorectal cancer using the *Apc*$^{Min/+}$ mouse model. They showed that EPA reduced the number and size of tumours associated with decreased tissue AA and prostaglandin levels, whereas AA supplementation to the EPA diet reduced anti-tumorigenic effect of EPA. The study of Shimizu *et al*. (2003) investigated the impact of highly purified EPA ethyl ester given daily for 2 months to children with UC in relapse. LTB_4 production from leukocytes and colon mucosa after 2 month of treatment was lower than before treatment. However, there was no control group, so it is difficult to associate the absence of relapses for the study period with the EPA ethyl ester administration.

The animal studies summarised here mainly involve chemically-induced colitis models. The effects of dietary PUFA supplementation on disease severity are related to the production of pro-inflammatory eicosanoids. Four studies have found decreased colon or ileum damage and inflammation compared to n-6 PUFA diets in a rat model of chemically-induced colitis (Vilaseca *et al*., 1990; Embey *et al*., 1991; Yuceyar *et al*., 1999; Nieto *et al*., 2002). Vilaseca *et al*. (1990) compared the effects of dietary supplementation with either sunflower (n-6) or cod liver (n-3) oil on the development of chronic granulomatous lesions in the colon of a TNBS rat model. Luminal eicosanoid levels increased after TNBS challenge in both groups. The eicosanoids PGE_2 and LTB_4 production declined after a few days, whereas the eicosanoid TXB_2 continued to rise in the n-6 diet group. In the n-3 group the colon lesions were markedly reduced after 30 days and inflammation, as well as ulceration, were almost absent by 50 days. The study concluded that the fish oil diet prevented the TXB_2 increase in the chronic stage of inflammation. Further evidence for protective effects of a fish oil (EPA)-enriched diet is shown by histological improvements in colon and ileum mucosa of rats challenged with 4% acetic acid to induce colitis (Embey *et al*., 1991).

The therapeutic effects of n-3 PUFA on TNBS-induced colitis was also investigated in a rat model of CD (Shoda *et al*., 1995). The rats were fed a diet enriched with 2% n-3 PUFA-rich perilla oil and compared to a diet enriched with 2% n-6 PUFA-rich safflower oil. In rats from the n-3 PUFA group, plasma LTB_4 concentrations and colon damage were reduced compared to those in the n-6 PUFA group. This study also suggested that supplementation with 2% n-3 PUFA α-linolenic acid, the precursor of EPA and DHA, may be more therapeutically effective in controlling intestinal inflammation in experimental CD than either EPA or DHA.

In a TNBS-induced rat colitis model, the protective role of fish oil (n-3 PUFA) (both as an enriched diet, or administered intrarectally) was investigated (Yuceyar *et al*., 1999). The n-3 PUFA enriched diet showed a protective effect by decreasing production of LTB_4 and LTC_4, and by decreasing activity of the myeloperoxidase, an enzyme which is stored in granules of polymorphonuclear neutrophils and macrophages and released into extracellular fluid in the setting of inflammatory processes. In a study by Campos *et al*. (2002), parenteral lipid emulsions enriched with n-3 PUFA reduced diarrhoea, attenuated morphological changes and decreased colon concentrations of inflammatory mediators in a rat model of acetic acid-induced colitis.

Nieto *et al*. (2002) studied biochemical and histological alterations in rats with TNBS-induced ulcerative colitis fed with mono- and/or polyunsaturated fatty acids (enriched with n-3 and n-6 PUFA). The fish oil group showed less colon damage, lower alkaline phosphatase and myeloperoxidase activities and PGE_2 levels compared to the olive oil group.

Another two studies have investigated the effect of n-3 PUFA diets on pro-inflammatory cytokine production (Andoh *et al*., 2003; Whiting *et al*., 2005). In the former, the effects of n-3 and n-6 PUFA-rich diets (fed for 12 days prior to the induction of enteritis by TNBS) on intestinal inflammation and modulation of early responses were evaluated in rats. Mucosal changes were more severe and serum levels of the pro-inflammatory cytokine IL6 higher in rats fed the n-6 PUFA diet compared with those in the n-3 PUFA group, suggesting a possible suppression of mucosal inflammation by blockage of mucosal IL6 secretion, but no difference was seen in the levels of the pro-inflammatory cytokine TNFα between the diet groups (Andoh *et al*.,

2003). In the latter study (which used SCID mice fed a n-3-enriched or control diet before colitis induction by transplantation of $CD45RB^{high}$ T cells), the n-3 PUFA-fed SCID mice had reduced clinical colitis and colon immunopathology associated with decreased pro-inflammatory cytokine synthesis (TNFα, IL12, IL1ß). There was also reduced myeloid cell recruitment and activation, enhanced epithelial barrier function (epithelial ZO-1 protein, mucosal type I collagen) and induction of the mucosal wound healing mechanism (Whiting *et al*., 2005).

As is the case in human clinical trials, there are also conflicting results from studies with fish oil or PUFA-enriched diets using animal models of IBD. Models of chemically-induced acute colitis have primarily been used in these studies and have generally shown anti-inflammatory effects of fish oil (Hegazi *et al*., 2006). However, preliminary data in a DSS-induced mouse model of colitis (Ramakers *et al*., 2008) have shown that an AA-enriched (ethyl ester oil) diet increased colon AA content, but did not result in more colon inflammation compared with the fish oil (EPA/DHA) and oleic acid (OA, sunflower seed oil) diet groups. The AA-enriched diet even exerted protective effects in colitis (reduced weight loss and diarrhoea scores) than observed in the other two groups.

There are also conflicting data from studies using genetic models such as the *Il10*$^{-/-}$ mouse. One study with *Il10*$^{-/-}$ mice found that fish oil (n-3 PUFA-enriched) reduced colon inflammation compared to n-6 PUFA-rich corn oil (Chapkin *et al*., 2007). Another study investigated the effects of fish oil (n-3 PUFA) and olive oil (n-9 monounsaturated fatty acid) compared with control corn oil (n-6 PUFA) on the severity of chronic colitis in *Il10*$^{-/-}$ mice. COX2 expression was shown as a possible mediator of the immunomodulatory effects of dietary fatty acids on chronic colitis (Hegazi *et al*., 2006). The fish oil diet, however, increased chronic colitis in the colon, and had inhibitory effects on intestinal COX2 expression. Inhibition of COX2 expression resulted in decreased production of anti-inflammatory eicosanoids, e.g. PGE_3.

The pathogenesis of chemically induced colitis may be different compared to genetically induced colitis. Furthermore, the development of colitis in *Il10*$^{-/-}$ mice is in some studies dependent on the presence of commensal luminal bacteria. In particular, inoculation with pure isolates of *Enterococcus* species (either in isolation (Balish &

Warner, 2002), or combined with a mixed "complex intestinal flora" derived from healthy control mice (Roy *et al*., 2007)) appears to play a critical role in establishing consistent inflammation in the intestinal mucosa. It may be that some of the conflicting data in genetic-based mouse models could be due to variations in bacterial colonisation of the gastrointestinal tract. Additional studies are therefore warranted to investigate the mechanisms of PUFA on intestinal bacteria, e.g. PUFA can affect mucosal adhesion sites for intestinal bacteria, and better characterise the role of bacteria in inflammation using genetic-based animal models of colitis.

The findings from previous rodent studies examining the effectiveness of n-3 and n-6 PUFA are inconsistent or conflicting. The discrepancy may be caused by confounding factors of the diet or PUFA supplements. Variation can arise from the study design, diets and feeding procedures, including impurity or unwanted oil components, flavour, sensitivity to oxidation, diet storage, duration of diet change, other macro- or micronutrients etc., which can affect the n-6/n-3 fatty acid ratio (Hudert *et al*., 2006).

Furthermore, because of the disparity in results from both chemically induced and genetic models of colitis, it is necessary that further studies are conducted to elucidate differential effects of PUFA on the time-course of colitis development.

1.4.3.2 Studies on intestinal gene expression

Molecular targets for the protective actions of n-3 and/or n-6 PUFA on models of IBD are yet to be elucidated. Only a few studies have investigated intestinal gene expression changes after PUFA supplementation in animal models of IBD using single gene approaches. Studies of the molecular mechanism underlying the PUFA effect from human studies are also lacking.

Caplan *et al*. (2001) found a reduction of necrotising enterocolitis and intestinal inflammation in a neonatal rat model stressed with asphyxia (increases susceptibility to necrotising enterocolitis) and fed with PUFA formula containing AA and DHA. This study also revealed that PUFA reduced the intestinal mRNA expression of the platelet activating factor (PAF)-synthesising enzyme PLA2 and the PAF receptor, a marker of intestinal inflammation. Similar to their previous study (Caplan *et al*., 2001), PUFA supplementation reduced the incidence of necrotising enterocolitis and also inhibited

intestinal PAF receptor and TLR4 gene expression compared with the controls in a rat model. The rats were fed AA/DHA, egg phospholipids or DHA only. All PUFA supplementations down-regulated PAF receptor expression in ileum and colon, suggesting an interaction between the PAF receptor and TLR signalling as a possible mechanism for the protective effect of PUFA on intestinal injury (Lu *et al*., 2007).

The effect of conjugated linoleic acid (CLA) and n-3 PUFA alone and in combination to prevent or ameliorate IBD in a pig model of DSS-induced colitis has been evaluated (Bassaganya-Riera & Hontecillas, 2006). Feeding CLA to the pigs delayed the onset of IBD, with less severe colitis and attenuated growth suppression which correlated with induction of colon PPARγ, over-expression of PPARγ coactivator 1α and down-regulation of TNFα. n-3 PUFA supplementation alone or in a mixture with CLA resulted in an early disease onset because of a marked induction of the PPARδ-response gene uncoupling protein 3. CLA and n-3 PUFA synergistically up-regulated colon keratinocyte growth factor expression but n-3 PUFA blocked CLA-induced PPARγ activation. In this pig study n-3 PUFA failed to protect from IBD but accelerated colon regeneration and clinical remission by activating PPARδ.

Another group has studied n-3 PUFA-derived lipid mediators in the *fat-1* transgenic mouse with DSS-induced colitis (Hudert *et al*., 2006). These mice express the *Caenorhabditis elegans fat-1* gene coding for an n-3 fatty acid desaturase and thus are able to produce n-3 PUFA from n-6 PUFA. These mice had lower colon inflammation than wildtype mice, and this was associated with changes in n-3-derived anti-inflammatory mediators and a decrease in expression of genes involved in colon inflammation (NFκB, TNFα, inducible NO synthase and IL1β). Increased mRNA levels for genes important to membrane integrity, such as trefoil factor 3, the intercellular tight junction protein zonula occludens 1 and Toll-interacting protein (an inhibitor of the TLR pathway mediating inflammatory response) were also observed.

1.5 Conclusions and future perspectives

This review describes the complex interactions among dietary PUFA, inflammatory and immune responses and regulation of gene expression that underlie the multifactorial aetiology of IBD. Many studies have focused on the anti-inflammatory effects of n-3

PUFA as indicated by reports of histological assessments, circulating PUFA concentrations or *ex vivo* production of inflammatory mediators. A range of animal models of acute chemically-induced colitis show primarily anti-inflammatory effects of fish oil rich in n-3 PUFA. In contrast, the increased colitis associated with dietary fish oil suggests that the pathogenesis of spontaneous colitis may be different. Most of the studies reviewed here animals were fed higher fat diets (10% or more) compared to a normal laboratory rodent diet with 5% fat, and the use of fish oil means there was a mixture of various fatty acids and other lipidic constituents (e.g. vitamins) present in these diets. Thus, any observed anti-inflammatory or immunomodulatory effects have been assigned to a fatty acid mix rather than pure PUFA. It is difficult to interpret animal studies with impure dietary constituents (e.g. oils), so that the observed effect can not precisely be defined to the true bioactive component of the diet. The hypothesis that effects are due to n-3 versus n-6 competition and series-2 versus series-3 eicosanoids, is somehow flawed. The use of pure dietary components (e.g. pure fatty acid ethyl esters) is preferred.

A large part of the evidence regarding anti- and pro-inflammatory and immunomodulatory effects of n-3 and n-6 PUFA, respectively, has arisen from cell culture studies where single fatty acids have been added. In order to define effects of dietary PUFA on IBD, animal model systems with diets of well defined fatty acid composition and purity are needed. Few animal studies of IBD have applied genome-wide expression profiling to define the molecular mechanisms underlying the disease-phenotype when PUFA diets are fed. Such studies should incorporate 'omics' tools (e.g. transcriptomics and proteomics) and knowledge derived from the human genome project to provide a more comprehensive understanding of the multiple pathways by which PUFA can regulate signalling and metabolic processes. Translating this complex amount of data represents a major challenge for nutrigenomics research. Once hub genes that are differentially expressed in response to PUFA have been identified by transcriptome analyses, other techniques (including chromatin immunoprecipitation and transactivation assays) can be applied in order to define direct targets of the PUFA-responding central genes.

1.6 Aim, approach and outline of the thesis

As presented in this introduction, there has been considerable research in human IBD and experimental colitis in linking genetic, immunologic, and environmental (e.g. diet, bacteria) components to the disease pathogenesis. The hypothesis of this thesis was that dietary n-3 PUFA EPA reduces and dietary n-6 PUFA AA induces colon inflammation in a genetically susceptible host and alters the bacterial profile during colitis, thus contributing to their effects on colon mucosal responses. The effects of dietary EPA and AA on inflammatory and immunomodulatory processes and changes in caecal bacteria community in the *Il10*$^{-/-}$ mouse model of colitis were investigated using genome-wide expression analysis and bacterial profiling.

To be able to study the effect of specific food components on colitis development, it is important to define the time at which colitis is developing. Bacterial inoculation of conventionally housed *Il10*$^{-/-}$ mice on the C57BL/6J background produces a more consistent colon inflammation and may also accelerate colitis development in these mice compared to a previous study of conventional non-inoculated C57BL/6J *Il10*$^{-/-}$ mice (Kennedy *et al*., 2000; Roy *et al*., personal communication). The aim of the time-course study in **Chapter 3** was to characterise the onset and progression of colon inflammation in these mice at specific time points using histopathological assessment in combination with transcriptomics and proteomics to define expression profiles before and after onset of colitis.

Based on the lack of effect of the n-9 monounsaturated fatty acid OA on inflammatory mediators, it remains to be confirmed for studies using transcriptomics and proteomics that OA is an appropriate control fatty acid for dietary PUFA intervention studies. The hypothesis of the next study was that whole transcriptome and proteome profiles associated with colon inflammation of *Il10*$^{-/-}$ mice (relative to C57 mice) fed a standard AIN-76A diet clustered with that of *Il10*$^{-/-}$ mice (relative to C57 mice) fed an OA ethyl ester-enriched AIN-76A diet. **Chapter 4** describes the similarities in gene and protein expression profiles in the colon of *Il10*$^{-/-}$ mice on a AIN-76A or OA diet compared to their C57 counterparts on the same diet. A comparison and clustering analysis between transcriptomic and proteomic profiles was performed to find overlaps between both profiles.

Based on literature evidence, it was hypothesised that the dietary n-3 PUFA EPA may reduce, and dietary n-6 PUFA AA may aggravate colon inflammation. **Chapters 5** and **6** describe the effects of dietary EPA and AA on colon inflammation in the *Il10*$^{-/-}$ mouse model using histopathological and transcriptome analysis. Both fatty acids were incorporated into the base diet in the form of a pure ethyl ester.

The time-course and PUFA intervention studies were also used to investigate the hypotheses that the lack of IL10 protein may cause changes in intestinal metabolic and regulatory processes and environment which in combination with the genetic defect may alter bacterial communities not only before the onset of colitis, but also with development of colitis. As n-3 and n-6 PUFA can reduce or induce inflammatory processes, respectively, they may also alter the bacterial community during colitis, thus contributing to their effects on colon mucosal responses. The aims in **Chapter 7** were to identify differences in bacterial diversity between *Il10*$^{-/-}$ and C57 mice at 7 weeks (no colitis) and 12 weeks (severe colitis) of age, and to identify changes in bacterial diversity in response to dietary EPA and AA during colitis development.

As PPARα arose as a key gene from the genome-wide analysis in the colon of *Il10*$^{-/-}$ mice in response to dietary EPA (Chapter 5), it was hypothesised that there were PPARα-target genes among the up-regulated colonic genes in EPA-fed *Il10*$^{-/-}$ mice. **Chapter 8** aimed to identify putative new PPARα target genes in response to dietary PUFA in the colon and to better understand the regulatory role of PPARα during colon inflammation.

In **Chapter 9**, the main findings are summarised and discussed. An outline of the thesis and experimental chapters are shown in Fig. 1.11.

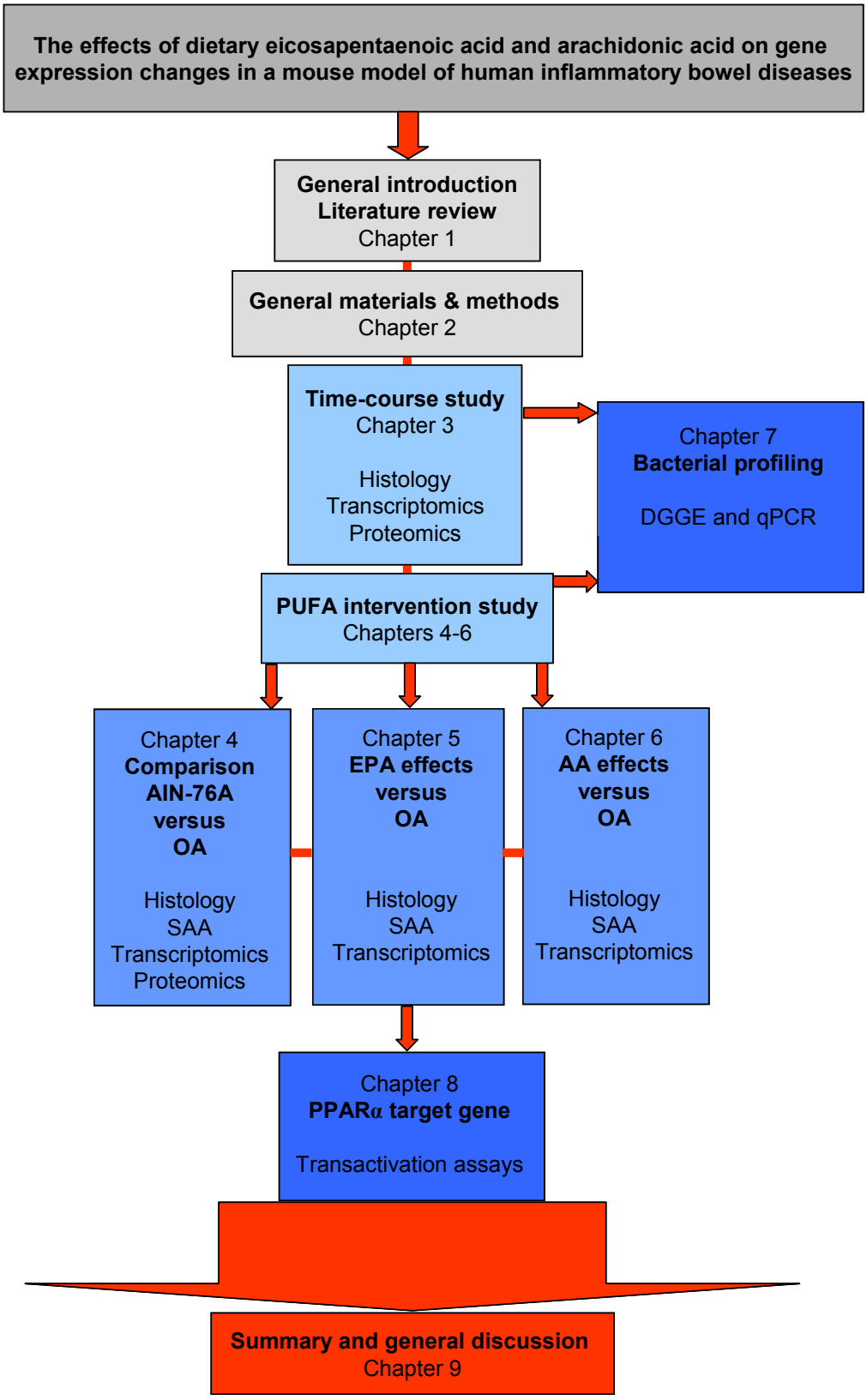


Fig. 1.11 Outline of the thesis and experimental chapters.

Chapter 2

General materials and methods

2.1 Animals

The experimental procedures for the time-course and PUFA intervention study were reviewed and approved by the AgResearch Ruakura Animal Ethics Committee in Hamilton, New Zealand, according to the Animal Protection Act and Animal Protection Regulations and amendments.

2.1.1 Housing

Male *Il10*$^{-/-}$ (C57BL/6J background, formal designation B6.129P2-*Il10*<tm1Cgn>/J) and C57 wildtype (C57BL/6J) mice were used. All mice were between 28 and 35 days of age at the start of the experiment, and were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). For convenience and consistency in reporting, their age was defined as 35 days or 5 weeks of age. Mice were individually housed in standard shoebox size cages (332 × 150 × 130 mm) containing untreated wood shavings (Hi Tech Security Disposals Ltd., Auckland, New Zealand) and a plastic tube for environmental enrichment. They were maintained under conventional conditions with a temperature of approximately 22°C, 50% relative humidity and a 12-hour light-dark cycle. Throughout the experimental period, dietary intake was estimated daily by collecting and weighing uneaten food. The dietary intake was adjusted daily to equal the mean amount of feed consumed by the *Il10*$^{-/-}$ mice on the previous day. Water was provided *ad libitum* and refreshed twice a week. All mice were weighed three times a week and carefully monitored for disease symptoms (weight loss, soft faeces, inactivity).

2.1.2 Induction of colitis

The inoculation procedure has been described previously (Roy *et al*., 2007). Four days after arrival, mice were inoculated by oral gavage (200 μL) with a mix of *E. faecalis* and *E. faecium* strains and commensal bacteria to obtain a more consistent and reproducible intestinal inflammation. It has been shown previously that a clinical isolate of *E. faecalis* can induce IBD, dysplasia, and carcinoma in *Il10*$^{-/-}$ mice (Balish & Warner, 2002). Six pure cultures of *E. faecalis and E. faecium* isolated from healthy calf and chicken (1.2×10^8 cfu/200 μL inoculum) were generously provided by Dr Adrian Cookson, from the Food Nutrition Genomics Team at AgResearch Grasslands in Palmerston North. To increase the likelihood of the enterococci and other bacteria to

colonise and induce inflammation, complex intestinal microflora were also collected from healthy, age-matched C57 mice. Briefly, 72 h prior to inoculation, the *Enterococcus* strains were subcultured from slope tubes onto fresh Slanetz & Bartley medium (Oxoid, Hampshire, UK) and incubated at 42°C for 48 h. A single colony from each culture was transferred to 5 mL of Todd-Hewitt Broth (Oxoid, Hampshire, UK) and incubated at 37°C for 24 h. All *Enterococcus* cultures were centrifuged (3000×g, 10 min, 4°C), resuspended in 5 mL sterile 1× PBS and pooled for inoculation (*Enterococcus* solution). Complex intestinal flora was derived from healthy, age-matched C57 mice which were humanely euthanised using CO_2 and cervical dislocation and the gastrointestinal tract (from stomach to caecum) removed. Digesta were collected from the intestine and caecum by washing with sterile 1× PBS, then suspended in a total of 30 mL PBS. After gentle inversion and a settling period of approximately 5 min, the complex intestinal flora suspension was mixed with the *Enterococcus* solution (6×10^7 cfu) in a 1:1 (v/v) solution to obtain the bacterial inoculum. All *Il10*$^{-/-}$ and C57 mice were inoculated with the same bacterial mixture to ensure that any difference between the bacteria community of the mouse genotypes was because of the intestinal inflammation in *Il10*$^{-/-}$ mice.

2.1.3 Experimental design

The experimental design of the time-course and PUFA intervention studies are shown in Fig. 2.1. For both studies, *Il10*$^{-/-}$ and C57 mice were fed experimental diets from 35 days or 5 weeks of age. In the time-course study, the diet was AIN-76A for all groups, while for the PUFA intervention study the diets are as shown in the figure. All mice were inoculated at 5 weeks of age with a mixture of pure *E. faecalis* and *E. faecium* strains and complex intestinal flora derived from healthy C57BL/6J mice raised under conventional conditions. Tissue sampling was performed at 7, 8.5, 10, 12 and 14 weeks of age for the time-course study and at 11 weeks of age for the PUFA intervention study.

The time-course study followed a 2×5 factorial and the PUFA intervention study followed a 2×4 factorial design with mouse strain and time or mouse strain and diet as factors, respectively. Multifactor experimental designs involve two or more factors. In

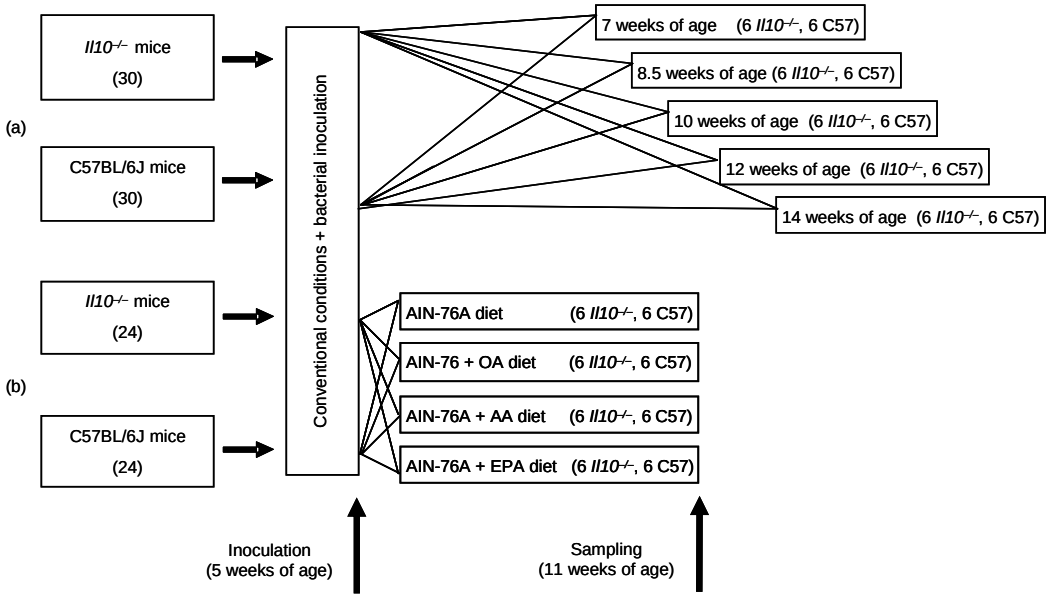


Fig. 2.1 Experimental design of the (a) time-course and (b) PUFA intervention study (adapted from Roy *et al*., 2007).

the present studies, one factor was genotype and the other factor was time or diet. Each factor can have two or more levels, e.g. in the present studies the levels represent either different time-points or diets (Yang & Speed, 2002). Factorial designs are used to study differences caused by single factors and also by the correlated effects between factors, the interaction effect and thus are more powerful than randomised block designs.

2.1.4 Fast-feed period and sampling

On the day prior to sampling, all mice were randomly divided into sampling days and groups. Mice were fasted overnight for 14 h, then feed was reoffered for 2 h and again removed for the 2 h immediately before mice were sacrificed (Fig. 2.2). This was done to minimise the variation in time between the last dietary intake and tissue sampling (Park *et al*., 1997).

Animals were euthanised by CO_2 asphyxiation and cervical dislocation. Blood was sampled via cardiac puncture (0.5 to 1 ml) and centrifuged to separate cells from plasma. The plasma was immediately frozen in liquid nitrogen and stored at –85°C for serum amyloid A (SAA) analysis. The intestine was quickly removed, cut open lengthwise, flushed with 0.9% sodium chloride to remove digesta, layed out on an ice-cold stainless steel tray and divided into duodenum, jejunum, ileum and colon. The latter intestinal sections were divided in half. The epithelial layer of the proximal first half of each intestinal section was scraped off and the mucus frozen at –85°C. The second half of the intestinal section was used for subsequent analyses. Of this half, one piece was stored at room temperature in 10% phosphate-buffered formaldehyde for histopathological assessment of inflammation. Another subsample of each intestinal section was also frozen in liquid nitrogen and stored at –85°C for gene and protein expression profiling.

2.2 Diet compositions

Mice were randomly assigned to either time-points (time-course study, 2×5 factorial design, n=6) or to dietary groups (PUFA intervention study, 2×4 factorial design, n=6). All diets used were based on a non-sterile, powdered AIN-76A-base diet and prepared in-house (Table 2.1). In the time-course study, all mice received AIN-76A diet. The experimental diets for the PUFA intervention study (AIN-76A, OA, EPA and AA diet)

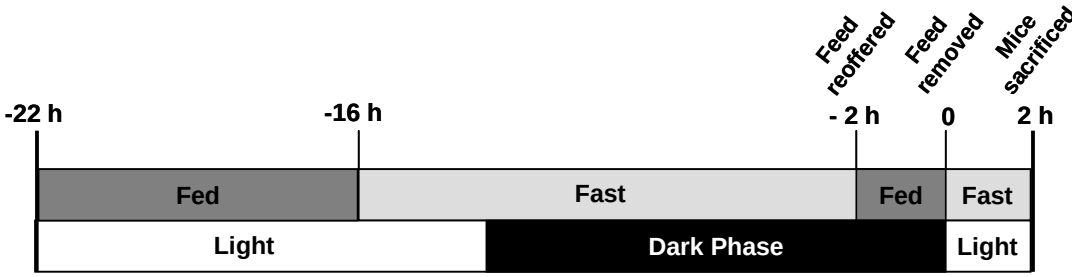


Fig. 2.2 Fast-feed regimen prior to tissue sampling (adapted from Park *et al*., 1997).

Table 2.1 Ingredient composition and determined chemical analysis of the experimental diets

	AIN-76A diet	OA diet	EPA diet	AA diet
Ingredient (%)				
Casein[1]	20	20	20	20
DL-methionine[2]	0.3	0.3	0.3	0.3
Sucrose[3]	50	50	50	50
Dextrin[4]	15	15	15	15
Arbocel, non-nutritive bulk[5]	5	5	5	5
Corn oil[6]	5	1	1	1
Linoleic acid (LA) 99%+	0	0.2	0.2	0.2
α-linolenic acid (ALA) 99%+	0	0.06	0.06	0.06
Oleic acid (OA) 99%+[7]	0	3.43	0	0
Eicosapentaenoic acid (EPA) 98%+[7]	0	0	3.43	0
Arachidonic acid (AA) 99%+[7]	0	0	0	3.43
Choline bitartrate[2]	0.2	0.2	0.2	0.2
AIN-76A Mineral Mix[8]	3.5	3.5	3.5	3.5
AIN-76A Vitamin Mix[8]	1	1	1	1
Ethoxyquin	0.01	0	0	0
TBHQ[9]	0	0.001	0.001	0.001
Analyte				
Gross energy (MJ/kg)	18.07	18.14	18.17	18.16
Nitrogen (%)	2.79	3.27	2.68	3.01
Lipid and fat-soluble compounds (%)	5.05	5.15	5.01	5.18
Fatty acids (%)[10]				
Palmitic acid (C16:0)	0.58	0.16	0.14	0.14
Stearic acid (C18:0)	0.13	0.05	0.04	0.04
Linoleic acid (C18:2n-6)	2.21	0.62	0.58	0.58
α-linolenic acid (C18:3n-3)	0.03	0.06	0.05	0.05
Oleic acid (C18:1n-9)	1.46	3.56	0.30	0.30
Arachidonic acid (C20:4n-6)	0	0	0	3.01
Eicosapentaenoic acid (C20:5n-3)	0	0	3.09	0
Other identified minor fatty acids	0.11	0.03	0.08	0.04

Both diets were prepared in-house using ingredients from the suppliers listed, and according to the compositions shown. [1]Alacid, lactic casein 30 mesh, NZMP Ltd., Wellington, New Zealand; [2]Sigma, Sigma–Aldrich Inc., St. Louis, MO, USA; [3]Caster sugar, Chelsea, New Zealand Sugar Company Limited, Auckland, New Zealand; [4]Wheaten cornstarch, Golden Harves, Primary Foods Ltd., Auckland, New Zealand; [5]Arbocell B600, J. Rettenmaier & Sohne GmbH+ Co, Rosenberg, Germany; [6]Tradewinds, Davis Trading, Palmerston North, New Zealand; [7] purified OA, EPA and AA ethyl esters as 3.7%; [8]Prepared in-house based on the AIN-76A diet formulation (Reeves *et al*., 1993); [9]TBHQ, Tertiary butylhydroquinone as antioxidant; [10]as analysed in the complete diets.

were formulated on the basis of the AIN-76A diet, and were as follows: (1) AIN-76A (5% corn oil), (2) AIN-76A (fat-free) + 1% corn oil + 3.4% OA, (3) AIN-76A (fat-free) + 1% corn oil + 3.4% EPA and (4) AIN-76A (fat-free) + 1% corn oil + 3.4% AA. OA, EPA or AA were added as purified ethyl ester oils to the fat-free AIN-76A powder. The 1% corn oil was supplemented with purified LA and ALA to prevent any essential fatty acid deficiency (Anonymous, 1977). Appropriate amounts of antioxidants were added to prevent lipid peroxidation in the ethyl ester oils. One reason for using 5% fat diets (normal fat content in the AIN-76A diet) was to compare to other mouse studies testing the effect of dietary components on the intestinal transcriptome associated with inflammation by performing meta-analysis (outside the scope of this thesis). A diet with a fat concentration of 5% has been shown to be adequate although may not support maximal growth and reproduction, as satisfactory for absorption of carotene and vitamin A and provides sufficient essential fatty acids (Nutrient Requirements of Laboratory Animals, 1995). The OA, EPA and AA diets were isocaloric, contained equal amounts of protein and varied only in lipid composition, each containing 5% fat (w/w) as in the AIN-76A base diet. Extraction and analysis of fatty acids in the OA, EPA and AA diets were performed before the study began to confirm lipid content and fatty acid composition in accordance with methods described previously (Sukhija & Palmquist, 1988; Wu *et al*., 1994). The diets were aliquoted in daily portions, sealed airtight in plastic bags and stored in containers at –85°C to reduce lipid oxidation. The diets were supplied fresh daily (3.5 to 4.0 g/mouse). Ethyl esters of OA, AA, linoleic and α-linolenic acid (all 99% purity, NuCheck Inc., Elysian, MN, USA) and EPA (98% purity, Brainfats Biotechnology Ltd, Auckland, New Zealand) were used. The ethyl ester of OA was used as the control fatty acid in an OA-enriched diet and was substituted by EPA and AA in the PUFA-enriched diets.

2.3 Histology

Local inflammation was evaluated by histopathological assessment of the intestinal sections and systemic inflammation levels by analysis of plasma serum amyloid A (SAA).

2.3.1 Tissue processing and staining

Tissue processing, staining and the histopathological assessment of inflammation for the time-course and the PUFA intervention study were performed by Dr Shuotun Zhu, a pathologist at The University of Auckland. Briefly, after fixation of the intestinal sections in 10% phosphate-buffered formaldehyde, the sections were placed into small plastic cassettes which hold the tissue while being processed into a paraffin block to facilitate thin sectioning. The water was removed from the wet fixed tissues by dehydration with a series of ethanols (70% to 80% to 95% to 100%) with an incubation time of 1 h for each step. The next step involved the removal or clearing of dehydrant with xylene (1 h) being mixable with the embedding medium (paraffin). Last, the tissues were infiltrated with paraffin. The tissues were removed from each cassette, placed into a metal tray and embedded with molten paraffin. Thereafter, 5 µm-thin sections were cut from the paraffin blocks using a microtome (sharp knife with mechanism to advance the block to the knife). The cut sections were floated on a warm water bath to smoothen the tissue section and placed on a glass microscopic slide. The slides were dried in an oven overnight to help the tissue adherence to the slide.

For the staining with haematoxylin and eosin, the sections were deparaffinised with xylene twice for 10 to 20 min, rehydrated with 100% and 95% ethanol for 1 to 2 min and rinsed in tap water and distilled water. Then the sections were stained with haematoxylin for 3 to 5 min, rinsed in tap water and placed into 1% HCl in 70% alcohol, observed under the microscope for the extent of differentiation. The sections were rinsed in running tap water for 15 min and placed into 95% ethanol before staining with eosin for 1 to 4 min. The stained sections were dehydrated twice in 95% and 100% ethanol, cleared twice in xylene and enclosed with mounting medium (Permount). The sections were covered with a thin plastic to preserve the tissue and for viewing under the microscope.

2.3.2 Histopathological assessment

The haematoxylin and eosin stained intestinal (duodenum, jejunum, ileum and colon) sections were evaluated by the pathologist who had no knowledge of the experimental protocols. The pathologist looked for indications of inflammation, including inflammatory cell infiltration (monocytes, neutrophils, fibrin exudation and

lymphangiectasis), tissue destruction (enterocyte or crypt loss, ballooning degeneration, oedema and mucosal atrophy) and tissue repair (hyperplasia, angiogenesis, granulomas and fibrosis). A rating score between 0 (no change from normal tissue section) and 3 (lesions involving most areas and all layers of the tissue section including mucosa, muscle and omental fat) was given for each aspect of inflammatory cell infiltration, tissue destruction, and tissue repair. The histological injury score (HIS) was calculated as: HIS=(inflammatory cell infiltration score ×2) + tissue destruction score + tissue repair score. Monocyte and neutrophil infiltration represented the main characteristic of the observed intestinal inflammation; therefore the sum of inflammatory cell infiltration was multiplied by 2 to give more weight to this value. A total intestinal HIS less than or equal to 3 was regarded as no inflammation, more than or equal to 7 as severe inflammation and intestinal HIS between 3 and 7 as moderate inflammation.

2.3.3 Serum amyloid A (SAA)

SAA, a systemic inflammatory marker was measured in the plasma to compare to the local inflammatory features using a murine specific SAA kit (Tridelta Development Ltd, Maynooth, County Kildare, Ireland), according to the manufacturer's instructions. Briefly, 50 µL of anti-SAA/streptavidin-horseradish peroxidase conjugate and 50 µL of diluted plasma sample or standard (ranging from 0 to 0.950 µg/mL) were added to each well (coated with a monoclonal antibody specific for SAA). After 1 h incubation (37°C) the plates were washed four times with 400 µL diluted buffer to wash off all unbound material. Tetramethylbenzidine substrate solution (100 µL) was added to produce the colour change in the reaction and the plates were incubated at room temperature for 5–10 min before adding 100 µL of stop solution. The absorbance of each well was read at 450 nm with 630 nm as reference using a plate reader (VersaMax, Molecular Devices Corp., Sunnyvale, Calif., USA). The intensity of the colour produced was proportional to the concentration of SAA present in the original sample.

2.4 Transcriptomics

Microarray technology is an extension of hybridisation-based methods used to identify and quantify nucleic acids in biological samples (Alwine *et al*., 1977; Kafatos *et al*., 1979). Microarrays utilise gene-specific probes that represent individual genes and are arrayed on an inert substrate. One of the most commonly applied array platforms

manufactured by Agilent (Wolber *et al*., 2006) was used. The experimental procedure involves isolation of RNA from the biological sample, followed by labelling with a fluorescent dye marker. After hybridisation to the array and subsequent washing steps, the arrays are scanned for fluorescence signal intensities to determine the extent of hybridisation to each gene-specific probe. Then the data are normalised and statistical tests applied to identify differences in gene expression between the experimental samples. In Fig. 2.3 the microarray experimental steps are illustrated.

2.4.1 Design of the microarray experiment

High-density oligonucleotide microarrays from the Agilent platform were used representing a two-colour array system. Short oligonucleotide sequences (probes of 60-mer length) representing a single gene or family of gene splice-variants are printed directly onto the array surface instead of intact sequences (Pease *et al*., 1994). For both mouse studies, the microarray experiments were performed using a reference design which is illustrated in Fig. 2.4. Reference designs are widely used, especially when comparing more than two treatments and they have the advantage of relatively easy analysis. The target sample is hybridised against a universal reference sample, thus comparisons are to a common reference (Yang & Speed, 2002). This is an indirect gene expression comparison between target and reference sample. The reference sample should contain transcripts for all probes on the array to avoid loss of information for that probe, because non-represented probes will have a value of zero for the reference channel (Sterrenburg *et al.*, 2002). The reference can be used as a vehicle to analyse and compare between the treatment samples. In the case of a limited number of arrays and resources and when the reference measurements are not of interest, a single orientation design in which the target sample is always labelled with one dye and the reference always with the other dye, is preferred. In that case, performance of a dye-swap for each hybridisation is not necessary (Sterrenburg *et al.*, 2002). It is impossible to separate bias of each dye from the sample variation, but the dye bias affects each sample equally and thus will not affect comparisons between the treatment samples. This design type has the advantage of easy and more robust analysis and interpretation (Dobbin *et al.,* 2003).

The focus of the present experiments was on the effects of different points in time or diets in a disease model, therefore the reference design has been chosen. The common

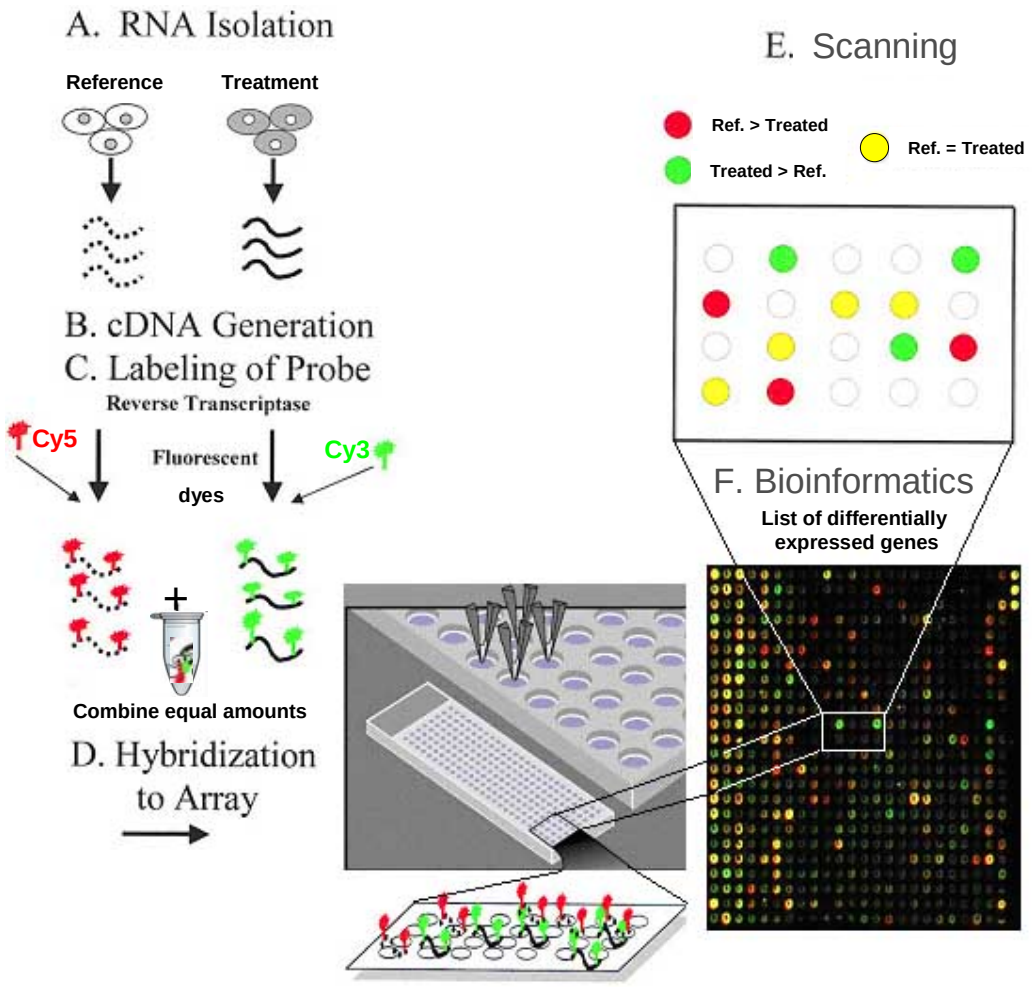


Fig. 2.3 Schematic representation of the steps performed in the microarray experiment. Adapted from the Food and Agriculture Organization (FAO) of the United Nations, Corporate Document Repository.

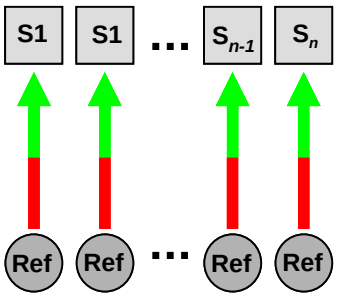


Fig. 2.4 Illustration of reference design used in microarray experiments (adapted from Yang & Speed, 2002). Single orientation design in which the treatment sample (S, n=number of samples) for instance is always labelled with Cy3 (green) and the reference sample (Ref) always with Cy5 (red).

reference RNA was made using an equal amount of total purified RNA extracted from several organ tissues (small intestine, colon, kidney, liver and fetuses) of normal, healthy mice to include most of the transcripts for the probes that are present on the array. The treatment samples were always labelled with green-fluorescent dye Cyanine3 (Cy3) and hybridised with the common reference always labelled with red-fluorescent dye Cyanine5 (Cy5). The logarithm (usually to the base 2) of the ratio of the measured red (R) and green (G) fluorescence signal intensities for each spot gives the relative abundance of the corresponding probe in the two nucleic acid samples ($\log2(R/G)$).

In the time-course study, colon RNA of individual mice was hybridised with the reference RNA using 15 microarrays. For this microarray experiment, the 7 weeks (no colon inflammation) and 12 weeks (severe colon inflammation) of age time points were selected with three to four biological replicates per experimental group (mouse strain/time point). In the PUFA intervention study, an equimolar pool of colon RNA extracts from two to three mice per experimental group (mouse strain/diet) were prepared and hybridised with the reference RNA onto 16 microarrays. The decision to pool was mainly based on reducing the number of arrays, but also to minimise individual variation.

2.4.2 RNA isolation

Total RNA from colon tissue was isolated by homogenising the samples in TRIzol (Invitrogen, Auckland, NZ; a modification of the acid guanidinium thiocyanate-phenol-chloroform extraction method) according to the manufacturer's instructions. Briefly, the colon sample homogenised in TRIzol was centrifuged and chloroform added to the supernatant to form an upper aqueous layer (RNA), middle white layer (DNA) and lower pink layer (protein). The upper aqueous layer was removed and 100% isopropanol added to precipitate the RNA. After incubation, centrifugation and removal of the supernatant, the RNA pellet was washed with 75% ethanol, air-dried and resuspended in 20 μL RNase/DNase-free water. RNA levels and subunit ratios were quantified with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity assessed with an RNA 6000 Nano Labchip kit using the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). Isolated RNA was purified using RNeasy Mini kit with spin columns (QIAGEN, San

Diego, CA, USA). Only total RNA with an OD 260/280 ratio greater than 2.0, a Bioanalyser 28s/18s peak ratio greater than 1.2 and an RNA integrity number (RIN) superior to 8 was used for microarray hybridisation. The optical density or OD ratio of 260/280 indicates the purity of the RNA sample. Contamination with protein (or phenol) which shows a maximum absorbance at 280 nm can reduce the 260/280 ratio. Nucleic acid has a maximum absorbance at 260 nm (Cantor & Schimmel, 1980). The RIN, a software tool of the Bioanalyser, is an extension of traditional measurement of the ratio of ribosomal 28s and 18s bands and reflects the presence or absence of degradation products in the RNA sample. The quality of eukaryotic total RNA is classified from 1 to 10, with 1 being the most degraded and 10 the most intact RNA profile (Schroeder *et al*., 2006).

2.4.3 Synthesis of labelled cRNA, microarray hybridisation and scanning

The Low RNA Input Linear Amplification kit (Agilent Technologies) was used for cDNA and cRNA synthesis according to the manufacturer's instructions. Briefly, 500 ng of purified total RNA from each individual or pool sample was amplified, reverse transcribed *in vitro* to cDNA using T7-polymerase and subsequently labelled with either Cy3-CTP (treatment) and Cy5-CTP (reference) dyes (10mM; Perkin-Elmer/NEN Life Science (NEL 580, 581), Boston, MA, USA). Dye incorporation was monitored spectrophotometrically using a Nanodrop ND-1000. The labelled cRNA (750 ng of sample combined with 750 ng of reference cRNA and control probes to ensure correct hybridisation) was hybridised to Agilent Technologies 44k (G4122-60510) mouse 60mer oligonucleotide arrays using the *in situ* hybridisation plus kit (Agilent Technologies) and following the manufacturer's protocols. These arrays comprise 44,290 probe sets representing 33,994 unique genes. Control probes were hybridised with RNA spike-ins and the hybridisation measurements between both was used to normalise to the measurements for the target probes. After hybridisation, the slides were washed in solutions I, II and III (Agilent Technologies), air-dried and scanned immediately with a GenePix Professional 4200A scanner (Molecular Devices Corp.). Spots were identified and quantified using GenePix 6.0 software (Molecular Devices, Corp.). Irregular features were identified by plotting the high and low intensities for

each slide against each other, for both the green and red dyes. Outliers were flagged as poor quality spots and eliminated before analysis.

2.4.4 Microarray analysis

Quality assessment and statistical analysis of the microarray data were performed using linear models for microarray analysis (limma) within the Bioconductor framework (Smyth, 2005). Data quality was assessed on diagnostic plots (boxplots and density plots) and spatial images generated from the raw (non-processed) data, but no background correction was necessary due to homogeneous hybridisation (Smyth & Speed, 2003). Arrays that passed the quality control were included in the analyses. Intensity ratios for all microarray spots were normalised using a local linear regression analysis (LOESS) to remove the effect of systematic variation in the microarrays. The data were log-transformed before analysis and the mean difference between treatments calculated resulting in a log-ratio for each probe. MA plots (plotting log-ratio *vs.* intensity of each spot after data normalisation) were generated to check that there was no dependence of the log-ratio on the intensity for any slide. For each probe the significance of the log-ratio was determined by calculating one moderated t-statistic per probe using an empirical Bayes approach (Smyth, 2004). Then the probability values were corrected for multiple testing using the Benjamini-Hochberg correction and a false discovery rate (FDR, q-value) calculated (Benjamini & Hochberg, 1995). Probes with a $q<0.05$ (time-course study) or a moderated $P<0.01$ (moderated P-value is without adjustment for multiple testing; PUFA intervention study) were considered to be differentially expressed between the experimental groups. Log-ratios have been converted to fold change in the gene lists.

2.5 Validation of microarray results by quantitative real-time PCR

The assessment of gene expression levels by using microarray technology is widespread in biology. The validation of microarray data using independent mRNA quantitation techniques including quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) remains a desirable and critical element of a microarray experiment (Dallas *et al*., 2005).

Total RNA from the same individual or pool samples as used for the microarrays was reverse transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, for each RNA sample, the following components were mixed in a RNase- and DNase-free PCR tube: 0.6 µg total RNA, 1 µL oligo$(dT)_{18}$ primers (50 µM) and PCR-grade water to complete the volume to 13 µL. The mixes were incubated at 65°C for 10 min to denature RNA secondary structures if present. Then a mastermix was prepared containing the following components: 4 µL 5× transcriptor RT reaction buffer, 0.5 µL protector RNase inhibitor (40 U/µL), 2 µL deoxynucleotide mix (10 mM each) and 0.5 µL transcriptor reverse transcriptase, and 7 µL of the mix added to the previous sample tubes to get 20 µL final volume. These mixes were incubated at 55°C for 30 min, followed by the inactivation of transcriptor reverse transcriptase by heating to 85°C for 5 min.

The resulting single-stranded cDNA was amplified in a PCR using sequence-specific primers. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, Mannheim, Germany) was used for detection and quantification of the genes (up- and down-regulated or un-changed genes) according to the manufacturer's protocol on a LightCycler 480 (Roche Diagnostics, Mannheim, Germany). Each 20 µL PCR contained 5 µL of 50× diluted cDNA and 15 µL of PCR mix (containing 1 µL of each 10 µM primer, 10 µL of 2× ready-to-use master mix and 3 µL PCR-grade water). The PCR amplification conditions were 95°C for 5 min pre-incubation, then 35 cycles at 95°C for 15 s, 63°C for 10 s and 72°C for 15 s. Melting curve analyses were performed by increasing the temperature 1°C/s from 65 to 95°C, with continuous fluorescence acquisition.

Cycles to threshold values were obtained in triplicate for each sample using a 96-well plate. A negative control (cDNA template replaced by PCR-grade water) was included in each run to assess the overall specificity of the PCR assay. Samples were standardised to *Canx* and/or cyclophilinA. Standard curves for all selected genes and reference genes were generated using serial dilutions of a pool of cDNA from all samples. LightCycler 480 Relative Quantification Software (Roche Diagnostics, Mannheim, Germany) was used to calculate PCR efficiencies of each set of primers and

mRNA expression levels for each sample based on the appropriate standard curves and normalised ratios (target/reference).

Statistical analysis of the expression differences between treatment and control groups were assessed using ANOVA in GenStat (10th edition, VSN International, Hemel Hempstead, UK, 2007), on log-transformed data where necessary in cases of unequal variances.

2.5.1 Primer sequences

The primer sequences used for qRT-PCR in Chapters 3 to 6 are listed in Table 2.2. First a panel of reference genes such as calnexin (*Canx*), cyclophilinA, hypoxanthine guanine phosphoribosyl transferase1 (*Hrpt1*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), was evaluated amongst all experimental groups. *Canx* was chosen as suitable normalisation reference gene. In Chapter 5, cyclophilinA was used as a second reference gene. *Canx* and cyclophilinA gene expression levels were shown to be consistent within murine intestinal samples (Dommels *et al*., 2007; Bünger *et al*., 2007). Most of the primer sequences were obtained from the PrimerBank at Harvard University (Wang & Seed, 2003), except *Ppara* and *Ces2* which were designed using Primer3 software (Rozen & Skaletsky, 2000). Primers for *Il1b* and *Srebf1* were selected from Universal Probe finder version 2.43 (Roche) and *Canx* was previously described (Dommels *et al*., 2007). Annealing conditions for each primer pair were optimised and amplicons sequenced at "The Allan Wilson Centre Genome Service" (Massey University, Palmerston North, NZ) to confirm identity (>98% sequence homology). Specificities of the PCR reactions were verified in melting curve analyses and by electrophoresis in 1% agarose gels.

2.6 Proteomics

Sample collection and preparation are a challenge in proteomic studies in order to avoid protein turnover, modifications or hydrolysis (Tao, 2009). The following paragraphs describe the process of total protein isolation and purification, protein identification and analysis by liquid chromatography and mass spectrometry (LC-MS).

Table 2.2 Primer sequences used for qRT-PCR

Gene symbol	GenBank accession [PrimerBank ID]	Forward primer	Reverse primer	Amplicon size (bp)
Abcb1a	NM_011076.1 [6755048a1]	CAGCAGTCAGTGTGCTTACAA	ATGGCTCTTTTATCGGCCTCA	205
Aldh1a1	NM_013467.2 [7304881a1]	ATACTTGTCGGATTTAGGAGGCT	GGGCCTATCTTCCAAATGAACA	192
Ces2	NM_145603.1	CACGGAACCAACTACATAAC	AGACATAGGGAAGGAAGACA	362
Fabp2	NM_007980.2 [6679737a1]	GTGGAAAGTAGACCGGAACGA	CCATCCTGTGTGATTGTCAGTT	117
Igfbp5	NM_010518.2 [6754312a2, 6754312a3]	TTGCCTCAACGAAAAGAGCTAC	CACAGTTGGGCAGGTACACAG	362
Il1b	NM_008361.3 [6680415a1], Roche universal probe no. 5	GCAACTGTTCCTGAACTCAACT	GATACTGCCTGCCTGAAGCT	191
Mmp13	NM_008607.1 [6678896a1, 6678896a3]	CTTCTTCTTGTTGAGCTGGACTC	GTTCGTTTGGGACCATTTGAGT	331
Ppara	NM_011144.2	GGTGTGTATGAAGCCATCT	AGCCACAAACAGGGAAAT	206
Srebf1	NM_011480.3 [Roche universal probe no. 83, 62]	TTACTCGAGCCTGCCTTCA	CAGTGTTTGTTCTAGGGGCTG	182
Sult1a1	NM_133670.1 [5420463a3]	CAGCCCCACGGATCATTAAG	ACGACCCATAGGACACTTTC	216
Reference gene				
Canx	NM_007597.2	CTGAAGGCTGGCTAGACGACGAA	GCTGACTCACACTTGGGGTTGG	130
CyclophilinA	NM_008907.1	CACTGCCAAGACTGAATG	CTACAGAAGGAATGGTTTGA	200

Primers for *Abcb1a*, *Aldh1a1*, *Fabp2, Igfbp5*, *Il1b*, *Mmp13*, *Srebf1 and Sult1a1* were selected from PrimerBank (Wang & Seed, 2003), for *Ppara*, *Ces2* and *Cyclophilin* designed using Primer3 software (Rozen & Skaletsky, 2000), for *Il1b* and *Srebf1* selected using Universal Probe finder version 2.43 (Roche) and *Canx* (previously described by Dommels *et al*., 2007) was used as reference gene. *Abcb1a* - ATP-binding cassette subfamilyB member1, *Aldh1a1* - aldehyde dehydrogenase1 family member A1, *Ces2* - carboxylesterase2, *Fabp2* - fatty acid binding protein2, *Igfbp5* - insulin-like growth factor binding protein5, *Il1b* – Interleukin1 beta, *Mmp13* – matrix metallopeptidase13, *Ppara* - peroxisome proliferator-activated receptor alpha, *Srebf1* – sterol regulatory element binding protein1, *Sult1a1* - sulfotransferase family 1A phenol-preferring member1, *Canx* – calnexin and *Cyclophilin* – CyclophilinA or peptidylprolyl isomeraseA.

2.6.1 Protein isolation

The protein-containing lower layer of the same TRIzol processed sample from which RNA was derived was used for protein isolation. Briefly, 100% ethanol was added to the lower layer to remove any traces of DNA. After centrifugation, the supernatant from each sample was combined with 100% isopropanol to precipitate the protein. The samples were incubated, centrifuged and the supernatant removed. The pellets were washed trice in 0.3M guanidine hydrochloric acid in 95% ethanol, incubated and centrifuged. The protein pellet was resuspended with 0.3M guanidine hydrochloric acid in 95% ethanol and stored at –20°C.

The stored protein pellets were processed through two washes and a final ethanol wash, then allowed to air-dry. Resolubilisation buffer (7 M urea (BioRad), 2 M thiourea (Sigma), 4% CHAPS (BioRad), 40 mM Tris (Invitrogen)) was added and samples incubated at 22°C, 600 rpm, overnight in a Thermomixer (Eppendorf). After centrifugation supernatants were used to determine protein concentration using the Bradford protein assay with BSA as a standard (Bradford, 1976). Volumes of samples that required pooling were calculated to produce total aliquots of 50 µg per treatment. Gels were run as duplicate biological replicates using the same pooled samples as described for the microarray design. The 50 µg total protein from treatment and control samples were labelled with 200 pmol of Cy5 and Cy2 dyes (GE Healthcare), respectively as described by the manufacturer. The labelled treatment and control samples were combined to make 100 µg protein and run on Immobiline Drystrips (GE Healthcare, 18 cm, pH 3 to 11 non-linear) in an equal volume of 7 M urea, 2 M thiourea, 4% CHAPS, few grains of Bromophenol Blue (Sigma), 2% pH 3-11 NL IPG buffer (GE Healthcare) and 65 mM DTT buffer (Sigma) to separate the proteins in the first dimension. The first dimension, equilibration and second dimension were performed as previously described (Barraclough *et al.*, 2004). Precision Plus protein standard plugs (BioRad) were used as molecular weight markers. Immediately after electrophoresis, the gels were washed in double-distilled H_2O and visualised using a Typhoon (TM) 9400 imager (GE Healthcare). The Cy2 images were scanned using a 488 nm laser and a 520 nm of band pass (BP) 40 emission filter, whereas Cy5 images were scanned using a 633 nm laser and a 670 nm BP 30 emission filter. All gels were scanned at a resolution of 200 µm, and analysed using Phoretix 2D Evolution software

(Non-Linear Dynamics). Staining was performed by placing gels into modified Neuhoff colloidal Coomassie stain (17% ammonium sulphate, 3% phosphoric acid, 34% methanol, 0.1% Coomassie G-250) (Neuhoff *et al*., 1988), after which gels were dried on glass plates at room temperature under cellophane and stored.

Differentially expressed proteins were only flagged as significant if the fold abundance change for each biological replicate changed in the same direction and either each replicate had a value either <-1.5 or >1.5 fold, or one replicate had a value <-2 or >2 fold difference and the other replicate <-1.3 or >1.3. Significant protein spots were excised from the dried gels, rehydrated in deionised water and digested with trypsin. Briefly, 25 mM ammonium bicarbonate in 50% acetonitrile was added to the gel pieces which were then incubated in a Thermomixer (1400 rpm, 22^{o}C, 10 min) with up to 2 repeats of this step depending on the density of the original staining. The gel pieces were then dried in a vacuum centrifuge (Speedyvac) and rehydrated at room temperature in a trypsin/HCl mix (20 µL trypsin of a stock made from 25 µg vial of Roche modified trypsin (sequencing grade) in 50 µL 1 mM HCl (BDH), 200 µL NH_4HCO_3 (Sigma) pH 8.0, 10 µL 100% acetonitrile (BDH)). Following overnight incubation in the Thermomixer (37^{o}C, 600 rpm), 30 µL of 5% formic acid (Pierce) in 50% acetonitrile (Sigma) were added to the gel pieces which were sonicated for 5 min. The samples were briefly centrifuged and the supernatent removed. This was repeated twice. Two additional extractions in formic acid/acetonitrile were repeated and pooled. Recovered peptides were concentrated by reducing the final volume of the extracts to approximately 10 µL in a vacuum centrifuge, followed by resuspension to a volume of 20 µL with formic acid/acetonitrile. The peptide solutions were stored at –20ºC until MS was performed.

2.6.2 LC-MS analysis of peptides

Tryptic peptides were separated and analysed using an Ettan™ multi dimensional liquid chromatography system (GE Healthcare, Uppsala, Sweden) coupled to a LTQ linear ion trap MS with a nanospray ionisation interface (ThermoQuest, Finnigan, San Jose, CA, USA). Samples (2 µL) were injected onto a 300 µm ID x 5 mm trap column (Zorbax 300-SB C18) for in-line desalting and separated on a nanoscale reverse phase chromatography (RPC) column 75 µm ID x 150 mm, 3 µm, (LC Packings, San

Francisco, CA, USA) in high-throughput configuration at 280 nL/min with a linear gradient from 0 to 60% B over 50 min (A: 0.1% formic acid; B: 84% acetonitrile and 0.1% formic acid). Data was acquired using a top 3 experiment in data-dependent mode with dynamic exclusion enabled. A data-dependent loss scan function is applied in tandem MS (MS/MS) for identification of post-translational modifications such as phosphorylation and glycosylation that are found in low abundance within cells. The identification for less intense peptides is limited by inadequate peptide fragmentation and leads to a loss of sequence ion information. The data-dependent neutral loss algorithm triggers MS scans on MS/MS fragment ions after detection of a specific, prominent neutral loss ion, resulting in robust peptide fragmentation and peptide identification. Basically, the top 3 MS method does a full scan looking at the top 3 most intense peptide ions, fragments (MS/MS) the most intense, followed by the second most intense and then the third most intense ions.

2.6.3 MS/MS data processing and analysis

MS/MS data were analysed using TurboSEQUEST protein identification software (Yates *et al*., 1995; Eng *et al*., 1994) and spectra were searched against the NCBI *Mus musculus* database. Modifications were set to allow for the detection of oxidised methionine (+16) and carboxyamidomethylated cysteine (+57). The modification of cysteine to cysteine (+57) is necessary to be able to digest the protein and analyse the cysteine. Double bonds are cleaved, and to stop them reforming they are alkylated by an alkylating agent which adds 57 to the molecular weight of cysteine. This difference is taken into account for analysis as all cysteines are treated as if they are +57. Methionine oxidation (+16) can occur naturally in the gel. As some methionines can be oxidised and some not, and the analysis will consider methionine to be either unmodified or modified and takes both possible molecular weights into account. The criteria used for a positive peptide identification for a doubly-charged peptide were a correlation factor greater than 2.0, a delta cross-correlation factor greater than 0.1 (indicating a significant difference between the best match reported and the next best match) and a high preliminary scoring. For triply-charged peptides the correlation factor threshold was set at 2.5. All matched peptides were confirmed by visual examination of the spectra.

2.7 Bioinformatics analysis

Bioinformatics tools are necessary in trancriptomics, proteomics or other 'omics' technologies to manage and analyse the huge amount of data and to compare and correlate gene and protein expression profiles. The following paragraphs describe Ingenuity pathway analysis, EASE and FUNC which are publicly available bioinformatics programs used in Chapters 3 to 6.

2.7.1 Ingenuity pathway analysis (IPA)

Ingenuity Pathway Analysis software (IPA, Version 7.0, Ingenuity Systems Inc., Redwood City, CA, USA) was used for pathway, functional and network analyses of differentially expressed genes and proteins in the datasets. The data files containing gene and protein identifiers (gene and protein accession number) and the corresponding changes in expression levels (fold change (FC), moderated *P*-value or q-value) were uploaded into the IPA program. In IPA, probes from the microarray dataset that satisfied the cut-off criteria for FC≥1.3 (up- or down-regulated) and moderated $P<0.01$ (PUFA intervention study), and FC≥1.5 (up- or down-regulated) and $q<0.05$ (time-course study) were considered for analysis. The differentially expressed proteins (≥1.5-fold difference) from the LC-MS/MS analysis were uploaded into IPA and no further cutoffs were applied in IPA. Each gene and protein was mapped to its corresponding gene and protein in the Ingenuity Pathway Knowledge Base.

Canonical pathway analysis determined which signalling and metabolic pathways were most significant to the experimental dataset. The significance of the association between the genes/proteins in the dataset and the canonical pathways was estimated in two ways: (1) The ratio of the number of genes/proteins from the dataset that mapped to the canonical pathway divided by the total number of genes/proteins mapping to the same canonical pathway; (2) Fisher's exact test was used to calculate the probability of the association between the genes/proteins in the dataset and the canonical pathway. Either the most significantly regulated (e.g. top 5 or top 10) gene pathways were considered or pathways with $P<0.01$, where expression levels of at least 10% of the genes from a particular pathway changed in the dataset.

Functional analysis identified biological functions and diseases that were most relevant to the dataset. Genes/proteins from the dataset that met the FC and *P*-value cut-offs and were associated with biological functions and diseases in the Ingenuity Pathway Knowledge Base were considered for the analysis. Fisher's exact test was used to calculate the probability for each biological function and disease process that were most relevant to the experimental dataset.

IPA was also used to generate biological interaction networks to address the regulatory relationships that exist between the genes/ proteins in the dataset. The Ingenuity Pathway Knowledge Base was queried for interactions between differentially expressed genes/proteins (focus genes/proteins) and all other gene/protein objects stored in the knowledge base, and generates networks. The interactions were supported by literature findings available online. IPA computed a score for each network according to the user's significance cut-offs for genes and/or proteins. The score was derived from the probability of the focus genes/proteins being found together in a network as a result of random chance.

2.7.2 FUNC analysis

For the FUNC approach, genes with changed expression level (moderated $P<0.01$, $FC\geq1.3$) in each comparison were analysed for over-represented gene ontology (GO) terms using the hypergeometric test and subsequent refinement provided by the FUNC package (Prüfer *et al*., 2007). GenBank IDs were used as the basis for the analysis, and duplicate GenBank IDs were consolidated into one count. Only GO terms annotated to at least 10 genes were considered. GO terms with FDR or $q<0.05$ according to the hypergeometric test were passed to data refinement, and those with $P<0.05$ after refinement were considered over-represented in the list of differentially expressed genes, compared with other genes on the array.

2.7.3 EASE analysis

Another bioinformatics program, Expression Analysis Systematic Explorer (EASE, software version 2.0, National Institutes of Health, USA), provides statistical methods for discovering enriched biological themes within gene lists using GO category over-representation analysis (Hosack *et al*., 2003). A stringent set of gene probes

differentially expressed (FC≥1.5, moderated $P<0.01$ or $q<0.05$) according to the microarray analysis were uploaded into EASE along with a list of all genes on the microarray to test for over-representation of annotation classes. An EASE score (adjusted Fisher exact test for statistical significance) was calculated for likelihood of over-representation of hierarchical categories based on biological processes, molecular functions and cellular components using the GO public database. Gene categories with an EASE score <0.05 and a FDR or $q<0.05$ were considered to be significantly over-represented.

2.8 Bacterial profiling

Culture-independent, molecular methods have become an important tool in gastrointestinal microbiology to study diversity, abundance and population dynamics of complex bacterial communities in humans and animals. The following paragraphs explain the DGGE fingerprinting technique involving DNA isolation, 16S rRNA gene-based amplification, generation and analysis of DGGE profiles, cloning and sequencing of isolated DNA fragments and absolute real-time PCR quantification. In Fig. 1.7 of Chapter 1 section 1.3.3.2.1, the workflow for the DGGE technique is illustrated.

2.8.1 DNA isolation

Bacterial genomic DNA was isolated from frozen caecum using the QIAamp DNA Stool mini kit (Qiagen, Hilden, Germany). Briefly, caecal samples (caecum and digesta) were homogenised in 1.4 mL buffer ASL and heated at 95°C for 5 min to lyse bacterial cells. After removal of potential inhibitors by incubation with an InhibitEx tablet, the 200 µL supernatants were treated with 15 µL proteinase K and 200 µL buffer AL at 70°C for 10 min to remove protein and polysaccharides. DNA was precipitated by 200 µL ethanol (96–100%), applied to a column provided in the kit followed by washes with 500 µL buffers AW1 and AW2. Then the extracted DNA of each sample was dissolved in 200 µL buffer AE and kept frozen at −20°C until used.

2.8.2 PCR amplification

The hypervariable region (V2-V3) of the bacterial 16S rRNA gene was amplified using HDA1-GC (GC-clamp) and HDA2 universal primers as previously described (Tannock *et al.*, 2000). Briefly, PCR amplifications were performed using a GeneAmp PCR

System 9700 thermal cycler (Applied Biosystems, CA, USA) in 50 µL volumes containing 5 µL 10x buffer, 1 µL deoxyribonucleotide triphosphate (dNTP) mix (200 µM each), 0.4 µM of each HDA primer, 100 ng of DNA template and 2.5 units of HotStarTaq DNA polymerase (Qiagen). The following amplification program was used: 95°C for 15 min; 30 cycles consisting of 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s; and then 30 min at 72°C. DNA from individual or pooled samples was used for DGGE analysis. PCR products, which were approximately 200 bp in length, were confirmed by electrophoresis using TBE buffer (50 mM Tris, 40 mM boric acid, 0.5 mM EDTA) and 2% (w/v) agarose gels before DGGE analysis.

2.8.3 Denaturing gradient gel electrophoresis (DGGE)

This electrophoresis was performed in a vertical polyacrylamide gel with a gradient of denaturants at elevated temperature (60°C) using a 4 place temporal temperature gradient gel electrophoresis (TTGE)/DGGE system (CBS Scientific, Del Mar, CA, USA). The best separation of V2-V3 DNA was achieved in 6% (w/v) polyacrylamide gels with a 35% to 60% denaturing gradient of formamide and urea that increased in the direction of electrophoresis. A 100% denaturing solution corresponded to 40% (v/v) formamide and 7.0 M urea. The polyacrylamide gels (200 × 175 × 0.75 mm) were cast between 2 glass plates separated by 0.75 mm spacers and clamped to a casting stand. The low (35%) denaturing solution consisted of: 3.75 mL polyacrylamide (acrylamide/bisacrylamide ratio, 37.5:1), 0.5 mL 50× TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 mM EDTA), 3.5 mL formamide (40%), 3.7 g urea (7 M) and distilled water up to a total volume of 25 mL which makes 2 gels. The high (60%) denaturing solution consisted of: 3.75 mL polyacrylamide, 0.5 mL 50× TAE buffer, 6 mL formamide, 6.3 g urea and distilled water up to a total volume of 25 mL. These solutions were prepared fresh from stock solutions, mixed and kept on ice. Before casting the gel, 95 µL of 10% APS (ammonium persulphate) and 55 µL of TEMED (N,N,N,N,-Tetramethyl ethyl diamine) were added to each denaturing solution. The denaturing gradient was formed using a standard gradient maker with a low flow rate (regulated by a peristaltic pump) from the gradient maker to the gel casting assembly. A 20-well comb was inserted and the gels were allowed to polymerise for 1 h.

Prior to loading the samples onto the gels, 1x TAE buffer (24 L, prepared from the 50× TAE buffer) was filled into the DGGE tank and allowed to equilibrate to 60°C. The wells were flushed with 1× TAE buffer to remove excess urea and the amplified DNA fragments combined with 2× loading dye (8 µL total) loaded into the wells. A control sample was included in each DGGE gel to compare sample profiles between gels. Electrophoresis was performed at constant voltage (65 V) and temperature (60°C) for about 14 h. After completion of the electrophoresis, gels were transferred carefully to the staining container and stained with SYBR Gold (Invitrogen, CA, USA, using 3 µL of 10,000× concentrated stain diluted in 600 mL deionised water). Gels were kept on a shaker for 10 min and destained in fresh deionised water for 30 min. Gels were viewed by UV transillumination and photographed using a Gel Logic 200 imaging system (Kodak, CT, USA).

2.8.4 Analysis of DGGE profiles

The digital images of the DGGE gels were analysed to detect DNA bands using the band-searching algorithm of BioNumerics software (Applied Maths, TX, USA). After normalisation of the gels relative to the control sample, similarities between the profiles within the same gel were analysed from the presence and absence of different bands using the unweighted pair group method of arithmetic averages (UPGMA) with the Dice similarity coefficient (D_{sc}). The position tolerance was set to 3% for all analyses.

2.8.5 Cloning and sequencing DNA from DGGE fragments

In order to distinguish the position of a certain operational taxonomic unit to the bacterial species (a single DGGE band can represent multiple bacterial species), the DNA band excised from the DGGE gel, is cloned and sequenced. DGGE bands were excised from the gel with a sterile razor blade, transferred into 40 µL of sterile water and incubated at 4°C overnight to elute the DNA. Eluted DNA was included as a template in a PCR using HDA1⁄2 primers. PCR products were purified using QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions, cloned into a plasmid vector (pCR 2.1) and transformed into *E. coli* TOP-10 cells using the TOPO-TA cloning system (Invitrogen, USA). Briefly, 0.5-4 µL fresh PCR product was incubated with 1 µL pCR 2.1 vector, 1 µL salt solution (from the TOPO-TA kit) and water to a total volume of 5 µL for 5 min at room temperature and the ligation reaction

placed on ice. To transform the chemically competent *E. coli* TOP-10 cells, 2 µL of ligation reaction were combined with 25 µL of TOP-10 cells and incubated for 5 min on ice. This step enables a close contact of the DNA with the cell membrane. The samples were then heat-shocked for 30 s at 42°C to allow DNA incorporation into the cell and placed back on ice for 2 min. The transformed cells were grown on Luria-Bertani (LB) agar (LB medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g L agar and deionised water up to 1 L, pH 7.0) plates containing 100 µg of ampicillin/mL (plasmid encodes expression of the ampicillin resistance gene) and 40 µg of X-Gal/mL (5-Bromo-4-Chloro-3-Indoyl-beta-D-Galactopyranoside) and incubated overnight at 37°C. A white single bacterial colony was picked from an agar plate and incubated in 2 mL of LB broth (see LB medium without L agar) containing ampicillin overnight at 37°C with shaking at 250 *g*. Plasmid DNA was extracted using the Zyppy Plasmid Miniprep Kit (ZYMO Research, Orange, CA, USA). Briefly, cells were pelleted from the culture, resuspended in water and the suspension lysed (100 µL 7× lysis buffer) and neutralised (350 µL neutralisation buffer). The neutralised supernatant containing the plasmid DNA was washed using spin columns and eluted (30 µL elution buffer). The DNA inserts were amplified with HDA1-GC/HDA2 primers and the PCR products were run in DGGE gels to confirm the melting behaviour of the original gel band. PCR products that migrated to the same position as the original band were selected for sequencing.

DNA sequencing was performed at the Allan Wilson Centre Genome Service at Massey University, using Applied Biosystems Inc. sequencing chemistries, i.e., BigDyeTM Terminator Version 3.1 Ready Reaction Cycle Sequencing kit. Purified plasmid DNA samples were diluted in water to a concentration of 300 ng/15 µL and the M13 forward primer (5'-CCCAGTCACGACGTTGTAAAACG-3') provided (3.2 pmol/µl) by the sequencing service. The sequences were compared against numerous 16S rRNA gene sequences available from the online database of the NCBI.

2.8.6 Real-time PCR quantification of total bacteria and selected bacterial groups

The primers used in the real-time quantification of total bacteria, *Bacteroides-Prevotella-Porphyromonas, Enterococcus* spp., *Clostridium perfringens* (*C. perfringens*) group, *E. coli* group, and *B. vulgatus* in the caecal DNA from *Il10*$^{-/-}$ and

C57 mice are listed in Table 7.1 of chapter 7. The presence of *Helicobacter* spp. was tested by conventional PCR using the primers H267f 5'-CTATGACGGGTATCCGGC-3' and H676r 5'-ATTCCACCTACCTCTCCCA-3' (Riley *et al*., 1996). This PCR (25 µL) contained 50 ng of DNA template, 1 µL dNTP mix, 0.4 µM of each primer and 2.5 units of HotStarTaq DNA polymerase (Qiagen). The following amplification program was used: 95°C for 15 min; 35 cycles consisting of 94°C for 5 s, 57°C for 5 s, and 72°C for 30 s; and then 10 min at 72°C. PCR amplicons were confirmed by electrophoresis using TBE buffer and 2% (w/v) agarose gels.

The bacterial strains used for preparing the standard curves of the respective genus-specific PCR were: *E. coli* ATCC 35150, *B. fragilis* NZRM 964, *E. faecalis* AGR 991, *C. perfringens* ATCC 13124 and *L. reuteri* DPC 16. *B. vulgatus* DSM 1447 was used in a species-specific PCR. All bacterial strains were grown anaerobically overnight at 37°C. Tryptone Soy Broth (Oxoid, SA, Australia) was used for *E. coli*, Man-Rogosa-Sharpe (MRS) broth (Oxoid) for *L. reuteri*, Todd-Hewitt broth (Oxoid) for *E. facecalis*; and Brain Heart Infusion broth (Oxoid) for *B. fragilis*, *B. vulgatus* and *C. perfringens*. Bacterial cells were counted using a Neubauer haemocytometer after 18 h incubation. The standard curves of each strain were prepared from genomic DNA extracted from a 10-fold dilution series (10^4 to 10^9 cfu/mL) of each reference strain. Briefly, bacterial cells were collected by centrifugation at 5000 *g* (10 min), resuspended in 180 µL of lysozyme (20 mg/mL in 10 mM Tris, pH 8.0 with HCl), and incubated for 18 h at 37°C. Genomic DNA was then extracted using the QIAamp DNA mini kit (Qiagen) according to manufacturer's instructions. The quantitative real-time PCR analysis was carried out in triplicate in a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) using 20 µL reactions containing 10 µL of Light-Cycler 480 SYBR Green I Master Mix (Roche Diagnostics), 0.5 µM of each primer and 5 µL of either template or water. The PCR product sizes were confirmed using agarose 1% gel electrophoresis.

The real-time PCR results (number of cells/g of wet caecum content) were log-transformed and statistical analysis was performed using two-way ANOVA, followed by the least significant difference *post hoc* test in GenStat (11^{th} edition, VSN International Ltd, Hertfordshire, UK). $P<0.05$ was considered significant.

2.9 PPARα-target gene identification and validation

The following paragraphs describe the steps for setting up an *in vitro* experiment to investigate if vanin1 is a direct PPARα target gene by pharmacological PPARα activation using the synthetic PPARα agonist WY14643. A conserved PPAR response element (PPRE) within the *Vnn1* gene was identified and a genomic region encompassing the PPRE cloned in front of a luciferase reporter to perform transactivation assays.

2.9.1 *In silico* analysis of PPAR response elements of a potential PPARα target gene

A PPRE contains direct repeat sequences separated by a single nucleotide spacer (AGGNCA_AGGTCA) which is bound by the PPAR/RXR heterodimer affecting target gene transcription (Ijpenberg *et al*., 1997). The *in silico* analysis of the mouse *Vnn1* gene using a published algorithm (Heinaniemi *et al*., 2007) identified conserved PPRE at 4175 and 4182 bp down-stream of the transcription start site (TSS). This method is based on experiment-derived evidence for DNA-binding strength of the PPAR subtypes to PPRE, the PPRE location relative to the TSS and the conservation of the PPRE across multiple species for a reliable PPRE prediction. For validation of the functionality of binding sites, the PPRE that are close to the TSS and/or conserved in its exact composition are chosen.

2.9.2 Plasmids and DNA constructs

Mouse genomic DNA (mouse strain C57BL6/J) was used to PCR-amplify 191 bp of the mouse vanin1 sequence containing two strong overlapping PPRE using a Platinum Taq DNA polymerase kit (Invitrogen) and forward mVnn1-F 5'-TCCCAGTGTGCTCAGTAATCCAG-3' and reverse mVnn1-R 5'-AACTTCCTTCGCTCCTTTCTCAG-3' primers. Then the 191 bp product was PCR-amplified using forward mVnn1-FKpnI 5'- CCGGGTACCTCCCAGTGTGCTCAG-3' and reverse mVnn1-RBglII 5'- CCCAGATCTAACTTCCTTCGCTCC-3' primers which include restriction sites for KpnI (New England Biolabs, Westburg BV, Leusden, The Netherlands) and BglII (Invitrogen, Breda, The Netherlands). The size of each PCR amplicon was confirmed on a 1% agarose gel and the DNA recovered from the agarose gel using a NucleoSpin Extract II kit (Macherey-Nagel, Bioke, Leiden, The

Netherlands) according to the manufacturer's instructions. The purified PCR amplicon with the restriction sites was then digested by combining 10 µL of DNA, 2 µL 10x Buffer2 (New England Biolabs, Westburg BV, Leusden, The Netherlands), 1 µL of each restriction enzyme and water to a total volume of 20 µL and incubated overnight at 37°C. The digested PCR product was confirmed on 1% agarose gel, DNA recovered and subcloned into the BglII and KpnI sites of a linearised pGL3-promoter vector (Promega Corp., Leiden, The Netherlands) which contains an SV40 promoter up-stream of the luciferase gene. To ligate the DNA insert with the vector, 4 µL of DNA (with cohesive ends), 1 µL of vector, 2 µL of 10× T4 DNA ligase buffer, 1 µL of T4 DNA ligase (New England Biolabs, Westburg BV, Leusden, The Netherlands) and water to a total volume of 20 µL were incubated overnight at 16°C.

Two site-directed mutations of the PPRE were introduced using two separate, partially overlapping PCR fragments obtained using the mVnn1 fragment as a template. Primer mVnn1-FKpnI combined with reverse primer mVnn1-Rmut 5'-TTCGTTTACCTTTGTCCCAATACCTCAGCAG-3' and the forward primer mVnn1-Fmut 5'-CTGCTGAGGTATTGGGACAAAGGTAAACGAA-3' combined with primer mVnn1-RBglII were used to generate the two partially overlapping PCR fragments. The two fragments with overlapping ends were combined in a final PCR amplified with the forward mVnn1-FKpnI and reverse mVnn1-RBglII primers. The final purified product containing the mutated PPRE in the mouse *Vnn1* sequence was digested and cloned into the BglII and KpnI sites of the pGL3-promoter vector as described above.

The recombinant plasmid was then transformed into competent DH5α *E. coli* cells following similar methods as described under 2.8.5. The transformed cells were grown on LB agar plates to form colonies. Small (similar method as described under 2.8.5 using the Zyppy Plasmid Miniprep Kit) and large scale preparation of plasmid DNA was performed using the Plasmid Maxi Kit (Qiagen, Leusden, The Netherlands). For the large scale preparation, a single colony was picked from the agar plate and incubated in 3 mL of LB broth containing ampicillin overnight at 37°C with shaking at 250 *g*. This starter culture was then used to inoculate 100 mL of LB broth containing ampicillin and cells were grown overnight at 37°C with shaking at 250 *g*. Cells were pelleted and DNA purified according to the manufacture's instructions. At the last stage DNA was redissolved in sterile distilled water.

All fragments were sequenced to confirm the integrity of the PPRE in the construct. The sequencing was performed by the Sequencing Service Facilities at Wageningen University. Purified plasmid DNA samples were diluted in water to a concentration of 200 ng/μL and the RV primer3 (5'-CTA GCA AAA TAG GCT GTC CC-3') was provided at a concentration of 5 ng/μL. The mPPARα expression plasmid, the (PPRE)3-LUC reporter plasmid containing three copies of acyl-CoA oxidase PPRE and the β-galactosidase reporter plasmid were kindly provided by Wageningen University.

2.9.3 Cell culture and transfections

Human hepatoma HepG2 cells obtained from ATCC (Manassas, VA, USA) were used because these cells have low PPAR expression levels and can readily be transiently transfected. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin (termed 'complete DMEM'), in an atmosphere of 5% CO_2/95% air at 37°C. Cells were maintained in 150 cm^2 cell culture flasks (Costar, Cambridge, MA, USA) and subcultured every 2-3 days at a ratio of 1:4, after they had reached 90% confluence.

HepG2 cells were transfected with PPARα expression and luciferase promoter reporter plasmids (PPRE)3-LUC as a positive control, or PPRE-Vnn1-LUC or PPRE-Vnn1mut-LUC) and co-transfected with a β-galactosidase reporter plasmid to normalise for differences in transfection efficiency using a NanoJuice transfection kit (Novagen, Nottingham, UK) according to the manufacturer's instructions. Briefly, 4×10^5 cells/well were seeded in 12-well cell culture plates and grown to 80% confluence. The HepG2 cells were incubated with a mix of NanoJuice transfection reagents and the DNA (500 ng/well in a 12-well plate) in serum-free DMEM medium. The transfection was terminated after 6 h by replacing the transfection mixture with complete DMEM. After transfection, cells were incubated in the presence or absence of PPARα ligand WY14643 at 50 μM or DMSO (vehicle) for 24 h prior to lysis. Luciferase activity was measured using the Promega luciferase assay kit (Promega Corp.) on a Fluoroskan Ascent FL apparatus (Thermo labsystems, Breda, The Netherlands). β-galactosidase activity was measured in the cell lysate by a standard assay using 2-nitrophenyl-BD galactopyranoside as substrate. The luciferase activity for each well was normalised to

the β-galactosidase activity obtained from the same well. Experiments were performed twice in triplicate.

Chapter 3

Transcriptomic and proteomic profiling to characterise onset and progression of colon inflammation in interleukin-10 gene-deficient mice

Part of the material presented in this chapter has been published as a paper:

Knoch B, Barnett MPG, Cooney J, McNabb WC, Barraclough D, Laing W, Zhu S, Park ZA, MacLean P, Knowles SO & Roy NC (2010) Molecular characterization of the onset and progression of colitis in inoculated interleukin-10 gene deficient mice: a role for PPARα. in press *PPAR Res* volume **2010**.

3.1 Abstract

Animal models of IBD are important for unravelling the complex interactions between diet, resident intestinal bacteria and the host's intestinal mucosa, and thus elucidating the mechanisms of initiation and progression of intestinal inflammation. It was shown that conventional *Il10*$^{-/-}$ mice (C57BL/6J background) developed increasing colon inflammation peaking at 12 weeks of age. Since the time-course of colitis in the bacterially-inoculated *Il10*$^{-/-}$ mouse model (C57BL/6J background) is undefined, the aim was to characterise colitis onset and progression in this model and to extend previous previous work with experimental colitis models by using a combined histopathological, transcriptomic and proteomic approach. The hypothesis of this study was that distinct gene and protein expression patterns could be defined in non-inflamed (7 weeks of age) and colitic (12 weeks of age) *Il10*$^{-/-}$ mice. Histopathological analysis performed at specific times indicated that bacterial inoculation resulted in severe colitis in *Il10*$^{-/-}$ mice from 10 to 12 weeks of age. No inflammation was observed for 7-week-old *Il10*$^{-/-}$ mice or any C57 mice. The combined transcriptomic (microarray) and proteomic (2D-DIGE coupled with LC/MS-MS) approach identified differences in colonic gene and protein expression profiles between 7 (no colitis) and 12 (severe colitis) weeks of age in *Il10*$^{-/-}$ relative to C57 mice. Comparative pathway, function and network analyses showed that more genes were differentially expressed in the colon tissue of 12-week-old *Il10*$^{-/-}$ relative to C57 mice compared to those at 7 weeks of age. Several aspects of innate and adaptive immune response (e.g. dendritic cell maturation) showed differences in gene expression between no and severe colon inflammation. Actin cytoskeleton dynamics and innate immunity-linked gene and protein expression data suggested a delayed remodelling process in the damaged colon of 12-week-old *Il10*$^{-/-}$ mice under inflammatory conditions.

3.2 Introduction

The exact disease etiology of IBD, including CD and UC is unknown, but there is evidence for genetic, environmental and dietary involvement in disease susceptibility (Sands, 2007). IBD is characterised by chronic and spontaneously relapsing, immunologically mediated inflammation of the distal gastrointestinal tract (Shkoda *et al*., 2007; Bouma & Strober, 2003). Animal models of IBD are important for unravelling the complex interactions among diet, resident intestinal bacteria and human

host, and thus elucidating the mechanisms of initiation and progression of intestinal inflammation.

Several rodent models are available that mimic different aspects of IBD, including gene knockout or defect, transgenic, spontaneous colitis, chemically-induced colitis and adoptive transfer models (Jurjus *et al*., 2004). Although no single model displays all the features of the human disease, they each reproduce key characteristics of intestinal inflammation (e.g., morphological changes, inflammation status and onset and progression of disease pathophysiology) and some have a well-defined genetic background and immune system. The animal models that have been widely used in pharmacological and pathophysiological studies are inducible colitis (chemicals, pathogenic bacteria) and genetically-modified rodent models. Genetically-modified mouse models have a defined gene defect or deficiency such as the *Il10*$^{-/-}$ mouse (Kühn *et al*., 1993) that develops colitis similar to human IBD. IL10 is an anti-inflammatory cytokine that can inhibit antigen presentation and subsequent release of pro-inflammatory cytokines to attenuate mucosal inflammation (Sanchez-Munoz *et al*., 2008). *Il10* gene mutation inactivates the IL10 protein that normally down-regulates T cell activity in these mice. This results in increased production of the Th1 pro-inflammatory cytokines IL12 and IFNγ in response to antigens from the intestinal flora (Kühn *et al*., 1993; Berg *et al*., 1996).

The inflammation level seen in *Il10*$^{-/-}$ mice varies with genetic background. Intestinal lesions are most severe on the C57BL/6J/129-Ola background and are of a more mild nature in the C57BL/6J strain (Berg *et al*., 1996). C57BL/6J *Il10*$^{-/-}$ mice develop a CD-like colitis by 12 weeks of age under conventional conditions (Berg *et al*., 1996), but not until 20 weeks of age when raised under specific pathogen-free conditions (McCarthy *et al*., 2003). Intestinal inflammation in this mouse model develops more consistently when germ-free *Il10*$^{-/-}$ (IL10 x 129SEV) mice are inoculated with *Enterococcus faecalis* (Balish & Warner, 2002) or when conventional *Il10*$^{-/-}$ (C57BL/6J) mice are inoculated with pure *Enterococcus* isolates (*E. faecalis* and *E. faecium*) combined with a mixed complex intestinal flora derived from healthy C57BL/6J mice (Roy *et al*., 2007). The timeline of the development of colitis is unknown in these inoculated C57BL/6J *Il10*$^{-/-}$ mice.

Multi-omic studies provide novel data on individual genes and proteins, pathways and biological functions that are altered in health and disease. Transcriptomic (Yamamoto *et al*., 2005; te Velde *et al*., 2007; Roy *et al*., 2007; Martinez-Augustin *et al*., 2008) and proteomic (Werner *et al*., 2007) methods have mostly been applied separately to intestinal tissue samples of various murine models of experimental colitis and of IBD patients. It is important to integrate gene and protein expression data to better predict phenotypic changes.

Studies to date have mostly applied transcriptomic (Yamamoto *et al*., 2005; te Velde *et al*., 2007; Roy *et al*., 2007; Martinez-Augustin *et al*., 2008) and proteomic (Werner *et al*., 2007) methods separately to intestinal tissue samples of various murine models of experimental colitis, and of IBD patients. It is important to integrate gene and protein expression data to get a more comprehensive understanding of phenotypic changes. A previous study showed that conventional non-inoculated *Il10*$^{-/-}$ mice (C57BL/6J background) developed increasing colonic inflammation peaking at 12 weeks of age (Kennedy *et al*., 2000). The hypothesis of this study was that distinct gene and protein expression patterns could be defined in non-inflamed (7 weeks of age) and colitic (12 weeks of age) *Il10*$^{-/-}$ mice. Since the time-course of colitis in the bacterially-inoculated *Il10*$^{-/-}$ mouse model (C57BL/6J background) used by Roy *et al*. (2007) is undefined, the aim was to characterise colitis onset and progression in this model and to extend previous previous work with experimental colitis models by using a combined histopathological, transcriptomic and proteomic approach.

3.3 Materials and methods

3.3.1 Animals and diet

A total of 30 male *Il10*$^{-/-}$ (C57BL/6J background, formal designation B6.129P2-*Il10*<tm1Cgn>/J) and 30 male C57 wildtype (C57BL/6J) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). For convenience and consistency in reporting, their age was defined as 35 days or 5 weeks of age at the start of the study. Animals had free access to water and were fed an AIN-76A standard powder diet prepared in-house. The diet composition is described in Materials and Methods chapter 2.2.

3.3.2 Induction of colitis

Preparation of the bacterial inoculum and induction of colitis are described in Materials and Methods chapter 2.1.2 and in the paper by Roy *et al*. (2007). All mice were inoculated at 5 weeks of age with a mixture of pure *E. faecalis* and *E. faecium* strains and complex intestinal flora derived from healthy C57BL/6J mice raised under conventional conditions.

Note: *The protocol for preparation of the bacterial inoculum was established by the microbiologist Dr Adrian Cookson at AgResearch Grasslands. The oral gavage with the bacterial inoculum was performed by the AgResearch Ruakura Small Animal Facility Manager, Ric Broadhurst.*

3.3.3 Experimental design

Mice were randomly assigned to 5 time points (7, 8.5, 10, 12 and 14 weeks of age). Housing, experimental design, fast-feed period and sampling are described in Materials and Methods chapter 2.1.1, 2.1.3 and 2.1.4. One piece of each intact intestinal tissue was stored at room temperature in 10% phosphate-buffered formaldehyde for histopathological assessment, another was immediately frozen in liquid nitrogen and kept at −85°C for transcriptomic and proteomic analysis.

3.3.4 Histology

The histopathological assessment of the full thickness intestinal sections (duodenum, jejunum, ileum or colon) and the scoring method are described in Materials and Methods chapter 2.3.1 and 2.3.2. The method produces a histological injury score (HIS) for each sample. It is the sum (total HIS) of principal histological aspects (inflammatory cell infiltration, tissue destruction and tissue repair).

Note: *Tissue processing and histopathological assessment was conducted by the pathologist Dr Shuotun Zhu at The University of Auckland.*

3.3.5 RNA isolation, microarray hybridisation and analysis

RNA isolation, microarray design, hybridisation and analysis are described in Materials and Methods chapter 2.4.2 to 2.4.4. The decision on the mice used for microarray was based on their histopathological assessment. The microarray experiment consisted of 15 arrays: 3 colon samples of *Il10*$^{-/-}$ mice at 7 weeks of age (no inflammation), 4 of *Il10*$^{-/-}$

mice at 12 weeks of age (severe inflammation), 4 of C57 mice at 7 weeks of age (no inflammation) and 4 of C57 mice at 12 weeks of age (no inflammation). At 7 weeks of age, one *Il10*$^{-/-}$ mouse died of unknown causes and two others were already showing moderate signs of colon inflammation and were thus excluded. Each individual RNA sample was hybridised with a reference sample onto the array. Array data were submitted to the Gene Expression Omnibus, accession number GSE17990.

The normalised data from the arrays of each time point group were averaged. For each comparison of interest (*Il10*$^{-/-}$ *vs.* C57 7 weeks and *Il10*$^{-/-}$ *vs.* C57 12 weeks), a list of differentially expressed genes was generated by calculating moderated t-statistics and FDR using limma within the Bioconductor framework as described in Materials and Methods chapter 2.4.4. Probes with a FDR or $q<0.05$ were considered to be differentially expressed.

Note: *The microarray analysis in Bioconductor (written in R) was conducted with assistance from AgResearch statistician Zaneta Park.*

3.3.6 Bioinformatics analysis of pathways and functions

IPA (Version 7.0, Ingenuity Systems Inc., Redwood City, CA, USA) was used for pathway and functional analyses of differentially expressed probes in the microarray dataset. EASE (software version 2.0, National Institutes of Health, USA) was used to identify enriched biological themes within gene lists using GO category over-representation analysis. These bioinformatics programs are described in Materials and Methods chapter 2.7.1 and 2.7.3. Probes from the microarray dataset that satisfied the cut-off criteria of FC≥1.5 (up- or down-regulated) and $q<0.05$ were considered for both analyses. Pathways were considered to be affected by colon inflammation when the probability value calculated by the Fisher's exact test was <0.01 and where at least 20% of the genes from a particular pathway were differentially expressed in the microarray dataset.

Note: *The bioinformatics analysis using EASE was conducted with assistance of AgResearch statistician Paul MacLean.*

3.3.7 Quantitative real-time PCR

qRT-PCR is described in Materials and Methods chapter 2.5. Ten genes (*Abcb1a*, *Aldh1a1*, *Ces2*, *Fabp2*, *Igfbp5*, *Il1b*, *Mmp13*, *Ppara, Srebf1* and *Sult1a1*) were used for quantification and microarray verification. Genes were selected to include both significantly and non-significantly expressed genes pertaining to pathways affected by inflammation, e.g., those related to detoxification, transport, immunity or metabolism. The primer sequences for target and reference genes are listed in Materials and Methods chapter Table 2.2. Calnexin (*Canx*) was chosen as a suitable normalisation reference gene.

3.3.8 Protein isolation and identification by LC-MS/MS data analyses

Protein isolation and identification by LC-MS/MS data analyses are described in Materials and Methods chapter 2.6.1 to 2.6.3. The protein-containing lower layer of the same TRIzol-processed sample from which RNA was derived, was used for protein isolation. Gels were run as duplicate biological replicates using the same pooled samples as described for the microarray design.

Note: *Protein isolation was conducted by the PhD candidate, protein identification and analysis was conducted with assistance from Diane Barraclough and Drs William Laing and Janine Cooney, Plant & Food Research and collaborators in Nutrigenomics NZ.*

3.3.9 Statistical analysis

All statistical analyses (body weight, dietary intake, HIS and qRT-PCR data) were performed using ANOVA in GenStat (10^{th} edition, VSN International, Hemel Hempstead, UK), on log-transformed data where necessary in cases of unequal variances. $P<0.05$ was regarded as significant. A trend was declared when $0.05<P<0.10$.

3.4 Results

3.4.1 Animal body weight and dietary intake

There was no difference between *Il10*$^{-/-}$ and C57 mice in terms of average body weight at the beginning of the experiment (16.5 ± 0.3 *vs.* 16.9 ± 0.2 g, Table 3.1). During the course of the experiment, *Il10*$^{-/-}$ mice gained weight more slowly (with a loss in body weight observed between 12 and 14 weeks of age) than C57 mice, resulting in a lower average body weight at 7, 8.5, 10, 12 and 14 weeks of age when compared to the C57

mice. This was significant (P<0.05) at 10, 12 and 14 weeks of age. Dietary intake was not different between *Il10*$^{-/-}$ and C57 mice which were pair-fed at any time point in the study (Table 3.1).

3.4.2 Development and characterisation of intestinal inflammation

Histological analysis showed that the average total HIS in *Il10*$^{-/-}$ mice was highest in the colon, and only two mice displayed moderate inflammation in the ileum. No signs of inflammation were observed in the duodenum or jejunum of *Il10*$^{-/-}$ mice or in any of the different intestinal sections of C57 mice (Fig. 3.1). Therefore, statistical analysis was limited to colon tissue of *Il10*$^{-/-}$ mice. There was a significant difference in the average total HIS in the colon between *Il10*$^{-/-}$ and C57 mice at all time points. This excludes the 7 week time point when the two *Il10*$^{-/-}$ mice which showed moderate signs of colon inflammation at 7 weeks of age, are not taken into account (Table 3.2). It became apparent that the average total colon HIS of *Il10*$^{-/-}$ mice increased over time peaking between 10 and 12 weeks of age, with no further increase at 12 and 14 weeks of age.

Colon inflammation was mainly characterised by inflammatory cell infiltration (monocytes and neutrophils), but also featured tissue destruction (crypt loss and oedema) and tissue repair (hyperplasia). The colon of *Il10*$^{-/-}$ mice was highly inflamed by 10 weeks of age and showed moderate inflammation by 12 and 14 weeks of age (Fig. 3.1). The total colon HIS in 10-week-old *Il10*$^{-/-}$ mice was significantly higher (P<0.05) and a trend was observed at 12 weeks of age (P=0.07) compared to 7-week-old *Il10*$^{-/-}$ mice. There was also less variability in the individual colon HIS at 8.5, 10 and 12 weeks compared to 7 and 14 weeks of age. The inflammatory lesions were transmural involving most layers of the intestinal wall. There was thickening of the mucosal layer and formation of crypt abscesses with loss of goblet cells (Fig. 3.2).

3.4.3 Inflammation-induced changes in expression profiles

Seven and 12 weeks of age were chosen to assess changes in colon gene expression because these two time points represented no inflammation (7 weeks of age) and severe inflammation (12 weeks of age) in *Il10*$^{-/-}$ mice. Pathway and network analysis using IPA were conducted on the transcriptome and proteome data. The transcriptome data

were also subjected to GO analysis using EASE to confirm and further support the IPA analysis. At the gene level, more changes were observed at 12 weeks than at 7 weeks of age in *Il10*$^{-/-}$ mice compared to C57 mice of the same age, with genes mostly being up-regulated at each time point in the colon of *Il10*$^{-/-}$ mice. Those gene changes are illustrated in Fig. 3.3 which shows the genes over-represented in the EASE analysis when comparing *Il10*$^{-/-}$ and C57 mice at 7 and 12 weeks of age. The mean expression of selected genes obtained by qRT-PCR mostly confirmed the expression changes from the microarray analysis. The changes in expression levels of *Ces2*, *Il1b* and of *Igfbp5*, *Srebf1* genes became significant in the colon of 7-week-old and 12-week-old *Il10*$^{-/-}$ mice, respectively by using the more sensitive qRT-PCR analysis (Table 3.3).

Only four consistent protein expression changes were identified at 7 weeks of age in a direct comparison of *Il10*$^{-/-}$ *vs.* C57 mice across the pooled biological replicates (pool 1 and pool 2), with two decreased and two increased in expression. Of these, only two met the threshold criteria and were visible after staining for spot picking and subsequent identification. At 12 weeks of age, 44 consistent protein expression changes were observed across the pooled biological replicates (pool 1 and pool 2) with 22 decreased and 22 increased in expression. Here, 42 protein expression changes met the threshold criteria and were selected for subsequent identification. The combined 44 spot-features identified over the two time points represented 40 unique proteins (excluding multiple isoforms due to post-translational modifications) and are shown in the gel image depicted in Fig. 3.4, and listed in Table 3.4. In seven cases, two or more proteins or protein isoforms were identified in the same 2D gel spot-feature.

3.4.4 Gene ontology, network/function and pathway analysis of colonic genes and proteins of *Il10*$^{-/-}$ and C57 mice at 7 weeks of age

Genes differentially expressed at 7 weeks in the colon of *Il10*$^{-/-}$ compared to C57 mice were classified into 1493 GO categories. Only 21 of these categories were over-represented based on an EASE score <0.05 and $q<0.05$; these are listed in Table 3.5. The EASE analysis of gene expression indicated that several biological processes were over-represented including: antigen presentation, carbohydrate and lipid metabolism for energy utilisation and steroid metabolism. Over-represented functional categories included MHC class II receptor activity. Most of the colonic genes in these GO

categories showed up-regulated expression in *Il10*$^{-/-}$ compared to C57 mice at 7 weeks of age.

In IPA, 50 networks were generated from the genes differentially expressed in the colon of *Il10*$^{-/-}$ mice relative to C57 mice at 7 weeks of age. The themes of the five highest scoring networks (P<0.05 using Fisher's exact test) which encompassed the highest number of differentially expressed genes in the transcriptome dataset were cancer, cell cycle, growth, proliferation and death and cell-mediated immune response (Table 3.6). As there were only two proteins (adenylate cyclase-associated protein1 and glutamate dehydrogenase1) that had lower abundance and one protein (peroxiredoxin) with higher abundance between *Il10*$^{-/-}$ and C57 mice (both at 7 weeks of age), no network analysis was carried out for protein in IPA.

The most significantly regulated pathways in IPA containing most of the differentially expressed genes (20%) included antigen presentation (MHC class II genes such as *Hla-dm*, *Hla-dq* and *Hla-dr* members) and interferon signalling pathway in which the expression levels of genes were mostly increased (Table 3.7).

3.4.5 Gene ontology, network/function and pathway analysis of colonic genes and proteins of *Il10*$^{-/-}$ and C57 mice at 12 weeks of age

At 12 weeks, differentially expressed genes were classified into 1570 GO categories and 32 of these categories were significantly over-represented with an EASE score <0.05 and q<0.05, and are listed in Table 3.8. Genes in biological process categories associated with defence response to biotic (e.g., pathogen) stimulus or stress were the most over-represented among the up-regulated genes, followed by humoral and innate immune response and antigen presentation. Genes assigned to protein activation, such as post-translational changes targeting membrane proteins, were also over-represented, and were mostly up-regulated in *Il10*$^{-/-}$ compared to C57 mice.

In IPA, 48 networks were generated from the colonic genes differentially expressed in *Il10*$^{-/-}$ compared to C57 mice at 12 weeks of age. In Table 3.6, the top 3 biological functions for the most significant transcriptomic and proteomic networks are shown. These transcriptomic and proteomic networks share the following biological functions:

cell-mediated immune response is represented in transcriptome 4 and proteome 1, cancer is represented in transcriptome 2 and proteome 3, and cell-to-cell signalling and interaction is represented in transcriptome 1 and 4. Thus, the theme linking transcriptomic and proteomic networks is cell migration and changes in tissue structure (implications for actin cytoskeleton dynamics) as well as cell death with associated inter- and intracellular signalling and initiation of the immune response.

The most significantly regulated pathways in IPA containing most of the differentially expressed genes (20-40%) represented signalling processes (Table 3.7). The signalling pathways included: antigen presentation pathway (MHC class I, e.g. *Hla* members and MHC class II genes, e.g. *Hla-dm*, *Hla-dq*, *Hla-dr* members); graft-versus-host disease signalling and allograft rejection signalling (e.g. *Il1b*, *Tnfa*), interferon signalling (e.g. *Ifng*), role of RNA-dependent protein kinase (PKR) in interferon induction and antiviral response (e.g. *Bcl*), dendritic cell maturation (collagen type I, e.g. *Col1a1*, *Col1a2* and type XVIII genes, e.g. *Col18a1*) and complement system, and the genes in those pathways were mostly up-regulated.

3.4.6 Pathway comparison analysis of 7- and 12-week-old *Il10*$^{-/-}$ mice relative to C57 mice at the same age

A gene pathway comparison analysis between *Il10*$^{-/-}$ and C57 mice at 7 weeks of age and *Il10*$^{-/-}$ and C57 mice at 12 weeks of age showed that ER stress and biosynthesis of steroids were more affected at 7 weeks of age. Genes associated with the dendritic cell maturation pathway were unchanged in *Il10*$^{-/-}$ compared to C57 mice at 7 weeks of age (especially collagens type I and XVIII), but were up-regulated in *Il10*$^{-/-}$ compared to C57 mice at 12 weeks of age. EASE analysis confirmed the over-representation of genes involved in steroid biosynthesis at 7 weeks of age and in collagen catabolism at 12 weeks of age as shown in Table 3.8.

Table 3.1 Body weight and dietary intake of *Il10*$^{-/-}$ and C57 mice at 7, 8.5, 10, 12 and 14 weeks of age

Weeks of age	Body weight (g)		Dietary intake (g)	
	C57 mice	***Il10*$^{-/-}$ mice**	**C57 mice**	***Il10*$^{-/-}$ mice**
7	18.8 ± 0.4	18.2 ± 0.8	3.4 ± 0.1	3.3 ± 0.2
8.5	20.3 ± 0.9	19.0 ± 0.3	3.6 ± 0.1	3.9 ± 0.3
10	22.5 ± 0.6	19.9 ± 0.4*	3.7 ± 0.0	3.9 ± 0.1
12	24.8 ± 0.7	21.0 ± 0.8*	3.6 ± 0.1	3.5 ± 0.2
14	25.3 ± 0.4	19.8 ± 0.7*	3.6 ± 0.1	3.5 ± 0.3

Data shown as mean ± standard error of mean (SEM) per group of mice sacrificed. Body weight was measured thrice weekly and dietary intake was estimated and adjusted daily to equal the mean amount of food consumed by *Il10*$^{-/-}$ mice on the previous day to ensure similar intakes between C57 and *Il10*$^{-/-}$ mice. *P<0.05 comparing *Il10*$^{-/-}$ vs. C57 mice of the same age.

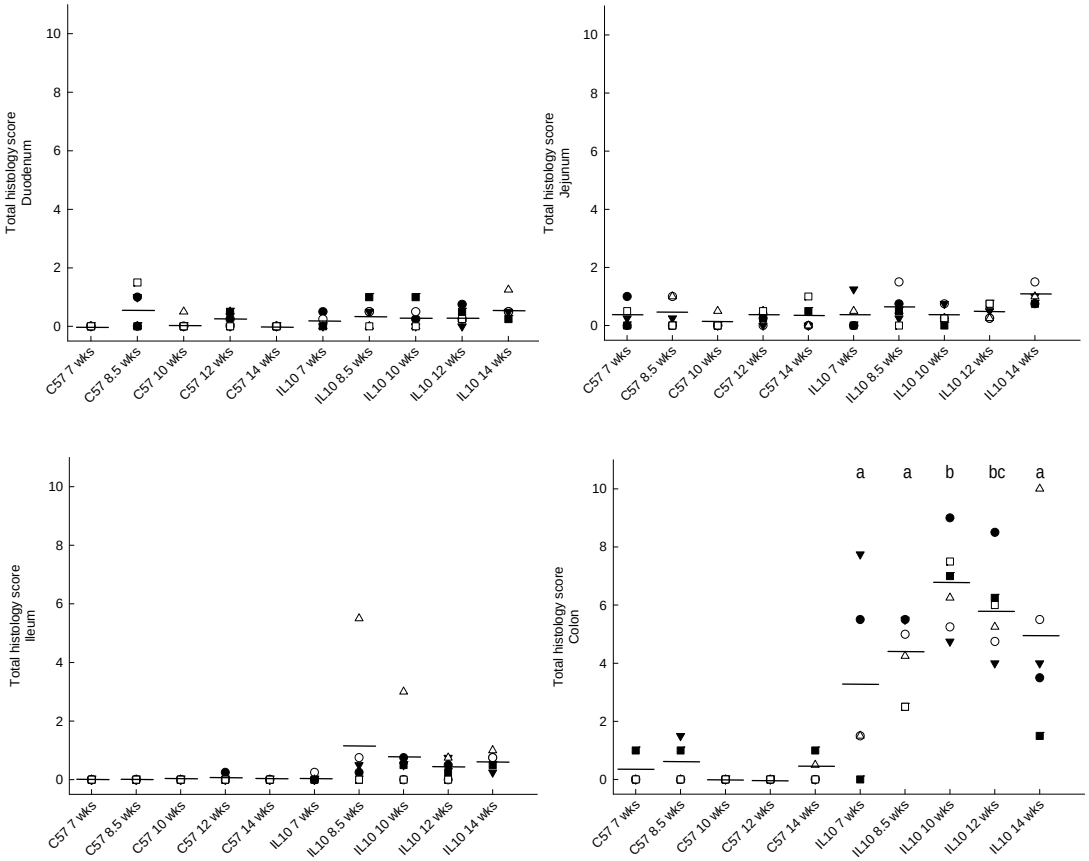


Fig. 3.1 Total histological injury score of duodenum, jejunum, ileum and colon of *Il10*$^{-/-}$and C57 mice at 7, 8.5, 10, 12 and 14 weeks of age. The individual scores and the average score (**—**) of each sampling group are shown. Statistical analysis was performed only for total colon HIS of *Il10*$^{-/-}$ mice due to many zero scores for C57 mice. Different letters mean that total colon HIS differs compared to 7-week-old *Il10*$^{-/-}$ mice (a,b, $P<0.05$ and c, $0.05<P<0.10$).

Table 3.2 Time of onset and incidence of colon inflammation in *Il10*$^{-/-}$ mice

Weeks of age	**7**	**8.5**	**10**	**12**	**14**
Number of mice sampled					
Il10$^{-/-}$mice (total 28[1])	5	6	6	6	5
C57 mice (total 30)	6	6	6	6	6
Number of mice with inflamed colon[2]					
Il10$^{-/-}$ mice	2	4	6	6	4
C57 mice	0	0	0	0	0
Incidence of colon inflammation (%)					
Il10$^{-/-}$ mice	40	67	100	100	80
C57 mice	0	0	0	0	0

[1]Two *Il10*$^{-/-}$ mice died during the study due to unknown causes. [2]Mice were regarded as inflamed when their total histological injury score (HIS) in the colon was >3. All C57 mice had a total colon HIS≤3.

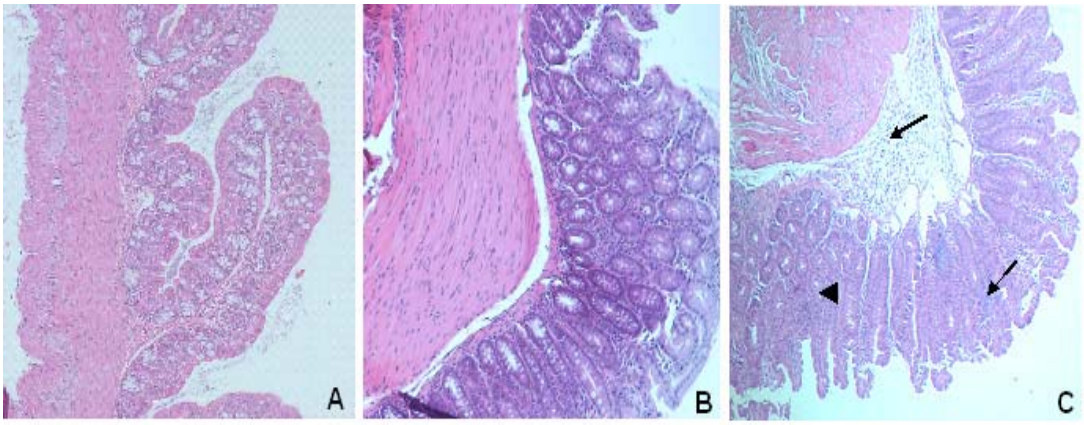


Fig. 3.2 Haematoxylin-eosin stained colon sections of *Il10*$^{-/-}$ and C57 mice. (A) Colon section (×20) from a non-inflamed C57 mouse at 12 weeks of age. (B) Colon section (×100) from an *Il10*$^{-/-}$ mouse at 7 weeks of age with no inflammation. (C) Moderate to severe inflamed colon section (×40) from an *Il10*$^{-/-}$ mouse at 12 weeks of age. Lesions involve most of the colon section with mainly monocyte and neutrophil infiltration (←), crypt abscesses and loss of crypt and goblet cells (◄).

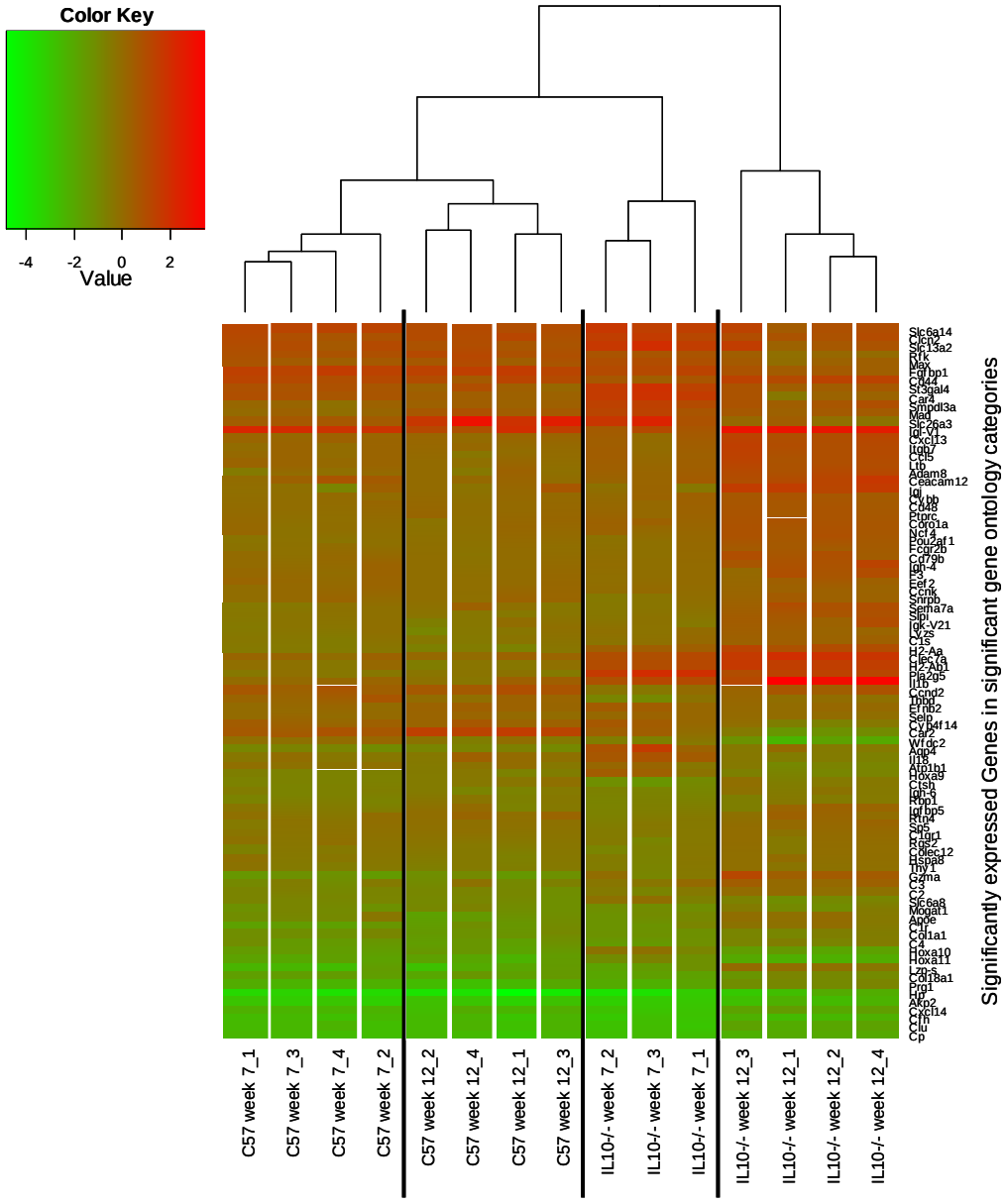


Fig. 3.3 Genes over-represented in EASE analysis comparing *Il10*$^{-/-}$ and C57 mice at 7 or 12 weeks of age. Heat map representing the expression levels of all differentially expressed genes in the significant GO categories (EASE score <0.05 and q<0.05). The groups are clearly defined within their clusters and differences in gene expression are seen for 12-week-old *Il10*$^{-/-}$ mice relative to 7-week-old *Il10*$^{-/-}$ mice and 12-week-old C57 mice.

Table 3.3 Validation of gene expression results from microarray analysis using qRT-PCR

Gene symbol	**Fold change microarray**		**Fold change qRT-PCR Canx**	
	***Il10*$^{-/-}$ vs. C57 7 weeks**	***Il10*$^{-/-}$ vs. C57 12 weeks**	***Il10*$^{-/-}$ vs. C57 7 weeks**	***Il10*$^{-/-}$ vs. C57 12 weeks**
Abcb1a	-3.4	-2.8	-5.2	-4.9
Aldh1a1	-1.5*	-3.0	-1.5$^{\#}$	-3.4
Ces2	-1.8*	-3.1	-2.9	-5.6
Fabp2	-1.9	-5.6	-1.5$^{\#}$	-9.8
Igfbp5	-1.5*	1.6*	-1.9$^{\#}$	2.8
Il1b	1.8*	6.4	3.4	8.5
Mmp13	1.0*	1.8	2.7$^{\#}$	9.9
Ppara	-2.0	-1.7	-5.6	-3.8
Srebf1	1.0*	1.0*	1.2$^{\#}$	1.8
Sult1a1	-3.2	-3.1	-3.6	-3.3

Canx (calnexin) reference gene used to normalise the data; *Microarray result was not significantly different using moderated t-statistics and false discovery rate (FDR) control in limma; genes that satisfied the criterion of FC≥1.5 and q<0.05 were considered to be significantly different; $^{\#}$qRT-PCR result was not significantly different using ANOVA.

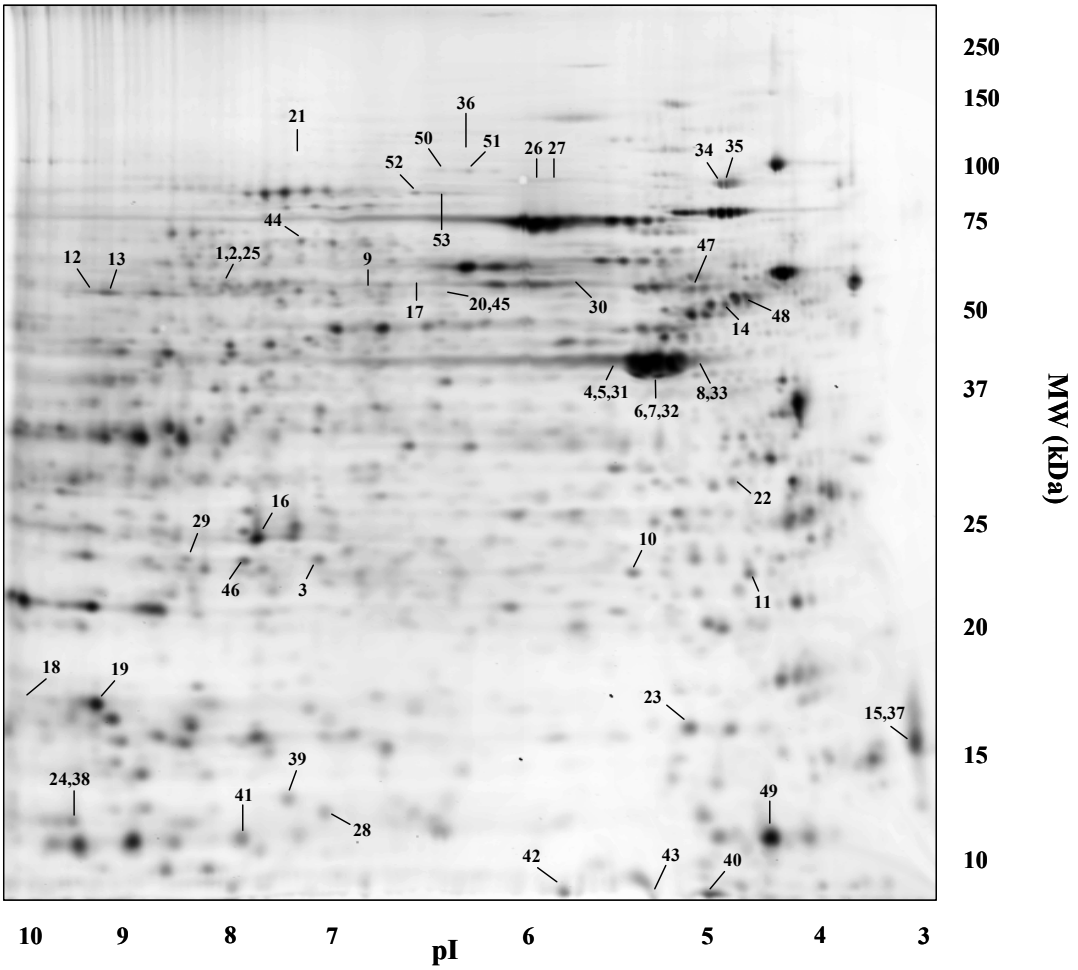


Fig. 3.4 2D-DIGE gel representing differentially expressed proteins identified in the colon tissue of *Il10*$^{-/-}$ mice compared to C57 mice at 7 and 12 weeks of age, respectively. Protein annotations are shown in Table 3.4. The approximate p*I* and molecular weight (MW) in kDa is given on the x and y axes.

Table 3.4 Proteins differentially expressed in the colon of *Il10*$^{-/-}$ relative to C57 mice at 7 and 12 weeks of age

Spot	Accession number	Protein description	Gene (MGI)	Theoretical M_r (kDa)	Theoretical p*I*	No. peptides matched	Sequence coverage %	SEQUEST P (pro)	Fold change
7 weeks									
1	gi|157951604	CAP, adenylate cyclase-associated protein 1	*Cap1*	51.5	7.29	2	4.2	9.50E-05	-2.9
2	gi|6680027	glutamate dehydrogenase 1	*Glud1*	61.3	7.96	3	6.1	2.85E-10	-2.9
3	gi|6671549	peroxiredoxin 6	*Prdx6*	24.8	5.96	5	27.2	3.99E-08	3.4
12 weeks									
4	gi|6752954	actin, gamma, cytoplasmic 1	*Actg1*	41.8	5.20	10	38.9	7.54E-12	-3.2
5	gi|6671507	actin, alpha 2, smooth muscle, aorta	*Acta2*	42.0	5.12	3	13.3	2.13E-06	-3.2
6	gi|6752954	actin, gamma, cytoplasmic 1	*Actg1*	41.8	5.20	9	30.7	2.76E-10	-7.4
7	gi|148666671	actin, gamma 2, smooth muscle, enteric	*Actg2*	41.9	5.20	4	20.7	1.50E-06	-7.4
8	gi|6752954	actin, gamma, cytoplasmic 1	*Actg1*	41.8	5.20	6	15.7	7.93E-07	-2.9
9	gi|21312260	aldehyde dehydrogenase 1 family, member B1	*Aldh1b1*	57.5	6.61	8	16.6	2.88E-09	-3.3
10	gi|160333304	apolipoprotein A-I	*Apoa1*	30.6	5.57	4	16.7	5.56E-12	2.4
11	gi|33563236	Rho, GDP dissociation inhibitor (GDI) beta	*Arhgdib*	22.8	4.80	4	22.5	4.11E-13	2.9
12	gi|6680748	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	*Atp5a1*	59.7	9.53	15	35.1	3.40E-12	-2.0
13	gi|6680748	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	*Atp5a1*	59.7	9.53	12	27.8	1.72E-12	-2.1
14	gi|31980648	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit	*Atp5b*	56.3	5.07	16	41.8	5.47E-12	-2.1
15	gi|6753244	calmodulin 1	*Calm1*	16.8	3.93	3	32.9	6.21E-11	5.0
16	gi|145301561	carbonic anhydrase 1	*Car1*	28.3	6.49	7	36.4	4.32E-10	2.3
17	gi|126521835	chaperonin subunit 2 (beta)	*Cct2*	57.4	5.96	11	26.7	2.76E-09	-2.2
18	gi|6680924	cofilin 1, non-muscle	*Cfl1*	18.5	8.20	4	18.8	8.89E-09	2.9
19	gi|6680924	cofilin 1, non-muscle	*Cfl1*	18.5	8.20	4	18.8	1.28E-09	2.5
20	gi|6755522	evolutionarily conserved signalling intermediate in Toll pathway	*Ecsit*	49.7	6.14	2	4.1	1.54E-05	-2.3
21	gi|33859482	eukaryotic translation elongation factor 2	*Eef2*	95.3	6.30	10	13.4	8.98E-12	-5.8
22	gi|123261836	EF hand domain containing 2	*Efhd2*	26.8	4.92	5	24.2	3.42E-08	2.0

23	gi|31712036	eukaryotic translation initiation factor 5A	*Eif5a*	16.8	4.94	3	20.8	9.81E-12	2.2
24	gi|14149635	fatty acid binding protein 4, adipocyte	*Fabp4*	14.6	8.74	5	48.5	1.28E-08	2.9
25	gi|6680027	glutamate dehydrogenase 1	*Glud1*	61.3	7.96	10	20.1	7.64E-11	-2.9
26	gi|123123547	gelsolin	*Gsn*	85.9	5.78	5	10.4	5.61E-07	-2.3
27	gi|123123547	gelsolin	*Gsn*	85.9	5.78	3	4.4	2.19E-07	-2.5
28	gi|56238582	histidine triad nucleotide binding protein	*Hint1*	13.8	6.41	2	16.7	1.39E-10	2.1
29	gi|62024583	Cr1 protein	*Igkv1-117*	26.3	7.97	2	11.3	2.05E-09	2.2
30	gi|114145561	keratin complex 2, basic, gene 8	*Krt8*	54.5	5.59	16	39.6	1.20E-11	-2.0
31	gi|123236748	keratin complex 1, acidic, gene 19	*Krt19*	44.5	5.15	6	12.9	6.39E-10	-3.2
32	gi|123236748	keratin complex 1, acidic, gene 19	*Krt19*	44.5	5.15	13	45.4	1.82E-08	-7.4
33	gi|123236748	keratin complex 1, acidic, gene 19	*Krt19*	44.5	5.15	9	25.3	8.96E-10	-2.9
34	gi|40556608	heat shock protein 1, beta	*Hsp90ab1*	83.2	4.82	15	22.1	2.47E-10	-2.8
35	gi|40556608	heat shock protein 1, beta	*Hsp90ab1*	83.2	4.82	17	25.3	1.01E-11	-2.1
36	gi|26984237	mitochondrial ATP-dependent protease Lon	*Lonp1*	10.6	6.20	4	4.0	5.94E-05	-3.0
37	gi|149266035	PREDICTED: similar to non-muscle myosin alkali light chain	*Myl6*	13.0	4.42	3	34.5	7.28E-06	5.1
38	gi|6755040	profilin 1	*Pfn1*	15.0	8.43	6	55.7	1.42E-12	2.9
39	gi|51772387	PREDICTED: similar to 40S ribosomal protein S12	*Rsp12*	14.5	6.94	2	12.1	4.18E-04	2.1
40	gi|33859624	S100 calcium binding protein A4	*S100a4*	11.7	5.07	2	17.8	1.43E-04	2.4
41	gi|6677837	S100 calcium binding protein A9 (calgranulin B)	*S100a9*	13.0	6.76	4	36.3	7.54E-06	4.0
42	gi|18314646	S100 calcium binding protein A11 (calgizzarin)	*S100a11*	11.1	5.15	1	16.3	1.65E-04	5.1
43	gi|18314646	S100 calcium binding protein A11 (calgizzarin)	*S100a11*	11.1	5.15	1	16.3	3.53E-05	3.2
44	gi|14389431	stress-induced phosphoprotein 1	*Stip1*	62.5	6.39	7	11.6	1.31E-05	-3.9
45	gi|27754031	sorting nexin 6	*Sx6*	45.0	5.47	3	7.14	2.62E-05	-2.3
46	gi|226958349	triosephosphate isomerase 1	*Tpi1*	26.7	7.07	3	19.3	6.59E-09	2.1
47	gi|6678469	tubulin, alpha 1C	*Tuba1c*	50.1	4.83	12	34.5	1.26E-11	-2.0
48	gi|7106439	tubulin, beta 5	*Tubb5*	49.7	4.64	9	34.7	1.30E-11	-2.2
49	gi|123210316	thioredoxin 1	*Txn1*	11.7	4.64	4	31.4	4.27E-07	2.5
50	gi|148877507	villin 1	*Vil1*	92.7	5.66	13	18.7	8.57E-09	-3.2
51	gi|148877507	villin 1	*Vil1*	92.7	5.66	20	26.4	4.52E-08	-4.6
52	gi|83921618	villin 2	*Vil2*	69.4	5.76	7	14.8	3.15E-09	-2.4
53	gi|83921618	villin 2	*Vil2*	69.4	5.76	8	15.9	1.99E-08	-2.6

Table 3.5 Categories of genes with expression increase of 2-fold or more in *Il10*$^{-/-}$ relative to C57 mice at 7 weeks of age using EASE analysis

System	Gene category	EASE score	FDR
GO Cellular Component	spindle cell	3.91E-04	<0.001
GO Molecular Function	MHC class II receptor activity	1.25E-03	<0.001
GO Biological Process	antigen processing, exogenous antigen via MHC class II	1.37E-03	<0.001
GO Biological Process	antigen presentation, exogenous antigen	1.37E-03	<0.001
GO Biological Process	alcohol metabolism	2.27E-03	<0.001
GO Biological Process	glucose metabolism	4.37E-03	<0.001
GO Biological Process	main pathways of carbohydrate metabolism	6.39E-03	<0.001
GO Biological Process	protein targeting	7.76E-03	<0.001
GO Biological Process	steroid biosynthesis	1.02E-02	<0.001
GO Biological Process	steroid metabolism	1.09E-02	<0.001
GO Biological Process	sterol biosynthesis	1.29E-02	<0.001
GO Cellular Component	microtubule cytoskeleton	1.75E-02	<0.001
GO Biological Process	sterol metabolism	1.82E-02	<0.001
GO Biological Process	carbohydrate metabolism	1.89E-02	<0.001
GO Biological Process	hexose metabolism	1.92E-02	<0.001
GO Molecular Function	oxidoreductase activity, acting on CH-OH group of donors	1.96E-02	<0.001
GO Biological Process	energy derivation by oxidation of organic compounds	2.47E-02	<0.001
GO Biological Process	catabolism	2.54E-02	<0.001
GO Cellular Component	soluble fraction	2.82E-02	<0.001
GO Molecular Function	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	2.92E-02	<0.001
GO Biological Process	monosaccharide metabolism	3.13E-02	<0.001

Table 3.6 Proteome and transcriptome network analysis in the colon of 7- and 12-week-old *Il10*$^{-/-}$ relative to C57 mice using IPA analysis

Network	Top functional categories
***Il10*$^{-/-}$ *vs.* C57 mice at 7 weeks of age**	
Transcriptome 1	Cancer, Dermatological disease and conditions, Endocrine system development and function
Transcriptome 2	Cardiovascular system development and function, Cancer, Tissue development
Transcriptome 3	Cancer, Cell death, Reproductive system disease
Transcriptome 4	Cell cycle, Cell-mediated immune response, Cancer
Transcriptome 5	Cellular growth and proliferation, Haematological system development and function, Haematopoiesis
***Il10*$^{-/-}$ *vs.* C57 mice at 12 weeks of age**	
Proteome 1	Tissue morphology, Cellular movement, Cell-mediated immune response
Proteome 2	Cell death, Haematological disease, Immunological disease
Proteome 3	Cancer, Tumour morphology, Cellular development
Proteome 4	Cardiovascular disease, Gene expression, Molecular transport
Transcriptome 1	Antigen presentation, Cell morphology, Cell-to-cell signalling and interaction
Transcriptome 2	Cancer, Gastrointestinal disease, Dermatological diseases and conditions
Transcriptome 3	Lipid metabolism, Small molecule biochemistry, Carbohydrate metabolism
Transcriptome 4	Inflammatory disease, Cell-to-cell signalling and interaction, Cell-mediated immune response

The top 3 biological functions for the most significant proteomic and transcriptomic networks (representing subsets of focus genes and proteins highly associated with those functions) are shown.

Table 3.7 Differentially expressed genes in the colon of *Il10*$^{-/-}$ relative to C57 mice at 7 and 12 weeks of age

Gene symbol	Gene name	GenBank accession	*Il10*$^{-/-}$ *vs.* C57 7 weeks of age		*Il10*$^{-/-}$ *vs.* C57 12 weeks of age	
			FC	FDR	FC	FDR
Apaf1	apoptotic peptidase activating factor 1	AK220340	1.6	<0.001	1.7	<0.001
B2m	beta-2-microglobulin	NM_009735	-1.1	0.506	2.3	<0.001
Bcl3	B-cell CLL/lymphoma 3	NM_033601	1.6	0.044	1.7	0.015
C1qa	complement component 1, q subcomponent, A chain	NM_007572	1.1	0.67	1.6	0.003
C1qc	complement component 1, q subcomponent, C chain	NM_007574	1.3	0.29	1.8	0.005
C1r	complement component 1, r subcomponent	NM_023143	1.5	0.018	2.0	<0.001
C1s	complement component 1, s subcomponent	NM_144938	1.3	0.059	1.9	<0.001
C2	complement component 2	NM_013484	1.2	0.337	2.6	<0.001
C3	complement component 3	NM_009778	1.8	0.015	4.5	<0.001
C4b	complement component 4B (Chido blood group)	NM_009780	-1.0	0.976	1.6	0.005
Casp7	caspase 7, apoptosis-related cysteine peptidase	NM_007611	2.0	<0.001	1.4	0.002
Ccl5	chemokine (C-C-motif) ligand 5 (Rantes)	NM_013653	1.1	0.70	2.3	<0.001
Cd74	CD74 molecule, major histocompatibility complex, class II invariant chain	NM_010545	4.0	<0.001	5.5	<0.001
Col18a1	collagen, type XVIII, alpha 1	NM_009929	-1.2	0.396	1.7	0.002
Col1a1	collagen, type I, alpha 1	NM_007742	-1.1	0.816	2.1	<0.001
Col1a2	collagen, type I, alpha 2	NM_007743	-1.2	0.51	1.6	0.02
Ebp	emopamil binding protein (sterol isomerase)	NM_007898	1.6	<0.001	1.0	0.89
Fas	Fas (TNF receptor superfamily, member 6)	NM_007987	1.9	<0.001	1.5	<0.001
Fcgr1a	Fc fragment of IgG, high affinity Ia, receptor (CD64)	NM_010186	1.3	0.139	1.6	0.002
Fcgr2a	Fc fragment of IgG, low affinity IIa, receptor (CD32)	NM_010188	1.2	0.297	1.5	0.001
Fcgr2b	Fc fragment of IgG, low affinity IIb, receptor (CD32)	NM_010187	1.1	0.691	1.7	0.003
Fdps	farnesyl diphosphate synthase (farnesyl pyro-phosphate synthetase, dimethylallyltranstrans-ferase, geranyltranstransferase)	NM_134469	1.7	0.003	1.3	0.152
Gsn	gelsolin	NM_146120	-1.8	0.006	-2.0	<0.001
Gzmb	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	NM_013542	1.4	0.015	1.9	<0.001
Hla-a	major histocompatibility complex, class I, A	NM_010391	1.4	0.201	2.3	<0.001
Hla-b	major histocompatibility complex, class I, B	NM_008199	-1.4	0.104	1.9	<0.001
Hla-c	major histocompatibility complex, class I, C	NM_010380	1.4	0.005	2.3	<0.001

Hla-dma	major histocompatibility complex, class II, DM alpha	NM_010386	1.5	<0.001	2.1	<0.001
Hla-dmb	major histocompatibility complex, class II, DM beta	NM_010387	4.9	<0.001	6.6	<0.001
Hla-dqa1	major histocompatibility complex, class II, DQ alpha 1	NM_010378	1.8	<0.001	2.9	<0.001
Hla-dqb2	major histocompatibility complex, class II, DQ beta 2	NM_010379	3.1	<0.001	5.3	<0.001
Hla-drb1	major histocompatibility complex, class II, DR beta 1	NM_010382	2.5	<0.001	4.5	<0.001
Hla-g	major histocompatibility complex, class I, G	NM_013819	1.4	0.013	2.0	<0.001
Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	NM_008255	1.7	0.005	1.5	0.005
Icam1	intercellular adhesion molecule 1	BC008626	1.7	<0.001	2.1	<0.001
Idi1	isopentenyl-diphosphate delta isomerase 1	NM_177960	2.0	0.002	1.2	0.322
Ifng	interferon, gamma	NM_008337	1.4	0.071	1.7	0.003
Igh-1a	immunoglobulin heavy chain 1a (serum IgG2a)	XM_484178	-1.2	0.7	3.7	<0.001
Il1b	interleukin 1, beta	NM_008361	1.8	0.211	6.4	<0.001
Il1rn	interleukin 1 receptor antagonist	NM_031167	1.3	0.442	1.8	0.024
Irf1	interferon regulatory factor 1	NM_008390	2.4	<0.001	2.2	<0.001
Irf8	interferon regulatory factor 8	NM_008320	2.0	<0.001	2.2	<0.001
Ltb	lymphotoxin beta (TNF superfamily, member 3)	NM_008518	1.1	0.591	2.0	<0.001
Ly75	lymphocyte antigen 75	NM_013825	1.3	0.042	1.5	0.001
Mapk13	mitogen-activated protein kinase 13	NM_011950	1.8	<0.001	1.7	<0.001
Mapk9	mitogen-activated protein kinase 9	NM_016961	1.5	0.004	1.6	<0.001
Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM_010907	1.6	0.002	1.6	0.001
Nfkbie	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	NM_008690	1.5	0.001	1.7	<0.001
Psmb8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	NM_010724	2.5	<0.001	5.9	<0.001
Psmb9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	NM_013585	2.7	<0.001	4.7	<0.001
Sc5dl	sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, S. cerevisiae)-like	NM_172769	1.7	0.001	1.2	0.539
Socs1	suppressor of cytokine signalling 1	NM_009896	3.0	<0.001	2.7	<0.001
Sqle	squalene epoxidase	NM_009270	1.8	0.001	1.2	0.146
Stat1	signal transducer and activator of transcription 1, 91kDa	NM_009283	2.6	<0.001	3.7	<0.001
Stat2	signal transducer and activator of transcription 2, 113kDa	NM_019963	1.6	0.025	1.8	0.005
Tap1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	NM_013683	1.8	0.003	2.1	<0.001
Tap2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	NM_011530	1.2	0.105	2.0	<0.001
Tlr2	toll-like receptor 2	NM_011905	1.1	0.857	1.9	0.001
Tlr9	toll-like receptor 9	NM_031178	1.9	<0.001	1.9	<0.001
Tnf	tumour necrosis factor (TNF superfamily, member 2)	NM_013693	1.3	0.064	1.7	<0.001

Tnfrsf1b	tumour necrosis factor receptor superfamily, member 1B	NM_011610	1.3	0.115	1.5	0.003
Tp53	tumor protein p53	NM_011640	1.5	0.001	1.6	<0.001
Tyrobp	TYRO protein tyrosine kinase binding protein	NM_011662	1.2	0.306	1.8	<0.001

Genes were grouped into one or more of the following IPA pathways: antigen presentation, graft vs. host disease signalling, allograft rejection signalling, interferon signalling, role of PKR in interferon induction and antiviral response, dendritic cell maturation, complement system, ER stress and steroid metabolism. Genes with FC≥1.5 and FDR or q<0.05 were considered for pathway analysis. Shaded cells identify genes that were not differentially expressed in the *Il10*$^{-/-}$ *vs.* C57 comparison at 7 or 12 weeks of age.

Table 3.8 Categories of genes with expression increase of 2-fold or more in *Il10*$^{-/-}$ relative to C57 mice at 12 weeks of age using EASE analysis

System	Gene category	EASE score	FDR
GO Biological Process	defence response	5.92E-14	<0.001
GO Biological Process	response to biotic stimulus	7.39E-14	<0.001
GO Biological Process	response to external stimulus	1.38E-12	<0.001
GO Biological Process	immune response	4.06E-12	<0.001
GO Biological Process	antigen presentation	4.14E-07	<0.001
GO Biological Process	antigen processing	4.52E-06	<0.001
GO Molecular Function	defence/immunity protein activity	6.14E-06	<0.001
GO Molecular Function	MHC class I receptor activity	2.67E-05	<0.001
GO Biological Process	response to pest/pathogen/parasite	1.43E-04	<0.001
GO Biological Process	physiological process	1.47E-04	<0.001
GO Molecular Function	antigen binding	2.31E-04	<0.001
GO Biological Process	response to stress	2.49E-04	<0.001
GO Biological Process	antigen presentation, endogenous antigen	1.21E-03	<0.001
GO Molecular Function	MHC class II receptor activity	1.56E-03	<0.001
GO Biological Process	antigen processing, exogenous antigen via MHC class II	1.67E-03	<0.001
GO Biological Process	antigen presentation, exogenous antigen	1.67E-03	<0.001
GO Cellular Component	extracellular space	1.75E-03	<0.001
GO Biological Process	protein targeting	2.57E-03	<0.001
GO Biological Process	humoral immune response	2.97E-03	<0.001
GO Cellular Component	plasma membrane	7.23E-03	<0.001
GO Biological Process	antigen processing, endogenous antigen via MHC class I	8.44E-03	<0.001
GO Biological Process	response to wounding	1.07E-02	<0.001
GO Molecular Function	oxidoreductase activity, acting on CH-OH group of donors	1.15E-02	<0.001
GO Molecular Function	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	1.71E-02	<0.001
GO Molecular Function	hydrolase activity	1.86E-02	<0.001
GO Biological Process	collagen catabolism	2.07E-02	<0.001
GO Biological Process	posttranslational membrane targeting	2.18E-02	<0.001
GO Biological Process	innate immune response	2.22E-02	<0.001
GO Cellular Component	vesicular fraction	2.51E-02	<0.001
GO Biological Process	response to chemical substance	3.06E-02	<0.001
GO Biological Process	inflammatory response	3.06E-02	<0.001

3.5 Discussion

This study characterises colitis onset and progression at the histopathological, transcriptome and proteome level in bacterially-inoculated *Il10*$^{-/-}$ mice (C57BL/6J background). Here, we show that most *Il10*$^{-/-}$ mice developed moderate colitis already by 8.5 weeks of age and severe colitis peaking between 10 and 12 weeks of age when compared to 7-week-old *Il10*$^{-/-}$ mice. The nature of colon inflammation was similar to that displayed by conventionally housed *Il10*$^{-/-}$ mice on the same genetic background not subjected to bacterial inoculation (Kennedy *et al*., 2000) and represented features characteristic of human CD. Several aspects of innate and adaptive immune responses were affected at the gene expression level before colitis onset (7 weeks of age) and at severe colitis (12 weeks of age) in *Il10*$^{-/-}$ mice. These findings agree with the inflammatory and immune responses observed in CD patients who have a defective interaction between their innate mucosal immune system and luminal bacteria (Strober *et al*., 2007). This study focuses on those pathways linking the gene and protein expression changes such as cytoskeletal rearrangement, cell migration and innate immunity that underlie colitis development in this *Il10*$^{-/-}$ mouse model.

Increased expression levels of membrane-bound *Tlr2* and *Tlr9* genes in the colon of 12-week-old *Il10*$^{-/-}$ compared to C57 mice are in agreement with their function. Peptidoglycan and lipoproteins from the cell wall of commensal bacteria are recognised by TLR2 (Strober *et al*., 2007). The underlying abnormal response between the innate immune system to bacterial structures is mediated via TLR and other pattern-recognition receptors which then regulate antigen-specific adaptive immune responses (Honda & Takeda, 2009). Dendritic cells, an important cellular component of the mucosal innate immune system, sample intestinal bacteria through pattern recognition with TLR (Mueller & Macpherson, 2006). The recognition of bacterial structures by TLR2 can lead to activation of NFκB-MAPK pathways, and interferon regulatory factor family members (Lee *et al*., 2006). This activation in turn induces differentiation of cells producing pro-inflammatory cytokines, such as IFNγ and IL17, responsible for CD-like inflammation (Strober *et al*., 2007). TLR9 stimulation in intestinal epithelial cells has been associated with maintenance of intestinal homeostasis (Lee *et al*., 2006). Increased mRNA abundance of the *Tlr9* gene in non-inflamed (7 weeks) and severely inflamed (12 weeks of age) *Il10*$^{-/-}$ mice compared to C57 mice indicated signalling

events probably to maintain colonic homeostasis. Increased expression levels of pro-inflammatory cytokine genes such as *Ifng*, *Tnfa* and *Il1b* genes were observed only in inflamed (12 weeks of age) *Il10*$^{-/-}$ mice. These TLR-dependent innate immune responses are important in colitis development of *Il10*$^{-/-}$ mice in order to adapt to luminal bacteria and their antigens.

The higher expression levels of MHC class I genes in the colon of *Il10*$^{-/-}$ compared to C57 mice at 12 weeks was likely to mount antigen-specific adaptive immune responses to bacterial invasion. Also the expression levels of the MHC class II genes were increased in 12-week-old and, to a lesser extent, in 7-week-old *Il10*$^{-/-}$ compared to C57 mice. In search of antigen-presenting dendritic cells, human $CD4^+$ T lymphocytes migrate within collagen matrices (Samstag *et al*., 2003). The increased expression levels of collagen genes type I and XVIII in the colon of the 12-week-old *Il10*$^{-/-}$ compared to C57 mice suggests elevated degradation of excess collagen in order to reorganise connective tissue and stabilise the intestinal barrier. Enhanced T cell migration as a consequence of the inflammatory state of the intestine is maintained by pro-inflammatory cytokines and has been associated with increased expression of gelsolin, a calcium-dependent actin-binding protein in intestinal smooth muscle cells (Ehrlich *et al*., 2000). The expression of *Gsn* gene coding for gelsolin (GSN) was decreased in the colon of the 12-week-old *Il10*$^{-/-}$ mice suggesting that colitis might be starting to decline. This was supported by the decrease in colon HIS from 12 weeks to 14 weeks of age and might be associated with reduced cell migration.

Most of the proteins differentially expressed in inflamed *Il10*$^{-/-}$ mice were involved in cytoskeletal rearrangement, cell-mediated immune response (e.g. Fcy receptor-mediated phagocytosis in macrophages and monocytes) and pathogen-influenced signalling (e.g. mechanisms of viral exit from host cells). The cytoplasmic actin gamma1 and smooth muscle actin gamma2 (ACTG1 and ACTG2) proteins were less abundant in the colon of *Il10*$^{-/-}$ compared to C57 mice at 12 weeks of age. Pro-inflammatory cytokines and bacteria can modify tight junctions interconnecting intestinal epithelial cells via the actin cytoskeleton, so disruption of actin leads to disruption of tight junctions and loss of barrier function with increased paracellular permeability (Shen & Turner, 2006). This is further supported by a study reporting decreased actin-binding gene expression levels in peripheral blood mononuclear cells of IBD patients suggesting disruptions of the

actin cytoskeleton might contribute to CD pathogenesis in humans (Mannick *et al*., 2004). The protein findings were in agreement with the decreased expression levels of actin genes, e.g. *Actg2* (1.6-fold) in the 12-week-old *Il10*$^{-/-}$ mice compared to C57 mice; their levels were unchanged in *Il10*$^{-/-}$ mice at 7 weeks of age when the intestinal barrier was presumably still intact.

At 12 weeks of age, differentially expressed proteins that are involved in the regulation of actin-based motility by calcium-dependent RhoGTPase (e.g. Rho, protein kinaseC) were GSN, cofilin1 (CFL1) and profilin1 (PFN1) . Rho proteins share growth-promoting and anti-apoptotic functions, regulation of gene expression through activation of signalling molecules, e.g. NFκB, and promote actin cytoskeleton reorganisation (Wennerberg & Der, 2004). Rho GDP dissociation inhibitor (GDI) beta (Rho GDIβ) protein, which is encoded by the *Arhgdib* gene (mRNA abundance was increased in *Il10*$^{-/-}$ mice at 12 weeks of age), regulates RhoGTPase activity by inhibiting GDP dissociation to leave RhoGTPases inactive (Huang *et al*., 2009). This suggests that in the inflamed colon of those *Il10*$^{-/-}$ mice, Rho protein activity is being inhibited by Rho GDIβ and actin cytoskeleton reorganisation does not get activated. Increased expression levels of GSN have been reported in smooth muscle cells in the small intestine of CD patients and correlated with increased migration of smooth muscle cells in response to inflammation (Ehrlich *et al*., 2000). However, the expression level of the *Gsn* gene and GSN protein reduced in the colon of 12-week-old *Il10*$^{-/-}$ mice which might reflect a declining inflammatory cell movement. Gelsolin-null mice have shown reduced neutrophil and fibroblast movement (Dos Remedios *et al*., 2003). The expression levels of the actin-binding proteins, CFL1 and PFN1 were increased in *Il10*$^{-/-}$ mice at 12 weeks of age, but the abundance of their transcripts were unchanged. Cofilin activated through dephosphorylation regulates actin polymerisation and depolymerisation and is an essential protein for cell viability and survival (Samstag *et al*., 2003). Cofilin has also been associated with bacteria-induced phagocytosis (Bierne *et al*., 2001) and with enhancing Na^+,K^+-ATPase activity thus linking the cytoskeleton with ion transporter activity (Lee *et al*., 2001).

It is still unclear how the proteins that control actin dynamics trigger T cell activation, which also depends on the physiological status (e.g. health or disease) in which the cellular immune response is being modulated (Burkhardt *et al*., 2008). The actin

cytoskeleton is a flexible system that is built up or broken down depending on antigen recognition, cell polarisation, and cell adhesion or cell migration. Actin-binding proteins are involved in surface receptor clustering during T cell activation and migration to dynamic cytoskeletal rearrangements at the interface between T cells and antigen presenting cells (Samstag *et al*., 2003). A study using an *in vitro* model of simulated ischemia–reperfusion showed an involvement of Rho-kinase-dependent cytoskeletal rearrangement in apoptosis initiation (van der Heijden *et al*., 2008). In the colon of the 12-week-old *Il10*$^{-/-}$ mice, Rho-kinase inhibition by Rho GDIβ may have inhibited or delayed actin rearrangement, and in part attenuated apoptosis, anti-apoptosis genes such as *Bcl2a1* gene were increased, presumably to enhance the survival of activated T cells and maintenance of inflammation. This is in line with the reported abnormal activation of mucosal T lymphocytes against bacterial antigens which triggers intestinal inflammation observed in CD patients (Shanahan, 2002).

In conclusion, the combined transcriptomic and proteomic approach identified distinct colonic gene and protein expression profiles for 7- and 12-week-old *Il10*$^{-/-}$ mice compared to C57 mice of the same age. Gene and protein expression data linked actin cytoskeleton dynamics and innate immunity and suggested a delayed remodelling process in the damaged colon tissue under inflammatory conditions. Immune response pathways were affected at both time points triggered by bacterial invasion, but particularly at 12 weeks of age. The gene and protein expression changes observed here mostly clustered in different pathways and represent complementary datasets. This may be due to post-transcriptional or post-translational modifications to genes and proteins, respectively, which can impact biological events and processes in cells.

Chapter 4

Comparison of transcriptomic and proteomic profiles of interleukin-10 gene-deficient and control mice fed AIN-76A or oleic acid-enriched diet

Part of the material presented in this chapter has been submitted as a paper:

Knoch B, Barnett MPG, Cooney J, McNabb WC, Barraclough D, Laing W, & Roy NC: Dietary oleic acid as control fatty acid for multi-omics studies: an investigation using interleukin-10 gene-deficient mice. Submitted to *Biotechnol J*

4.1 Abstract

Oleic acid (OA) has been used as a control fatty acid in dietary PUFA intervention studies due to its lack of effect on eiconasoid biosythesis. Since the use of OA as an appropriate control fatty acid remains to be confirmed for multi-omic studies, here the hypothesis was that colonic transcriptome and proteome profiles of colitic *Il10*$^{-/-}$ mice (compared to C57 mice) fed AIN-76A diet would cluster with *Il10*$^{-/-}$ mice (compared to C57 mice) fed an OA-enriched diet. A secondary aim was to identify overlapping colonic gene and protein expression patterns in *Il10*$^{-/-}$ and C57 mice fed AIN-76A or OA diets. Overall, a close clustering of colonic gene and protein expression profiles between the mice fed the AIN-76A or OA diet was observed. Inflammation-induced regulatory processes associated with cellular and humoral immune responses, cellular stress response and metabolic processes related to energy utilisation were identified in *Il10*$^{-/-}$ mice fed either diet. OA is a suitable unsaturated fatty acid for use in multi-omic studies. The general lack of overlap observed between transcriptomic and proteomic profiles for each diet comparison (*Il10*$^{-/-}$ compared to C57 mice) supports a systems biology approach to understand the effects of diet on gene expression.

4.2 Introduction

For a comprehensive understanding of metabolic processes, their complex interactions, and especially to address functional aspects in response to dietary intervention in health and disease, a systems biology approach, including gene and protein expression profiles, becomes important (de Roos & McArdle, 2008). Using oligonucleotide arrays, a number of mRNA expression and pathway changes that occur with the onset of colitis in interleukin-10 gene-deficient (*Il10*$^{-/-}$) mice have been reported (Roy *et al*., 2007). The *Il10*$^{-/-}$ mouse model of inflammatory bowel disease (IBD) is characterised by deregulated immune responses and inflammatory processes leading to intestinal tissue damage (Kühn *et al*., 19993). Studies have reported expression changes in intestinal epithelial cell-derived proteins isolated from germ-free and *E. faecalis*-monocolonised *Il10*$^{-/-}$ and wildtype mice (Werner *et al*., 2007; Hoffmann *et al*., 2008), and from IBD patients and healthy subjects (Shkoda *et al*., 2007). No single study has yet compared changes in both the transcriptome and proteome associated with colon inflammation *in vivo*.

OA, an n-9 monounsaturated fatty acid mainly found in olive oil, has been implicated (in combination with the antioxidant content of olive oil) in the reduction of cardiovascular disease and rheumatoid arthritis attributed to the Mediterranean diet (Wahle *et al*., 2004; Shaikh *et al*., 2008). The study by Shaikh *et al*. (2008) showed that OA-treatment of antigen-presenting cells had no effect on antigen presentation. OA prevented the inhibition of immune response driven through the MHC class I antigen presentation pathway normally observed as a result of exposure to palmitic acid. Other studies have shown that dietary OA does not alter tissue AA levels, has no effect on 2-series eicosanoid biosynthesis (McEntee *et al*., 2008), and is neutral to lipid metabolism (Rioux *et al*., 2008). In contrast, diets enriched in *trans* fatty acids and saturated fatty acids can increase plasma markers of inflammation in humans, such as pro-inflammatory cytokines, adhesion molecules and acute phase proteins, implicated as risk factors for cardiovascular diseases (Baer *et al*., 2004). The same study reported that the OA-enriched diet did not alter any of the inflammation markers compared to the control diet and pointed to the importance of a threshold intake of fatty acids necessary to increase or decrease inflammation, as diets high in OA (e.g., a Mediterranean-type diet), can have beneficial effects on health in high-risk populations (Wahle *et al*., 2004; Baer *et al*., 2004). Based on its lack of effect on eicosanoid biosynthesis, OA has been used as a control fatty acid to test the effect of other unsaturated fatty acids in several studies (Li *et al*., 1994; Hansen Petrik *et al*., 2000; McEntee *et al*., 2008; Ramakers *et al*., 2008), but the use of OA as an appropriate control fatty acid remains to be confirmed for multi-omic studies.

Here, the hypothesis was that colonic transcriptome and proteome profiles of colitic *Il10*$^{-/-}$ mice (compared to C57 mice) fed a standard AIN-76A diet would cluster with those of colitic *Il10*$^{-/-}$ mice (compared to C57 mice) fed an OA-enriched AIN-76A diet. Transcriptomic and proteomic profiles of *Il10*$^{-/-}$ mice (compared to C57 mice) fed the AIN-76A diet were compared with the profiles of *Il10*$^{-/-}$ mice (compared to C57 mice) fed an OA diet. A secondary aim was to identify overlapping gene and protein expression patterns in the colon of *Il10*$^{-/-}$ and C57 mice fed AIN-76A or OA diets.

4.3. Materials and methods

4.3.1 Animals and diets

Twelve male *Il10*$^{-/-}$ (C57BL/6J background, formal designation B6.129P2-*Il10*<tm1Cgn>/J) mice and 12 male C57 wildtype (C57BL/6J) mice, 35 days or 5 weeks of age at the start of the study were maintained for 6 weeks on an AIN-76A standard diet or on an AIN-76A-based diet enriched with OA. This was part of the PUFA intervention study described in Chapters 5 and 6. The OA diet was formulated to contain AIN-76A (fat-free) + 1% corn oil, supplemented with 0.2% purified LA and 0.06% ALA ethyl esters and 3.4% OA ethyl ester. The diets were isocaloric and varied only in lipid composition, both diets containing 5% fat (w/w), the usual fat content of the AIN-76A diet. The diet compositions are described in Materials and Methods chapter 2.2. The killing of the mice at the end of the PUFA intervention study was done in randomised order and the AIN-76A and OA group were used in this study for more detailed analysis.

Note: *The lipid and fatty acid analysis of the diets was performed by Michael Agnew at AgResearch and Fliss Jackson at Massey University, respectively, before the start of the experiment*

Induction of colitis, the fast-feed period and sampling procedure are all described in Materials and Methods chapter 2.1.2 and 2.1.4. Plasma was separated from the blood, frozen in liquid nitrogen and stored at −85°C for serum amyloid A (SAA) analysis. One full-thickness colon section was stored at room temperature in 10% phosphate-buffered formaldehyde for histopathological assessment, and another immediately frozen in liquid nitrogen and stored at −85°C for gene and protein expression profiling. Histological analysis of the colon and SAA measurement in the plasma are described in Materials and Methods chapter 2.3.1 to 2.3.3.

Note: *Tissue processing and histopathological assessment was conducted by Dr Shuotun Zhu at The University of Auckland. The SAA analysis was performed by Kim Oden at AgResearch.*

4.3.2 RNA isolation, microarrays and qRT-PCR

RNA isolation, microarray design, hybridisation and analysis and qRT-PCR are all described in Materials and Methods chapter 2.4.1, 2.4.2, 2.4.3, 2.4.4 and 2.5. Equimolar pools of colon RNA extracts (2-3 samples/pool) were used in the reference microarray design. Array data were submitted to the Gene Expression Omnibus, accession number GSE12028. Ten genes (*Abcb1a*, *Aldh1a1*, *Ces2*, *Fabp2*, *Igfbp5*, *Il1b*, *Mmp13*, *Ppara,*

Srebf1 and *Sult1a1*) from the microarray experiment were validated by qRT-PCR. The primer sequences for target and reference genes are listed in Materials and Methods chapter Table 2.2. Calnexin (*Canx*) was chosen as a normalisation reference gene.

4.3.3 Protein isolation and identification by LC-MS/MS data analyses

Protein isolation and identification by LC-MS/MS analyses are described in Materials and Methods chapter 2.6.1 to 2.6.3. The protein-containing lower layer of the same TRIzol-processed sample from which RNA was derived was used for protein isolation. Gels were run as duplicate biological replicates using the same pooled samples as described for the microarray design.

Note: *Protein isolation was conducted by the PhD candidate, LC-MS analysis of peptides and MS/MS data processing and analysis was conducted with assistance from Diane Barraclough and Drs William Laing and Janine Cooney from Plant & Food Research, all collaborators in Nutrigenomics NZ.*

4.3.4 Bioinformatics analysis

The bioinformatics analysis is described in Materials and Methods chapter 2.7.1. For the experimental comparisons (AIN-76A diet *Il10*$^{-/-}$ *vs.* C57 mice, OA diet *Il10*$^{-/-}$ *vs.* C57 mice and *Il10*$^{-/-}$ mice OA *vs.* AIN-76A diet), a candidate list of differentially expressed genes and proteins was generated. Probes from the microarray dataset that satisfied the cut-off criteria for FC≥1.3 (up- or down-regulated) and $P<0.01$ were considered for the pathway and functional analysis using IPA (Version 7.0, Ingenuity Systems Inc., Redwood City, CA, USA). This FC was applied in a recent study showing that a FC>1.6 can underestimate the number of differentially expressed genes regulated by dietary treatment (Sülzle *et al*., 2004).

Note: *The microarray analysis in Bioconductor (written in R) was conducted with assistance of Zaneta Park, a statistician at AgResearch.*

4.3.5 Statistical analyses

SAS (9.1.3 Service Pack 4, SAS Institute Inc., Cary, NC, USA) was used for statistical analyses of body weight and dietary intakes, and GenStat (10th edition, VSN International, Hemel Hempstead, UK) used to analyse HIS, SAA and qRT-PCR data. Analysis of repeated measurements with ante-dependence and auto-regressive variance-

covariance structures was used to fit the body weight and dietary intake data and to test for treatment differences in each mouse strain over the experimental period. For ANOVA analyses, log-transformations of raw data were performed in cases of unequal variances. Differences in HIS between AIN-76A and OA diets for each mouse strain were analysed using an ANOVA with pooled variance. A constant was added to the SAA data prior to log-transformation to avoid negative values found in C57 mice. Differences were considered significant at $P<0.05$. One *Il10*$^{-/-}$ mouse from the OA diet group died during the experiment due to unknown causes. This animal was not scored for histological signs of inflammation and was not included in the statistical analysis of HIS.

4.4 Results

4.4.1 Phenotypic effects of AIN-76A and OA diet

Body weight and dietary intake of the mice during the development of colon inflammation are shown in Fig. 4.1. There was no difference in mean body weight between AIN-76A- and OA-fed *Il10*$^{-/-}$ or C57 mice over time. The dietary intake of the AIN-76A *Il10*$^{-/-}$ group was higher compared to the OA *Il10*$^{-/-}$ group at days 47, 58, 61, 63, 68 and 70, and between AIN-76A- and OA-fed C57 mice at days 61, 70 and 75. This did not affect the bodyweight. The analysed level of OA ethyl ester was 3.6% resulting in an intake of 0.14 g/d of OA when mice were offered approximately 4 g of diet daily.

The colon sections of *Il10*$^{-/-}$ mice fed AIN-76A and the OA diet showed signs of high (total colon HIS: 7.75) and moderate (total colon HIS: 7.00) inflammation, which was mostly derived from inflammatory cell infiltrates. The colons of AIN-76A- and OA-fed C57 mice showed no sign of inflammation, with a mean total HIS score of 0.17 and 0.13, respectively. No statistical difference was observed for total colon HIS or any of the inflammatory aspects scored between the diets for *Il10*$^{-/-}$ and C57 mice (Table 4.1).

The mean plasma SAA concentration from *Il10*$^{-/-}$ mice fed AIN-76A diet was 787 µg/mL, and 538 µg/mL for the OA diet. In C57 mice fed either the AIN-76A (63 µg/mL) or the OA (24 µg/mL) diet, the SAA concentration was lower than the *Il10*$^{-/-}$ mice fed the same diet ($P<0.05$). Plasma SAA was not different ($P>0.05$) between the

two diets within each strain. Thus, both local (tissue histology) and systemic (SAA) inflammation levels for *Il10*$^{-/-}$ or C57 mice on AIN-76A and the OA diet showed similar patterns (Table 4.1).

4.4.2 Gene expression effects of AIN-76A and OA diet in *Il10*$^{-/-}$ compared to C57 mice

To investigate the effects of AIN-76A and OA diet on expression of genes involved in colon inflammation, whole genome microarray data of colon tissue from *Il10*$^{-/-}$ mice on the AIN-76A or OA diet were compared with C57 mice fed the same diet. To focus on the predominant changes in metabolic and regulatory processes, the top 5 regulated pathways of the AIN-76A and the OA diet comparison (*Il10*$^{-/-}$ *vs.* C57 mice) were considered. The top 5 pathways for the *Il10*$^{-/-}$ *vs.* C57 mice comparisons on either the AIN-76A diet or the OA diet comprised 162 probes corresponding to 119 unique genes. Of these, 35 genes were differentially expressed with the AIN-76A diet (*Il10*$^{-/-}$ *vs.* C57), 41 genes with the OA diet (*Il10*$^{-/-}$ *vs.* C57) comparison, and about 40% of genes (43) showed expression changes common to both diets. Pathway analysis showed that the most affected pathways (P<0.05) in those comparisons were signalling processes with 25 to 40% of genes involved in these pathways. These signalling pathways are shown as heatmaps (Fig. 4.2). Changes in colon gene expression levels seen in the AIN-76A diet (*Il10*$^{-/-}$ *vs.* C57) and the OA diet (*Il10*$^{-/-}$ *vs.* C57) comparison were associated with interferon signalling, fibrosis development and antigen presentation pathways. ER stress pathways and death receptor signalling pathways were primarily affected in the AIN-76A diet (*Il10*$^{-/-}$ *vs.* C57) comparison, whereas complement system and IL10 signalling were among the top 5 regulated pathways in the OA diet (*Il10*$^{-/-}$ *vs.* C57) comparison.

Signalling processes common to both diet comparisons showed increased expression levels of genes in cellular and humoral immune response and cytokine signalling. Detailed analyses of pathways of cellular immune response such as interferon signalling showed increased expression levels of interferon alpha receptor 2 (*Ifnar2*), *Ifng* and *Stat1* genes. The cytokine signalling pathways, e.g., IL10 signalling included up-regulated Fc region of IgG receptor IIa and b (*Fcgr2a*, *Fcgr2b*), *Il10*, *Il1a, Il1b*, nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor alpha and epsilon

(*Nfkbia* and *Nfkbie*) and *Tnf* gene. Highly expressed genes of the humoral immune response included genes of the complement component system (e.g., *C1qa*, *C1qc*, *C1r*, *C1s*, *C2* and *C4b*) and histocompatibility class II (e.g., *Hla-Dma*, *Hla-Dmb*, *Hla-Dqa1*, *Hla-Dqb2*, *Hla-Drb1*), proteasome subunit beta type 8 and 9 (*Psmb8*, *Psmb9*) and transporter1 ATP-binding cassette subfamily B (*Tap1*) for the AIN-76A diet and the OA diet (*Il10*$^{-/-}$ *vs.* C57) comparison.

In contrast to the OA-fed *Il10*$^{-/-}$ *vs.* C57 mice, those fed the AIN-76A diet had increased expression levels of genes associated with cellular stress response (e.g., ER stress pathway) and apoptosis (e.g., death receptor signalling pathway) in the colon of *Il10*$^{-/-}$ relative to C57 mice.

Functional analysis conducted with IPA for the AIN-76A diet and the OA diet (*Il10*$^{-/-}$ *vs.* C57) comparison shows the top 5 biological functions for each category and are listed in Table 4.2. Confirmation of the microarray analysis was performed on ten genes using qRT-PCR. Similar expression patterns were observed for the mean concentration ratios (n = 5-6/group) and the microarray analysis of pooled samples (Table 4.3). Gene expression changes using individual samples were significant for all genes except *Pparα* and *Srebf1* due to higher variability in expression between individuals.

Comparing the OA and AIN-76A diet within *Il10*$^{-/-}$ mice showed 47 differentially expressed genes and ten differentially expressed proteins. Only 3% of the genes were associated with the top 5 regulated pathways and were mostly down-regulated in OA- compared to AIN-76A-fed *Il10*$^{-/-}$ mice. Due to the relatively low gene and protein expression changes in OA- compared to AIN-76A-fed *Il10*$^{-/-}$ mice, no further analysis was carried out.

4.4.3 Protein expression effects of AIN-76A and OA diet in *Il10*$^{-/-}$ compared to C57 mice

To determine the effects of the two diets on the expression levels of proteins involved in colon inflammation, a comparative proteomics analysis was conducted using 2D-DIGE technology coupled with LC-MS/MS. A total of 257 consistent protein changes within the pooled biological replicates were identified (either the AIN-76A diet pool 1 and

pool 2 or the OA diet pool 1 and pool 2), with 172 protein changes consistent across both diets (52 decreased and 120 increased in expression). Eighty-seven protein spots which met the threshold criteria were selected for identification. These spot-features represented 62 unique proteins (excluding multiple isoforms due to post-translational modifications). In five cases, two proteins were identified in the same 2D gel spot-feature. Apart from three proteins, all identified proteins showed the same direction of fold abundance change across both diets (Fig. 4.3, 4.4 and Table 4.4). There was no evidence that the diets were different based on ANOVA and FDR correction for multiple testing using Benjamini & Hochberg (1995).

As for the gene expression profiles, pathway analysis in IPA comparing both diets for the top 5 pathways indicated a widespread down-regulation of metabolism related proteins, and up-regulation of proteins involved in cellular stress and inflammatory responses. These metabolic pathways included carbohydrate metabolism (e.g., glycolysis/gluconeogenesis; glyceraldehyde-3-phosphate dehydrogenase, GAPDH and starch and sucrose metabolism; UDP-glucose dehydrogenase, UGDH), lipid metabolism (e.g., synthesis and degradation of ketone bodies; 3-hydroxy-3-methylglutaryl-CoenzymeA synthase 2, HMGCS2), amino acid metabolism (e.g., valine, leucine and isoleucine degradation; aldehyde dehydrogenases, hydroxyacyl-Coenzyme A dehydrogenase) and energy metabolism (e.g., nitrogen metabolism; carbonic anhydrases). The abundance of proteins of cellular stress (e.g., ER stress; heat shock 70 kDa protein 5, HSPA5) and inflammatory response (e.g., NRF2-mediated oxidative stress and acute phase responses; superoxide dismutase 1, SOD1; thioredoxin 1, TXN1; apolipoprotein A1; peroxiredoxin 1, PRDX5; regenerating islet-derived 3 gamma and S100 calcium binding protein A 9) were increased in the colon of *Il10*$^{-/-}$ compared to C57 mice fed AIN-76A or OA diets (Table 4.4).

4.4.4 Comparison of transcriptomic and proteomic data

Comparing transcriptomic with proteomic data within each diet revealed that about 90% of the proteins detected by MS could be matched to a microarray probe, while eight proteins were not represented on the microarray. In total, only eight out of 62 unique proteins showing changes in abundance could be assigned to a differentially expressed (FC≥1.3, P>0.01) gene for both diets as shown in Table 4.5. Seven out of eight matches

showed a FC in the same direction, whereas tropomyosin2 was down-regulated at the gene and up-regulated at the protein level.

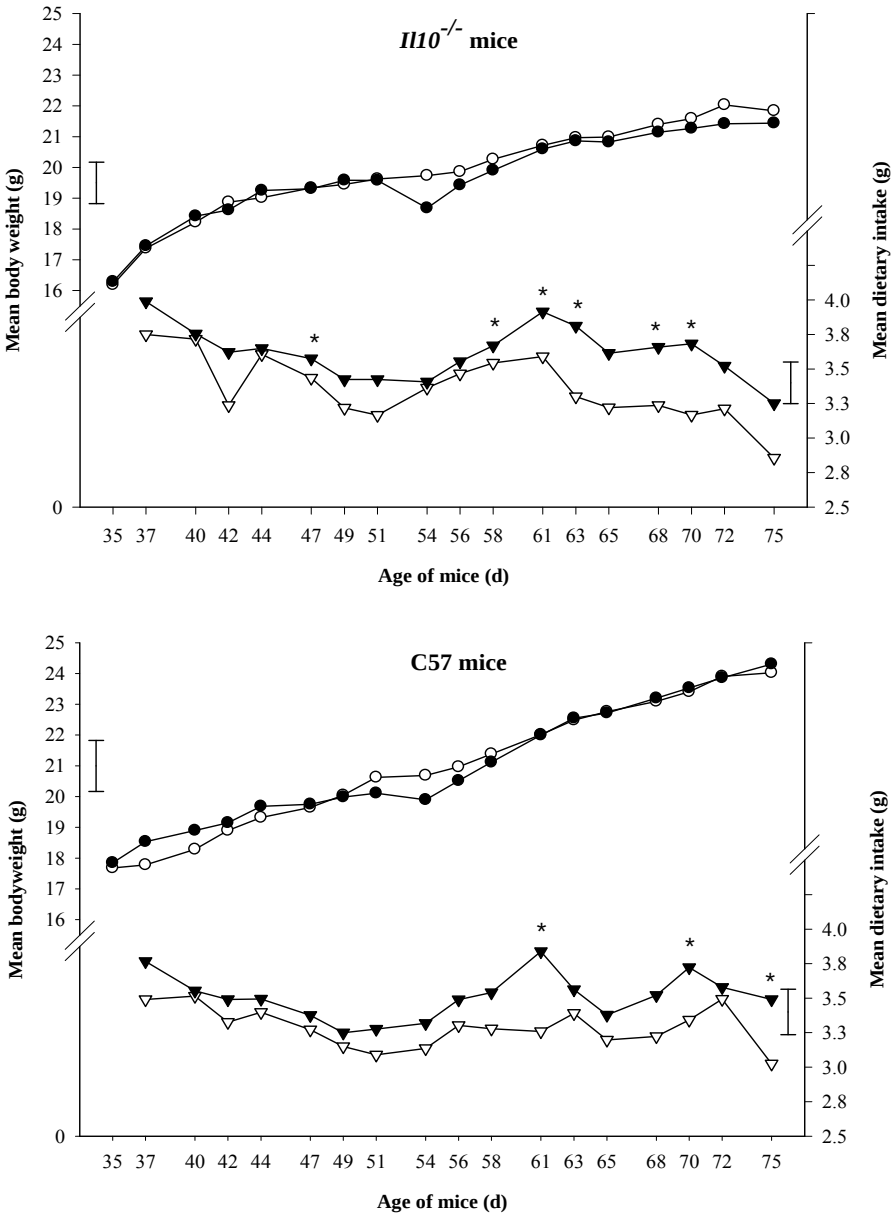


Fig. 4.1 Mean body weight (g) and dietary intake (g) of *Il10*$^{-/-}$ and C57 mice fed AIN-76A and OA diets (n=5-6/diet) for the experimental period. Error bars represent the least significant difference (5% level) over the time points within each strain. Asterisks indicate the difference is significant for that day (P<0.05). ○ = bodyweight OA group; ● = bodyweight AIN-76A group; Δ = dietary intake OA group; ▼ = dietary intake AIN-76A group.

Table 4.1 Histological injury score (HIS) in colon tissue and plasma serum amyloid A (SAA) in *Il10*$^{-/-}$ and C57 mice fed AIN-76A and OA diets

	C57 mice		***Il10*$^{-/-}$ mice**		
	AIN-76A diet	**OA diet**	**AIN-76A diet**	**OA diet**	**lsd**
Colon HIS	0.17	0.13	7.75	7.00	3.2
Inflammatory cell infiltrates	0.08	0.13	5.58	5.30	2.1
Tissue destruction	0.08	0	1.67	1.30	1.2
Tissue repair	0	0	0.50	0.40	0.5
SAA (µg/mL)	63.4 (4.72)	23.8 (4.28)	787.0 (6.73)	538.0 (6.38)	1.26

The total colon HIS with its scores for the inflammation types and SAA (log-transformed values in parentheses) are shown as mean with least significant difference (lsd).

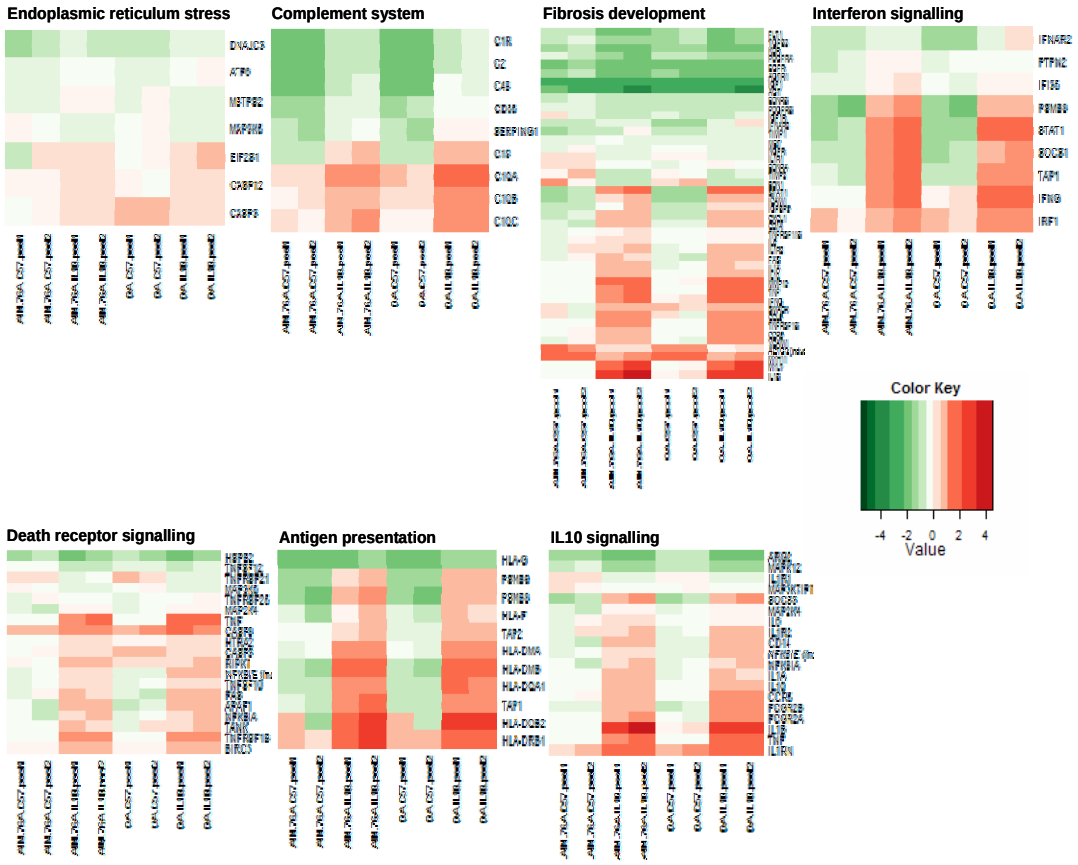


Fig. 4.2 Heatmaps showing the top 5 regulated pathways with genes differentially expressed in the colon of AIN-76A- or OA-fed *Il10*$^{-/-}$ relative to C57 mice on the same diet. These pathways include endoplasmic reticulum stress pathway, complement system, fibrosis development, interferon signalling, death receptor signalling, antigen presentation pathway and IL10 signalling. Increased gene expression levels are represented in red tones and decreased gene expression levels in green tones. Pathways with at least 30% of the constituent genes being significantly different in expression levels (FC≥1.3, P<0.01) in the pathway were considered in the top 5.

Table 4.2 Functional analysis for genes differentially expressed in the colon of *Il10*$^{-/-}$ relative to C57 mice fed the AIN-76A or OA diet using IPA analysis

Function	**Number of genes/function**	**Function**	**Number of genes/function**
AIN-76A *Il10*$^{-/-}$ *vs.* C57 mice		**OA *Il10*$^{-/-}$ *vs.* C57 mice**	
Diseases and disorders		Diseases and disorders	
Connective tissue disorders	172	Immunological disease	238
Inflammatory disease	258	Inflammatory disease	239
Skeletal and muscular disorders	201	Connective tissue disorders	159
Immunological disease	258	Skeletal and muscular disorders	181
Cancer	692	Metabolic disease	129
Molecular and cellular functions		Molecular and cellular functions	
Cellular movement	338	Cellular movement	275
Cellular growth and proliferation	536	Cell-to-cell signalling and interaction	283
Cell-to-cell signalling and interaction	328	Cellular growth and proliferation	393
Cell death	476	Cell signalling	513
Free radical signalling	48	Cell death	343
Physiological system development		Physiological system development	
Haematological system development and function	324	Immune response	300
Immune response	312	Haematological system development and function	264
Immune and lymphatic system development and function	254	Immune and lymphatic system development and function	239
Tissue morphology	236	Tissue morphology	182
Tissue development	227	Tissue development	206

Significant functions (P<0.0001) were grouped into three categories: Diseases and disorders, Molecular and cellular functions, Physiological system development. Numbers are the number of genes differentially expressed in the microarray experiment within each function.

Table 4.3 Microarray data validation by qRT-PCR

Genes	GeneBank	AIN-76A *Il10*$^{-/-}$ *vs.* C57 mice			OA *Il10*$^{-/-}$ *vs.* C57 mice		
		Microarray FC Pools	**qRT-PCR FC Pools**	**qRT-PCR FC Individ.**	**Microarray FC Pools**	**qRT-PCR FC Pools**	**qRT-PCR FC Individ.**
Abcb1a	NM_011076	-4.6	-6.2	-6.6	-3.3	-9.1	-8.2
Aldh1a1	NM_013467	-2.4	-3.9	-3.6	-2.1	-5.3	-5.3
Ces2	NM_145603	-3.6	-5.6	-4.4	-3.6	-7.7	-9.4
Fabp2	NM_007980	-2.7	-12.3	-15.5	-2.4	-14.3	-16.9
Igfbp5	NM_010518	1.9	3.1	3.9	2.0	3.2	3.1
Il1b	NM_008361	14.0	17.8	25.0	8.5	16.9	14.1
Mmp13	NM_008607	7.6	8.0	4.4	3.2	9.0	6.8
Ppara	NM_011144	-4.1	-6.5	-3.2[#]	-2.5	-5.0	-5.4
Srebf1	NM_011480	1.1*	3.3	3.9	1.1*	1.8	1.5[#]
Sult1a1	NM_133670	-4.0	-11.2	-9.6	-2.1	-8.1	-9.0

Calnexin (*Canx*) was used as a reference gene used to normalise the data; *Microarray result was not significantly different using the moderated t-statistics in limma; genes that satisfied the criterion of FC≥1.3 and P<0.01 were considered to be significantly different; [#]qRT-PCR result was not significantly different using ANOVA.

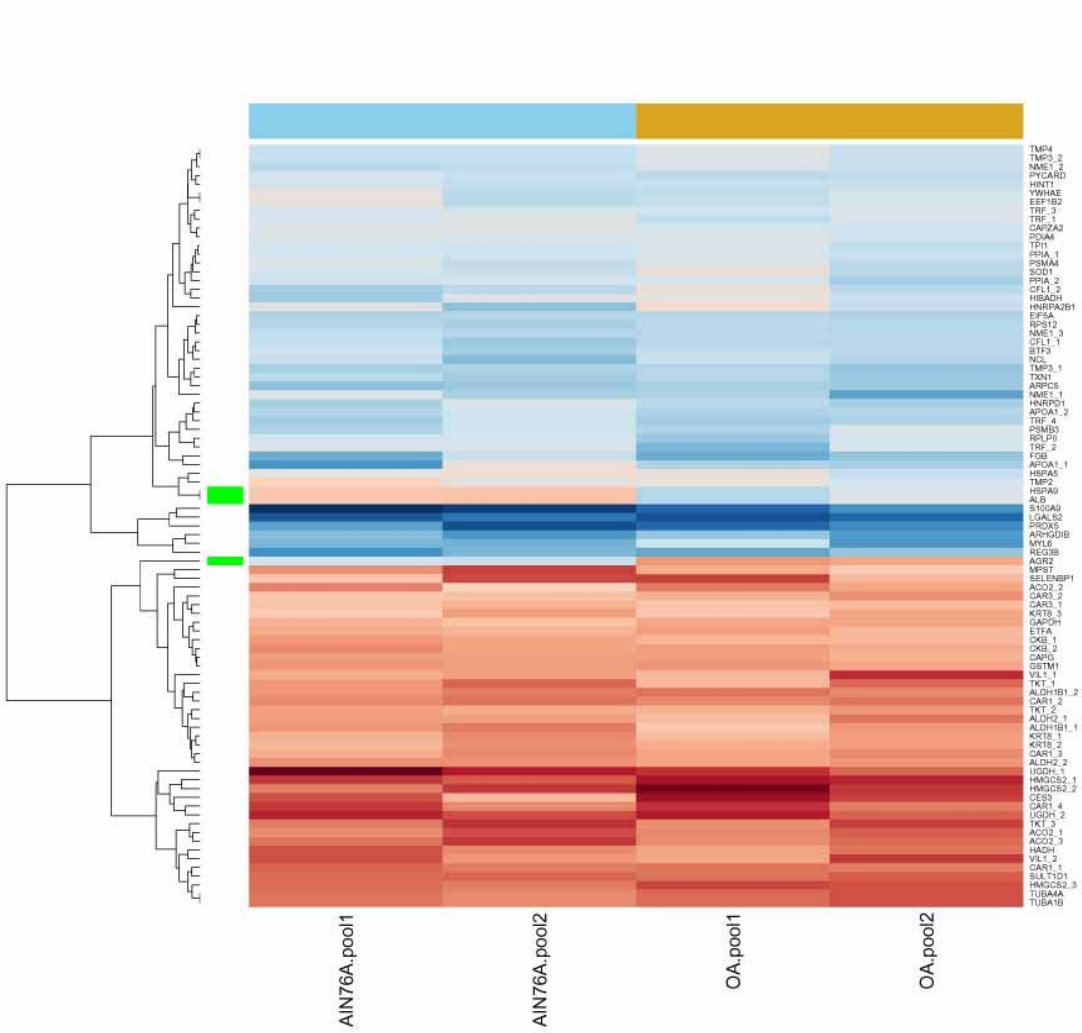


Fig. 4.3 Heatmap showing identified proteins differentially expressed in the colon of *Il10*$^{-/-}$ compared to C57 mice fed either the AIN-76A or OA diet. Increased abundance for proteins are shown in red and decreased abundance of proteins in blue. The green annotation highlights the three proteins whose fold difference was not in identical direction across the AIN-76A and OA diets. Protein expression levels were considered significantly affected by inflammation where the fold abundance for each biological replicate changed in the same direction and either both gave a value either <-1.5 or >1.5 fold, or where one biological replicate gave a value <-2 or >2 fold difference and the other biological replicate <-1.3 or >1.3.

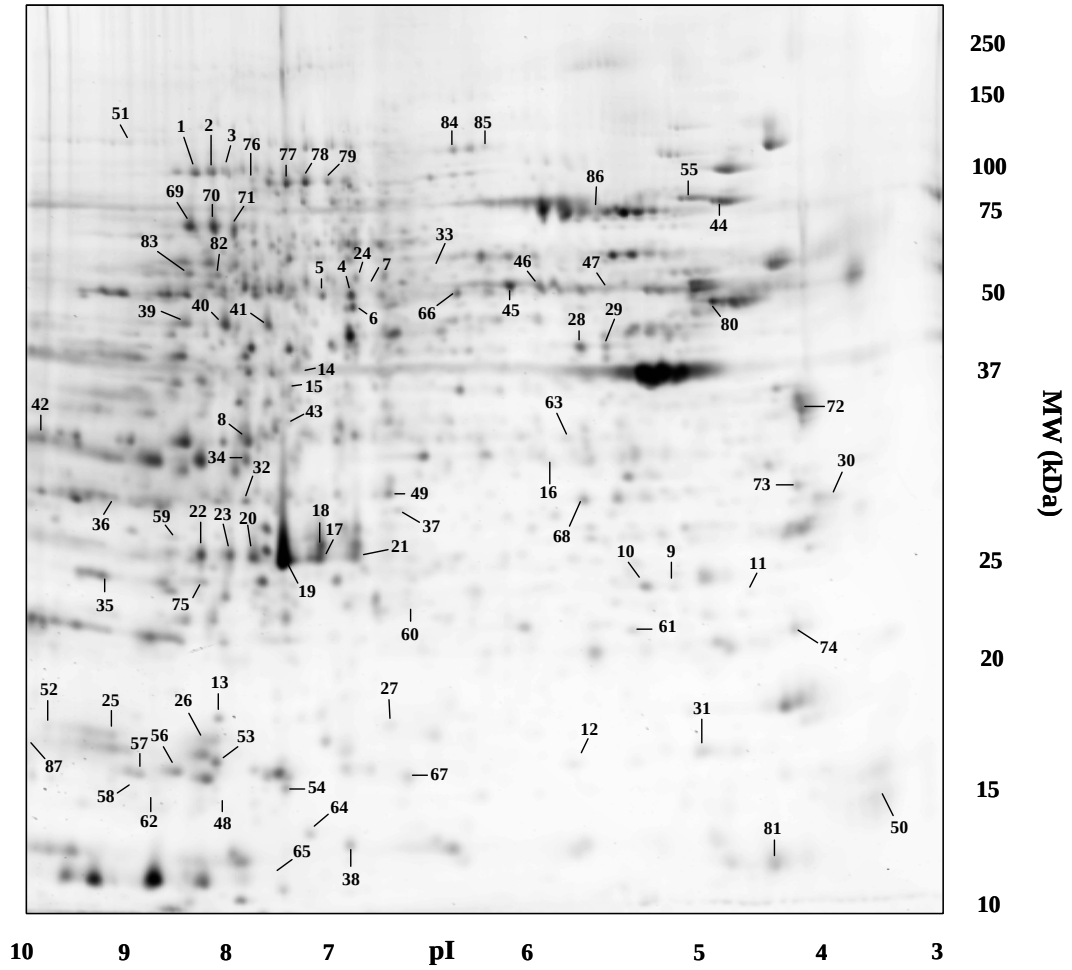


Fig. 4.4 Representative 2D-DIGE gel comparing the protein extracts from the colon of *Il10*$^{-/-}$ mice relative to C57 mice fed the AIN-76A or OA diet. The approximate isoelectric point (p*I*) and molecular weight (MW) in kDa is given on the *x* and *y* axes. Spots 86 and 87 represent the proteins for which the fold change expression was not in identical direction between the AIN-76A and OA diet comparisons.

Table 4.4 Proteins differentially expressed in the colon of *Il10*$^{-/-}$ relative to C57 mice fed the AIN-76A or OA diet

Spot	Accession number	Protein description	Gene (MGI)	Theoretic M_r (kDa)	Theoretic p*I*	No. peptides matched	Sequence coverage %	SEQUEST P (pro)	Fold change AIN-76A *Il10*$^{-/-}$ vs. C57	Fold change OA *Il10*$^{-/-}$ vs. C57
1	gi|18079339	aconitase 2, mitochondrial	*Aco2*	85.5	7.8	10	17.6	7.60E-10	-4.4	-4.1
2	gi|18079339	aconitase 2, mitochondrial	*Aco2*	85.5	7.8	10	15.9	4.70E-10	-5.6	-3.6
3	gi|18079339	aconitase 2, mitochondrial	*Aco2*	85.5	7.8	6	9.7	1.20E-09	-2	-2.8
4	gi|21312260	aldehyde dehydrogenase 1 family, member B1	*Aldh1b1*	57.6	6.61	5	10.6	6.10E-06	-3	-2
5	gi|21312260	aldehyde dehydrogenase 1 family, member B1	*Aldh1b1*	57.6	6.61	5	10.6	2.50E-07	-2.2	-3.4
6	gi|6753036	aldehyde dehydrogenase 2, mitochondrial	*Aldh2*	56.5	7.47	5	12.9	2.30E-09	-2.3	-2.8
7	gi|6753036	aldehyde dehydrogenase 2, mitochondrial	*Aldh2*	56.5	7.47	5	11.9	2.20E-09	-2.6	-2.5
8	gi|6996913	annexin A2	*Anxa2*	38.7	7.67	9	26.5	1.50E-08	2.4	2.3
9	gi|160333304	apolipoprotein A-I	*Apoa1*	30.6	5.57	1	3.4	2.10E-04	5.5	3.5
10	gi|160333304	apolipoprotein A-I	*Apoa1*	30.6	5.57	4	16.7	8.90E-09	2.7	3.1
11	gi|33563236	Rho, GDP dissociation inhibitor (GDI) beta	*Arhgdib*	22.8	4.8	5	36	2.60E-11	7.7	7.1
12	gi|62510460	actin related protein 2/3 complex, subunit 5	*Arpc5*	16.3	5.35	4	15.2	1.70E-05	4.7	3.9
13	gi|56605979	PREDICTED: similar to general transcription factor	*Btf3*	17.7	7.76	2	29	2.20E-11	3.1	2.8
14	gi|110227379	gelsolin-like capping protein	*Capg*	38.8	6.51	2	7.5	1.30E-08	-2.1	-2.1
15	gi|110227379	gelsolin-like capping protein	*Capg*	38.8	6.51	4	18.3	9.90E-10	2.3	3.2
16	gi|148681933	capping protein (actin filament) muscle Z-line, alpha 2, isoform CRA_c	*Capza2*	33	5.5	3	15	6.70E-11	1.7	2.1
17	gi|145301561	carbonic anhydrase 1	*Car1*	28.3	6.49	9	49	2.40E-09	-3.7	-3.7
18	gi|145301561	carbonic anhydrase 1	*Car1*	28.3	6.49	7	36.4	7.90E-11	-2.8	-4.4
19	gi|145301561	carbonic anhydrase 1	*Car1*	28.3	6.49	7	36.4	2.50E-11	-3.4	-3.4
20	gi|145301561	carbonic anhydrase 1	*Car1*	28.3	6.49	7	36.4	2.30E-08	-2.5	-2.6
21	gi|145301561	carbonic anhydrase 1	*Car1*	28.3	6.49	5	26.8	4.30E-10	-4.5	-5.5
22	gi|31982861	carbonic anhydrase 3	*Car3*	29.4	6.99	4	16.2	2.50E-06	-1.5	-1.5
23	gi|31982861	carbonic anhydrase 3	*Car3*	29.4	6.99	5	21.2	6.30E-06	-1.3	-2.4
24	gi|117553604	carboxylesterase 3	*Ces3*	61.8	6.16	4	9	3.00E-06	-3.2	-9.6
25	gi|149255823	PREDICTED: similar to Cofilin-1 (Cofilin,	*Cfl1*	18.5	7.78	4	27.1	1.70E-11	3.5	3

		non-muscle isoform)								
26	gi|149255823	PREDICTED: similar to Cofilin-1 (Cofilin, non-muscle isoform)	*Cfl1*	18.5	7.78	5	31.9	1.30E-13	2.5	2.4
27	gi|149255823	PREDICTED: similar to Cofilin-1 (Cofilin, non-muscle isoform)	*Cfl1*	18.5	7.78	4	19.9	6.60E-08	3.5	2.1
28	gi|10946574	creatine kinase, brain	*Ckb*	42.7	5.31	8	31.8	1.10E-11	-2.3	-1.7
29	gi|10946574	creatine kinase, brain	*Ckb*	42.7	5.31	8	31.8	2.50E-10	-2.4	-2.1
30	gi|31981925	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	*Ywhae*	29.2	4.48	4	17.3	7.20E-08	2.3	2.3
30	gi|31980922	eukaryotic translation elongation factor 1 beta 2	*Eef1b2*	24.7	4.38	3	17.8	1.40E-11	2.3	2.3
31	gi|31712036	eukaryotic translation initiation factor 5A	*Eif5a*	16.8	4.94	4	28.6	1.00E-30	3.3	3.2
32	gi|31981826	electron transferring flavoprotein, alpha polypeptide	*Etfa*	34.9	8.22	5	19.2	1.30E-08	-1.9	-2
33	gi|33859809	fibrinogen, B beta polypeptide	*Fgb*	54.7	6.71	4	8.7	3.70E-07	4.6	5.9
34	gi|52139064	glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	35.8	8.33	3	9	2.70E-04	-1.6	-2.2
35	gi|6754084	glutathione S-transferase, mu 1	*Gstm1*	26	8.11	4	20.6	2.70E-07	-2.4	-2.3
36	gi|111038118	L-3-hydroxyacyl-Coenzyme A dehydrogenase	*Hadh*	34.4	8.97	3	12.4	2.50E-10	-3.8	-3.2
37	gi|21704140	3-hydroxyisobutyrate dehydrogenase precursor	*Hibadh*	35.4	8.12	2	8.7	4.10E-10	2.8	1.9
38	gi|33468857	histidine triad nucleotide binding protein	*Hint1*	13.8	6.41	2	16.7	1.70E-10	2.4	2.4
39	gi|31560689	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	*Hmgcs2*	56.8	8.43	8	17.5	7.30E-08	-5.6	-10.4
40	gi|31560689	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	*Hmgcs2*	56.8	8.43	6	13	1.00E-07	-5.8	-13.2
41	gi|31560689	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	*Hmgcs2*	56.8	8.43	6	13.6	5.40E-08	-3.6	-6
42	gi|109134362	heterogeneous nuclear ribonucleoprotein A2/B1 isoform 1	*Hnrpa2 b1*	36	8.95	2	7.6	5.00E-10	3.4	1.7
43	gi|148664250	heterogeneous nuclear ribonucleoprotein D-like	*Hnrpd1*	46.2	9.87	3	7.6	4.80E-10	2.8	3.2
44	gi|31981722	heat shock 70kD protein 5 (glucose-regulated protein)	*Hspa5*	72.4	4.87	11	25.2	1.20E-12	1.4	2
45	gi|114145561	keratin complex 2, basic, gene 8	*Krt8*	54.6	5.59	23	48	1.50E-08	-2.4	-2.1

46	gi|114145561	keratin complex 2, basic, gene 8	*Krt8*	54.6	5.59	19	39.8	3.50E-09	-2.3	-2.2
47	gi|114145561	keratin complex 2, basic, gene 8	*Krt8*	54.6	5.59	10	22.4	4.00E-07	-1.8	-1.8
48	gi|13385082	lectin, galactose-binding, soluble 2	*Lgals2*	14.9	7.39	3	20.8	9.70E-07	21.4	26.4
49	gi|148697727	mercaptopyruvate sulfurtransferase	*Mpst*	33	6.12	5	20.9	2.90E-08	-5.2	-1.6
50	gi|149266035	PREDICTED: similar to non-muscle myosin alkali light chain	*Myl6*	13	4.42	5	44	1.30E-06	6.3	6
51	gi|84875537	nucleolin	*Ncl*	76.7	4.53	6	9.3	5.10E-10	4	2.8
52	gi|149274354	PREDICTED: similar to Nucleoside diphosphate kinase A (NDK A) (NDP	*Nme1*	17.1	9.38	5	20.7	3.70E-09	2.8	6
53	gi|149262292	PREDICTED: similar to Nucleoside diphosphate kinase A (NDK A) (NDP	*Nme1*	17.2	7.24	4	28.3	4.80E-08	2.7	2.1
54	gi|149262292	PREDICTED: similar to Nucleoside diphosphate kinase A (NDK A) (NDP	*Nme1*	17.2	7.24	7	37.5	1.30E-07	3	3.1
55	gi|86198316	protein disulfide isomerase associated 4	*Pdia4*	72.4	4.94	6	12.6	2.80E-14	1.9	1.9
56	gi|6679439	peptidylprolyl isomerase A	*Ppia*	18	7.97	3	17.7	3.10E-04	2.2	2.2
57	gi|6679439	peptidylprolyl isomerase A	*Ppia*	18	7.97	3	14	2.90E-07	2.2	2.8
58	gi|6755114	peroxiredoxin 5 precursor	*Prdx5*	21.9	9.06	4	22.4	8.90E-12	20.7	16.5
59	gi|6755196	proteasome (prosome, macropain) subunit, alpha type 4	*Psma4*	29.5	7.69	2	6.9	6.50E-06	2.2	2.3
60	gi|6755202	proteasome beta 3 subunit	*Psmb3*	23	6.15	3	23.4	2.60E-09	2.9	2.5
61	gi|165377185	PYD and CARD domain containing	*Pycard*	21.5	4.89	3	24.9	3.20E-08	2.3	2.7
62	gi|6754980	pancreatitis-associated protein	*Reg3g*	19.5	7.47	1	4.6	1.90E-04	8.1	6
63	gi|6671569	acidic ribosomal phosphoprotein P0	*Rplp0*	34.2	5.87	5	19.6	2.83E-07	2.1	3.3
64	gi|149272239	PREDICTED: similar to 40S ribosomal protein S12 isoform 1	*Rps12*	14.5	6.94	2	12.1	1.00E-04	3.4	2.9
65	gi|6677837	S100 calcium binding protein A9 (calgranulin B)	*S100a9*	13.1	6.76	2	18.6	1.30E-05	44.5	15.9
66	gi|22164798	selenium binding protein 1	*Selenbp 1*	52.5	5.85	4	9.3	7.20E-04	-4.1	-4.1
67	gi|148665969	superoxide dismutase 1, soluble, isoform CRA_b	*Sod1*	15.9	6.04	1	7.1	4.10E-07	2.3	2.1
68	gi|148706038	sulfotransferase family 1D, member 1	*Sult1d1*	36.1	5.47	3	10.9	7.60E-07	-4.3	-4.7
69	gi|6678359	transketolase	*Tkt*	67.6	7.22	14	34.5	6.70E-14	-3.5	-3.1
70	gi|6678359	transketolase	*Tkt*	67.6	7.22	8	18.3	2.00E-14	-2.1	-2.2
71	gi|6678359	transketolase	*Tkt*	67.6	7.22	7	13.5	1.90E-12	-6.2	-5.1

72	gi|50190	beta-tropomyosin	*Tmp2*	33	4.51	4	15.1	2.20E-08	1.3	1.8
73	gi|74226916	tropomyosin 3	*Tpm3*	29	4.6	4	19.4	3.90E-09	3.6	4
73	gi|74226916	tropomyosin 3	*Tpm3*	29	4.6	6	22.6	4.40E-10	2.5	2.1
74	gi|47894398	tropomyosin 4	*Tpm4*	28.5	4.5	4	11.3	2.50E-05	2.5	2.1
74	gi|47894398	tropomyosin 4	*Tpm4*	28.5	4.5	3	11.3	1.30E-05	3.6	4
75	gi|6678413	triosephosphate isomerase 1	*Tpi1*	26.7	7.07	5	24.5	7.30E-08	2.2	2.3
76	gi|20330802	transferrin	*Trf*	76.7	6.84	7	10.6	5.20E-06	3.1	3.8
77	gi|20330802	transferrin	*Trf*	76.7	6.84	11	16.6	3.30E-06	1.9	3.7
78	gi|20330802	transferrin	*Trf*	76.7	6.84	9	14.5	1.10E-07	2.1	2
79	gi|20330802	transferrin	*Trf*	76.7	6.84	9	14.5	1.40E-05	1.9	2.3
80	gi|6755901	tubulin, alpha 1	*Tuba1b*	50.1	4.81	5	16.6	7.50E-09	-3.2	-5.1
80	gi|6678467	tubulin, alpha 4	*Tuba4a*	49.9	4.79	5	16.7	6.80E-09	-3.2	-5.1
81	gi|123210316	thioredoxin 1	*Txn1*	11.7	4.64	4	31.4	4.10E-07	3.5	3.7
82	gi|6678499	UDP-glucose dehydrogenase	*Ugdh*	54.8	7.38	3	7.5	7.40E-10	-14.6	-6
83	gi|6678499	UDP-glucose dehydrogenase	*Ugdh*	54.8	7.38	4	9.9	7.80E-06	-7	-7.2
84	gi|190684696	villin 1	*Vil1*	92.8	5.66	5	6.2	2.40E-05	-2.2	-5.7
85	gi|190684696	villin 1	*Vil1*	92.8	5.66	9	12.3	2.30E-05	-3.6	-5.2
86	gi|33859506	albumin	*Alb*	68.7	5.69	8	16	6.40E-09	-1.3	2.5
86	gi|6754256	heat shock protein 9	*Hspa9*	73.5	5.85	4	8.7	8.00E-07	-1.3	2.5
87	gi|123236231	anterior gradient 2	*Agr2*	19.9	9.39	4	32	3.70E-12	2.3	-2.4

Table 4.5 Comparison of proteomic and transcriptomic profiles in the colon of *Il10*$^{-/-}$ relative to C57 mice fed the AIN-76A or OA diet

Protein description/gene symbol	**GI Number**	**AIN-76A *Il10*$^{-/-}$ vs. C57**	**OA *Il10*$^{-/-}$ vs. C57**	**GeneBank accession**	**AIN-76A *Il10*$^{-/-}$ vs. C57**	**OA *Il10*$^{-/-}$ vs. C57**
		Proteomics FC (*P*-value)			**Transcriptomics FC (*P*-value)**	
aldehyde dehydrogenase 2 family - *Aldh2*	6753036	-2.5 (2.3E-09)	-2.6 (2.3E-09)	NM_009656	-1.8 (4.39E-03)	-1.8 (7.72E-04)
Rho GDP dissociation inhibitor (GDI) beta - *Arhgdib*	33563236	7.7 (2.6E-11)	7.1 (2.6E-11)	NM_007486	3.0 (1.42E-05)	2.7 (1.00E-06)
actin related protein 2/3 complex, subunit 5, 16kDa - *Arpc5*	62510460	4.7 (1.7E-05)	3.9 (1.7E-05)	NM_026369	1.7 (4.84E-04)	1.3 (8.34E-03)
carboxylesterase 3 - *Ces3*	117553604	-3.2 (3.0E-06)	-9.6 (3.0E-06)	NM_053200	-5.4 (1.64E-04)	-4.8 (1.70E-05)
L-3-hydroxyacyl-Coenzyme A dehydrogenase - *Hadh*	111038118	-3.8 (2.5E-10)	-3.2 (2.5E-10)	AK199677	-2.4 (1.05E-04)	-1.7 (4.07E-04)
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 - *Hmgcs2*	31560689	-5.0 (4.3E-08)	-9.9 (4.3E-08)	NM_008256	-4.4 (6.47E-04)	-3.3 (4.84E-04)
S100 calcium binding protein A9 - *S100a9*	6677837	44.5 (1.3E-05)	15.9 (1.3E-05)	NM_009114	56.4 (2.45E-06)	20.7 (0.00E+00)
tropomyosin 2 (beta) - *Tpm2*	50190	1.3 (2.2E-08)	1.8 (2.2E-08)	NM_009416	-1.5 (1.11E-03)	-1.3 (3.82E-03)

Where multiple protein isoforms were detected due to post-translational modifications the mean fold change (FC) across the isoforms is given.

4.5 Discussion

The metabolic and signalling pathways most affected during colitis form the basis for comparing the effect of AIN-76A and OA diets on gene and protein expression profiles in the colon of *Il10*$^{-/-}$ compared to C57 mice. Colonic transcriptome and proteome profiles of *Il10*$^{-/-}$ mice (compared to C57 mice) fed AIN-76A diet clustered similarly with those of *Il10*$^{-/-}$ mice (compared to C57 mice) fed the OA diet. This result supports the use of OA as a control in dietary fatty acid intervention studies using multi-omic tools. No physiological differences were observed between the two diets with regard to body weight or local (colon HIS) and systemic (SAA) inflammation markers during colitis development.

Transcriptome and proteome analyses showed increased expression levels of colon genes and proteins involved in signalling processes related to inflammation in both diets. From pathway analysis it became clear that AIN-76A and OA diets (*Il10*$^{-/-}$ *vs.* C57 mice) showed similar colon transcript changes associated with cell-mediated and humoral aspects of the innate and adaptive immune system. As such, both diets affected interferon signalling, with the *Ifng* gene expression levels increased in the colon of *Il10*$^{-/-}$ compared to C57 mice. IFNG is one of the most important cytokines which stimulates macrophages to induce genes/proteins of antigen processing and presentation (TAP, MHC class I and II, DMA, DMB), antiviral, anti-proliferative, apoptotic (caspases, Fas (TNF receptor superfamily member 6), TNFα-receptor), antimicrobial (Fc receptor, complement proteins) mechanisms and leukocyte trafficking (adhesion molecules) during innate and acquired immune responses (Schroder *et al*., 2004). The binding of IFNγ to a receptor (e.g., *Ifnar2*), activates the JAK/STAT pathway (Schroder *et al*., 2004), which is in agreement with increased expression levels of *Stat1* gene in the colon of AIN-76A or OA-fed *Il10*$^{-/-}$ mice compared to C57 mice on the same diet. Transcripts of the antigen presentation pathway (*Hla-D*, *Hla-F*, *Hla-G*, *Psmb* and *Tap* members) were in higher abundance in the colon of *Il10*$^{-/-}$ compared to C57 mice, regardless of which diet was fed. In agreement, increased mRNA abundance of MHC class II antigens induced by INFG and TNF has been found in the colonic mucosa of IBD patients and is known as a key event in the onset of IBD (Mueller, 2002).

Interestingly, there were increased expression levels of genes associated with ER stress (*Atf6*, *Eif2s1*) and death receptor signalling (*Apaf1*, *Casp3*, *Casp8*, *Fas*, *Hspb2*, *Mapk3k5*, *Ripk1*, *Tank*, *Tnfrsf1b*) pathways in the colon of *Il10*$^{-/-}$ compared to C57 mice fed AIN-76A diet, but not when these mice were fed the OA diet. Death receptors (e.g., TNFR1, FAS or APO1) and their ligands play an important role in the regulation of apoptosis to maintain tissue homeostasis (Daniel *et al*., 2001). The death domain protein RIP is crucial for death receptor signalling, and also for TNFR1-mediated NFkB activation. Caspases are involved in the initiation (caspase-2, -8 and -9) and execution (caspase-3, -6 and -7) of apoptotic cell death (Daniel *et al*., 2001). The increased mRNA abundance of key regulators of death receptor signalling in colitic *Il10*$^{-/-}$ mice suggests that colitis develops in the absence of IL10 induced apoptosis, especially in AIN-76A-fed *Il10*$^{-/-}$ mice. Apoptosis can be caused by epithelial cell death and accompanying structural alterations of the epithelial barrier (Johnson & Barrett, 2006).

Pathway analysis further indicated fibrosis development in the inflamed colon of *Il10*$^{-/-}$ mice on both diets compared to C57 mice. Intestinal fibrosis is a process in which inflammation leads to an increase of local fibroblasts and various other cells and collagen depositions. It involves a complex interplay between those cell types and fibrotic mediators (Rieder & Fiocchi, 2009). The colon tissue of *Il10*$^{-/-}$ mice on both diets showed shortening and deformation, inflammatory cell infiltration (primarily monocytes) in all layers of the intestinal wall, disorganised crypts and low level of hyperplasia. Increased expression levels of known pro-fibrotic (*Egfr*, *Igf1*, *Il1b*, *Pdgfr*, *Myl7*, *Tgfb2*) and anti-fibrotic (*Ifng*) mediator genes in the colon of *Il10*$^{-/-}$ compared to C57 mice fed either diet indicated that colitis in *Il10*$^{-/-}$ mice, induced by bacteria, most likely progressed to early stages of fibrosis. A previous study has shown that *E. faecalis* (which was part of the bacterial inoculum used in the present study) induced IBD, dysplasia, fibrosis and even carcinoma in *Il10*$^{-/-}$ mice (129SEV background) (Balish & Warner, 2002). Fibrosis development as indicated by gene expression changes should be investigated further, and the role of the composition of enteric flora as a contributing factor to fibrosis evaluated because intestinal fibrosis might be reversible after bacterial changes.

Proteomics data showed that for both diets, a direct comparison between *Il10*$^{-/-}$ and C57 mice revealed changes in abundance of proteins associated with metabolism and cellular

stress and inflammatory responses. Metabolic proteins involved in carbohydrate, lipid, amino acid and energy metabolism were generally down-regulated, suggesting a reduced functionality of multiple biochemical pathways. The lower abundance of energy related proteins such as GAPDH, an important enzyme involved in glycolysis, and UGDH is of interest. GAPDH can interact with structural proteins such as actin (Jung *et al*., 2002) and low expression levels combined with widespread impaired metabolic functionality suggests that there is insufficient glycolytic ATP energy provided for organisation and remodelling of the actin cytoskeleton; maintenance of membrane integrity and function is thus impaired as a consequence of intestinal inflammation (Keshavarzian *et al*., 2003).

Altered colonic ketogenesis was implied by lower expression levels of three isoforms of the protein HMGCS2, which converts acetyl-CoA to ketone bodies, in *Il10*$^{-/-}$ compared to C57 mice. This effect seems to be more pronounced with the OA diet. This protein has been suggested as a key ketogenic agent and is down-regulated in colon cancer, indicating a role in tumour progression (Camarero *et al*., 2006). Altered energy metabolism (e.g., down-regulation of oxidative pathway regulatory genes) has been reported in colonocytes from patients with UC, known as an energy-deficiency disease (Fukushima & Fiocchi, 2004). Down-regulated enzymes associated with energy metabolism were also observed in intestinal epithelial cells from *Il10*$^{-/-}$ mice two weeks after bacterial inoculation (Werner *et al*., 2007).

In response to colitis, the abundance of cellular stress marker proteins was increased, e.g., HSPA5 in *Il10*$^{-/-}$ mice fed AIN-76A or the OA diet. Stress responses in these mice could be counteracted by increased expression levels of antioxidant proteins such as PRDX5, which was also reported as increased in intestinal epithelial cells of *E. faecalis*-monocolonised *Il10*$^{-/-}$ mice to protect cells from further ROS-mediated tissue damage (Werner *et al*., 2007). Other proteins (the cytosolic SOD1 and TXN1) which had increased abundance in *Il10*$^{-/-}$ compared to C57 mice fed either diet may be produced in order to detoxify ROS in colonocytes. In radiation-induced inflammation of mouse small intestinal mucosa, mitochondrial-associated antioxidant systems were shown to be induced by increased expression of SOD2 and TXN2 protein isoforms (Haton *et al*., 2007).

A comparison analysis between transcriptomic and proteomic data within the AIN-76A or OA diet comparisons (*Il10*$^{-/-}$ *vs.* C57 mice) showed only minimal overlap between gene and protein expression changes. This might be due to the different time scales associated with gene transcript production and protein expression, and to date is an issue that remains unresolved in multi-omic studies.

In conclusion, close clustering of colonic gene and protein expression profiles was observed between mice fed AIN-76A and the OA diet. Inflammation-induced regulatory processes associated with cellular and humoral immune responses, cellular stress response and metabolic processes related to energy utilisation were identified in *Il10*$^{-/-}$ mice fed either diet. The general lack of overlap between gene and protein expression profiles for each diet comparison (*Il10*$^{-/-}$ *vs.* C57 mice) underlines the importance of a systems biology approach in nutrigenomics research.

Chapter 5

Genome-wide analysis of dietary eicosapentaenoic acid- and oleic acid-induced modulation of colon inflammation in interleukin-10 gene-deficient mice

Part of the material presented in this chapter has been published as a paper:

Knoch B, Barnett MPG, Zhu S, Park ZA, Nones K, Dommels YEM, Knowles SO, McNabb WC & Roy NC (2009) Genome-wide analysis of dietary eicosapentaenoic acid- and oleic acid-induced modulation in colon inflammation of interleukin-10 gene-deficient mice. *J Nutrigenet Nutrigenomics* **2**, 9-28.

5.1 Abstract

Dietary n-3 PUFA can reduce inflammation via a range of mechanisms. This study tested the effect of dietary EPA on intestinal inflammation using *Il10*$^{-/-}$ mice. At 35 days of age, 12 weaned *Il10*$^{-/-}$ mice and 12 C57 mice were randomly assigned to one of two modified AIN-76A diets, supplemented with 3.4% purified ethyl esters of either EPA (n-3) or OA (control). To identify genes relevant to colon inflammation, gene expression (microarrays and qRT-PCR) and bioinformatics analyses were used. In this study, dietary EPA reversed the decrease in colon fatty acid β-oxidation gene expression levels observed in OA-fed *Il10*$^{-/-}$ compared to C57 mice. *Il10*$^{-/-}$ mice fed the OA diet showed decreased expression levels of antioxidant enzyme genes, as well as those involved in detoxification of xenobiotics, compared to C57 mice on the same diet. In contrast, dietary EPA increased the expression levels of these genes in *Il10*$^{-/-}$ mice. These data indicate that dietary EPA-induced endogenous lipid oxidation might have a potential anti-inflammatory effect on inflamed colon tissue. This is supported by the activation of the *Ppara* gene that down-regulates the expression of pro-inflammatory and immunomodulatory genes and proteins.

5.2 Introduction

Acute inflammation supports the host in eliminating foreign substances and invading microorganisms or repairing tissue damaged by infection. This complex and precisely regulated process is essential for pathogen detection and host defence, and its deregulation can lead to a chronic state of inflammation. This is characterised by increased production of pro-inflammatory mediators (such as cytokines, chemokines, prostaglandins and ROS and infiltration of immune cells, particularly granulocytes, monocytes, macrophages and lymphocytes, into sites of inflammation. Chronic inflammation underlies many conditions including gastric cancer, rheumatoid arthritis and CD which is one of the IBD. TNFα has been identified as a key pathogenic cytokine for immune-mediated inflammatory diseases such as CD. Other inflammation-associated cytokines include IL6, IL1β, IFNγ and TGFβ. The aetiology and pathogenesis of IBD is still unclear but appears to result from complex interactions between the immune system, the environment and the host's genes (Watkins *et al*., 2007). IL10 as an anti-inflammatory cytokine has been used to control inflammation initiated and perpetuated by pro-inflammatory mediators. Therefore, *Il10*$^{-/-}$ mice have

attracted interest as an animal model of inflammatory diseases and have been used in IBD studies (Murray, 2006).

Diet is an environmental factor with the potential to change the course of IBD (Bassaganya-Riera & Hontecillas, 2006). The n-3 and n-6 PUFA, as precursors of potent lipid mediators, can play an important role in the regulation of intestinal inflammation (Hudert *et al*., 2006). There is some evidence that n-6 PUFA promote inflammation, whereas n-3 PUFA such as EPA have anti-inflammatory properties. These properties are probably mediated through their ability to inhibit the formation of n-6 PUFA derived pro-inflammatory eicosanoids via the COX and LOX pathways (Hudert *et al*., 2006; James *et al*., 2000). Beneficial effects of n-3 PUFA on health have been associated with: inhibition of eicosanoid pathways leading to a down-regulation of inflammatory responses and protein expression; modulation of enzymes related to various signalling pathways involving normal and pathologic cell function; increased incorporation of n-3 PUFA into membrane phospholipids altering cell membrane functions (fluidity, permeability, activity of specific receptors), and changing gene transcription through their direct binding or the binding of their metabolites to ligand-dependent transcription factors (Deckelbaum *et al*., 2006, Seo *et al*., 2005). Expression of key proteins involved in inflammation, lipid metabolism and energy utilisation can also be affected by n-3 PUFA (Deckelbaum *et al*., 2006).

More recently, it was reported that fatty acids can act as metabolic regulators to control the activity of key transcription factors including PPAR (Wild *et al*., 2007). The PPARα and PPARγ isoforms in the intestine can be activated by PUFA to reduce intestinal inflammation (Bassaganya-Riera & Hontecillas, 2006). PPARγ has selectivity for unsaturated fatty acids including PUFA, whereas PPARα can be activated by lipids or their metabolites, e.g. oxidised phospholipids (Stulnig, 2003). The anti-inflammatory effect of n-3 PUFA in the intestine may be via activation of PPAR and inhibition of NFκB to reduce pro-inflammatory cytokine gene expression (Wild *et al*., 2007).

It has been reported that n-3 PUFA reduce intestinal inflammation in animal models of IBD (Nieto *et al*., 2002). However, it has also been shown that an n-3 PUFA-enriched diet failed to protect pigs from the onset of enteric disease in an IBD model, but did accelerate colonic regeneration and clinical remission, probably by activating PPARδ,

another isoform of PPAR (Bassaganya-Riera & Hontecillas, 2006). Another study by this group showed that dietary n-3 PUFA supplementation did not prevent DSS-induced colitis in mice (Bassaganya-Riera *et al*., 2004). It is difficult to rationalise the inconsistency in these studies because of the different experimental conditions used.

This study was designed to test whether dietary supplementation with the n-3 PUFA EPA reduced intestinal inflammation in the *Il10*$^{-/-}$ mouse model of IBD. Genome-wide transcription profiling (microarrays) was used to identify relevant pathways that may play a role in experimental colitis in *Il10*$^{-/-}$ mice (compared to healthy C57 mice). Differentially expressed genes were clustered into pathways using IPA. The identification of pathway-specific patterns of gene expression that further explain the role of dietary EPA compared to OA in experimental colitis is reported.

5.3. Materials and methods

5.3.1 Inoculated *Il10*$^{-/-}$ mouse model of IBD

Housing and induction of colitis are described in Materials and Methods chapter 2.1.1 and 2.1.2. Twelve male *Il10*$^{-/-}$ (C57BL6/J background, formal designation B6.129P2-*Il10*<tm1Cgn>/J) and 12 male C57 wildtype (C57BL/6J) mice were purchased from the Jackson Laboratory (Bar Harbor, Me., USA). All mice were 35 days or 5 weeks of age at the start of the experiment.

5.3.2 Experimental design and diets

Experimental design, diet compositions and fatty acid analysis are described in Materials and Methods chapter 2.1.3 and 2.2. All mice were maintained for 6 weeks on an AIN-76A-based diet enriched with OA or an AIN-76A-based diet enriched with EPA. This was part of the PUFA intervention study described in Chapters 4 and 6. The OA and EPA diets were formulated to contain AIN-76A (fat-free) + 1% corn oil, supplemented with 0.2% purified LA and 0.06% ALA ethyl esters and 3.4% OA or EPA ethyl ester. The diets were isocaloric and varied only in lipid composition, both diets containing 5% fat (w/w), the usual fat content of the AIN-76A diet. The killing of the mice at the end of the PUFA feeding study was done in randomised order and the OA and EPA group used here for more detailed analysis.

Note: *The lipid and fatty acid analysis of the diets was performed by Michael Agnew at AgResearch and Fliss Jackson at Massey University, respectively, before the start of the experiment.*

5.3.3 Tissue Collection

Tissue collection and fast-feed period prior to sampling are described in Materials and Methods chapter 2.1.4. Plasma was separated from the blood, frozen in liquid nitrogen and stored at −85°C for SAA analysis. One colon section was stored at room temperature in 10% phosphate-buffered formaldehyde for histopathological assessment, and another frozen immediately in liquid nitrogen and stored at −85°C for gene profiling.

5.3.4 Histology and SAA

Histological analysis of the colon and SAA measurement in the plasma are described in Materials and Methods chapter 2.3.1 to 2.3.3.

Note: *Tissue processing and histopathological assessment was conducted by Dr Shuotun Zhu at The University of Auckland. The SAA analysis was performed by Kim Oden at AgResearch.*

5.3.5 RNA isolation

RNA isolation and reference RNA preparation are described in Materials and Methods chapter 2.4.1 and 2.4.2. Equimolar pools of colon RNA extracts (2-3 samples/pool) were used in the reference microarray design.

5.3.6 Microarrays

Microarray reference design, synthesis of labelled cRNA, hybridisation and scanning are described in Materials and Methods chapter 2.4.1 and 2.4.3. Array data were submitted to the Gene Expression Omnibus, accession number GSE12028.

5.3.7 Analysis of microarray data

Statistical analysis of the microarray data is described in Materials and Methods chapter 2.4.4. Three comparisons were performed: (1) genotype comparison (inflammation), i.e. *Il10*$^{-/-}$ mice (inflamed) vs. C57 mice (not inflamed), all fed the OA diet; (2) diet comparison (EPA vs. OA) in *Il10*$^{-/-}$ mice, and (3) diet comparison (EPA vs. OA) in C57 mice. For each comparison, a list of differentially expressed probe sets that satisfied the

criteria of FC≥1.3 and moderated $P<0.01$ was generated. This FC was chosen because a recent study showed that a FC>1.6 could underestimate the number of differentially expressed genes regulated by dietary treatment (Sülzle *et al*., 2004). Two complementary methods (IPA and FUNC) were applied to relate changes in gene expression to functional changes. More details on these bioinformatics methods can be found in Materials and Methods chapter 2.7.1 and 2.7.2. For the FUNC approach, the differentially expressed genes (FC≥1.3, moderated $P<0.01$) from each comparison as used for IPA analysis were analysed for over-represented GO terms.

Note: *The microarray analysis in Bioconductor (written in R) was conducted with assistance of Zaneta Park, a statistician at AgResearch. FUNC analysis was conducted with assistance of Anar Khan, a bioinformatician at AgResearch.*

5.3.8 Quantitative real-time PCR

qRT-PCR is described in Materials and Methods chapter 2.5. Six key genes (*Abcb1a*, *Aldh1a1*, *Ces2*, *Fabp2*, *Igfbp5*, *Il1b*, *Ppara* and *Sult1a1*) responsive to EPA in *Il10*$^{-/-}$ mice were analysed by qRT-PCR to verify the microarray results. The primer sequences for target and reference genes are listed in Materials and Methods chapter Table 2.2. *Cyclophilin* (*CyclophilinA* or peptidylprolyl isomeraseA) and calnexin (*Canx*) were used as reference genes.

5.3.9 Statistics

Statistical analyses of body weight, dietary intake, HIS and SAA were performed using GenStat (9th edition, VSN International, Hemel Hempstead, UK). For the body weight data, antedependence modelling for repeated measures was used to test for significant differences between treatments in each strain separately and at each time point. As part of this analysis, the order of the data was first estimated, where the order of the experiment represents the number of preceding times which have an effect on the current time. Missing values were also estimated using this order before performing the analysis. Linear regression was applied to estimate body weight gain for each mouse comparing EPA with OA diets for *Il10*$^{-/-}$ and C57 mice. One sample t-test was applied to detect differences in dietary intake between days 51 and 61 for each diet/strain combination, as colon inflammation tends to start developing around 51 days of age. Differences in HIS between EPA and OA for both mouse strains were analysed using an

ANOVA with pooled variance. As expected, there were a high number of zero HIS for the C57 mice and so the data for the two strains was analysed separately. The plasma concentration of SAA was analysed by ANOVA with strain and diet as factors and a constant was added to the data prior to a log-transformation to avoid negative values found in C57 mice. Differences were considered significant when $P<0.05$. A trend was declared when $0.05<P<0.10$. One *Il10*$^{-/-}$ mouse from the OA diet group died for unknown causes. Two *Il10*$^{-/-}$ mice from the EPA diet group were euthanised before the end of the experiment because their weight loss exceeded that specified as acceptable under the ethical guidelines; this might have been due to inflammation. These animals have not been scored for histological signs of inflammation and were not taken into account for statistical analysis of HIS.

5.4 Results

5.4.1 Body weight and dietary intake

The average body weight and dietary intake from 35 to 75 days of age for C57 and *Il10*$^{-/-}$ mice fed the EPA and OA diets is shown in Fig. 5.1. Overall, average body weight increased over time in all experimental groups except for *Il10*$^{-/-}$ mice fed the EPA diet. *Il10*$^{-/-}$ mice gained significantly less weight than the C57 mice (0.12 *vs.* 0.16 g/d, $P=0.04$) on the OA diet. There was a significant reduction in body weight for *Il10*$^{-/-}$ mice fed the EPA diet at day 54 ($P=0.002$); from day 58 onward the mean weight of *Il10*$^{-/-}$ mice fed the EPA diet steadily declined, with an average weight loss from day 58 to day 75 of 0.06 g/d versus a weight gain of 0.1 g/d for OA-fed *Il10*$^{-/-}$ mice. This difference was significant ($P<0.01$). Weight loss in the EPA-fed *Il10*$^{-/-}$ mice was not more than 20% of the pre-trial body weight, the limit specified as acceptable under the ethical guidelines. There was also a significant difference in dietary intake at 51 days versus 61 days in the *Il10*$^{-/-}$ mice fed the EPA diet ($P=0.009$) but not with the OA-fed *Il10*$^{-/-}$ mice ($P=0.18$). It is not clear why the EPA-fed *Il10*$^{-/-}$ mice ate less and lost weight, as the EPA diet was well accepted by the C57 mice. The analysed levels of OA and EPA ethyl esters were 3.6% and 3.0% resulting in an intake of 0.14 g/d of OA and 0.12 g/d of EPA when offered approximately 4 g of diet.

5.4.2 Histology and SAA

As expected, the average of total intestinal HIS in *Il10*$^{-/-}$ mice was higher than for C57 mice in both diet groups. The colon was the most inflamed intestinal section. No signs of intestinal inflammation were observed for C57 mice (Table 5.1). All five *Il10*$^{-/-}$ mice fed the OA diet and three of the four EPA-fed *Il10*$^{-/-}$ mice showed histological signs of colon inflammation. The HIS for colon of *Il10*$^{-/-}$ mice fed the EPA diet was more variable and ranged from low to highly inflamed, compared to the OA group (Fig. 5.2). HIS for the colon tissue of *Il10*$^{-/-}$ mice on both OA and EPA diets mainly represented inflammatory cell infiltration (74%, monocytes and neutrophils) and to a lesser extent, tissue destruction (22%, crypt loss and oedema) and tissue repair (4%, hyperplasia); the inflammatory lesions only occurred in some areas of the colon (Fig. 5.3). There was no significant reduction in inflammatory cell infiltration or tissue destruction and repair in the colon of *Il10*$^{-/-}$ mice in the EPA compared to the OA group. There was no difference in the SAA plasma concentration (a marker for inflammation) between the EPA and OA diet groups in *Il10*$^{-/-}$ or C57 mice, but there was a significantly higher SAA plasma concentration in OA-fed *Il10*$^{-/-}$ compared to C57 mice (Table 5.1).

5.4.3 Gene expression in the colon of *Il10*$^{-/-}$ and C57 mice fed the OA diet

To study the underlying mechanism of inflammation in the colon, *Il10*$^{-/-}$ mice on the OA diet were compared to C57 mice on the same diet, and changes in global gene expression were analysed. The microarray analyses revealed 2,565 oligonucleotide probes differentially expressed (FC≥1.3, P<0.01) in the colon of *Il10*$^{-/-}$ and C57 mice fed the OA diet (1,404 up-regulated and 1,161 down-regulated). Additionally, qRT-PCR analyses were performed for selected genes from the colon of pooled mice, which in general confirmed the array results (Table 5.2).

In IPA, 1,340 differentially expressed genes were eligible for generating networks and of those, 1,238 were associated with functions and pathways. For the remaining 102 genes, molecular interaction information was lacking. Comparing *Il10*$^{-/-}$ and C57 mice fed the OA diet, genes of the most significant pathways (IL10 signalling, leukocyte extravasation signalling, LPS and IL1–mediated inhibition of RXR function, fibrosis development, xenobiotic metabolism, fatty acid metabolism and tryptophan metabolism) underlying the dataset were merged in IPA to form a biological network composed of 107 nodes and their direct interactions were supported by published information (Fig. 5.4). Furthermore, 8 nodes were identified as central genes; epidermal

growth factor receptor (*Egfr*), intercellular adhesion molecule1 (*Icam1*), interferon regulatory factor1 (*Irf1*), integrin αM (*Itgam*), matrix metallopeptidase9 (*Mmp9*), *Ppara, Stat* and *Stat3* connected many of the genes which were differentially expressed between the genotypes. Almost all down-regulated genes involved in fatty acid metabolism were linked to *Ppara* which itself was directly connected to *Mmp9* and through *Mmp9* to the other central genes *Icam1*, *Irf1*, *Itgam* and *Stat1* that control most of the genes up-regulated in *Il10*$^{-/-}$ mice. Genes involved in leukocyte extravasation signalling were linked to *Icam1* and *Itgam* genes, which are both part of that pathway. The *Stat1* gene was also linked to class II MHC transactivator *(CIIta)* that regulates some important genes from the antigen presentation pathway *(Hla-drb*, *Hla-dmb* and *Hla-dqa)*.

Pathway analysis in IPA revealed 65 metabolic and signalling pathways that showed significant changes in gene expression in *Il10*$^{-/-}$ compared to the C57 mice, both fed the OA diet. The top 10 pathways included IL10 signalling, leukocyte extravasation signalling, fibrosis development, xenobiotic metabolism, fatty acid metabolism and tryptophan metabolism (Table 5.3). Genes differentially expressed in these top pathways are shown in Table 5.4.

Parallel to IPA, FUNC was used to compare the underlying molecular functions and biological processes involved in colon inflammation. The significantly over-represented GO terms of genes are listed in Table 5.5. The predominant biological processes, antigen presentation, immune response and cell proliferation, identified in FUNC, were also in the top biological function list in IPA (Table 5.3), suggesting that these processes underlie colon inflammation in *Il10*$^{-/-}$ mice.

In *Il10*$^{-/-}$ mice fed the OA diet, the IL10 signalling pathway showed increased mRNA levels for genes coding for chemokine receptor5 (*Ccr5*), *Cd14* molecule (*Cd14*), *Il10*, interleukin-10 receptor alpha (*Il10ra*), *Il1a*, *Il1b*, interleukin-1 receptor type1 and 2 (*Il1r1*, *Il1r2*) and *Nfkb* inhibitors (*Nfkbia*, *Nfkbie*), all of which play a role in inflammatory and immune responses. Other genes with increased expression levels in the IL10 signalling pathway were *Stat3* (an acute phase response factor), *Tnfa* (a pro-inflammatory cytokine), and suppressor of cytokine signalling 3 (*Socs3*), all linked to inflammatory disorders.

Increased expression levels of genes in the colon from OA-fed *Il10*$^{-/-}$ mice were also involved in the leukocyte extravasation signalling pathway, which included *Icam1*, vascular cell adhesion molecule1 (*Vcam1*) and integrin beta2 (*Itgb2*) genes involved in cell adhesion and cell surface-mediated signalling. Other genes with increased expression levels associated with that pathway were chemokine ligands and receptors (*Cxcl12* and *Cxcr4*), matrix metalloproteinases (*Mmp3*, *Mmp7*, *Mmp9*, *Mmp10*, *Mmp13* and *Mmp14*) and tissue inhibitor of matrix metalloproteinases1 (*Timp1*). Claudin8 (*Cldn8*) and junctional adhesion molecule3 (*Jam3*) are required for maintaining epithelial tight junctions and their expression was down-regulated in the *Il10*$^{-/-}$ colon compared to the C57 mice, both fed the OA diet. Genes in pathways associated with fibrosis development were increased in expression, whereas oxidative stress response genes e.g., NAD(P)H oxidoreductase (*Nqo1*) were down-regulated in OA-fed *Il10*$^{-/-}$ mice.

Genes involved in xenobiotic metabolism and fatty acid metabolism were differentially expressed in the colon of OA-fed *Il10*$^{-/-}$ compared to C57 mice. A down-regulation was observed in cytochrome P450 genes (e.g., *Cyp2c9*, *Cyp2c37*, *Cyp2d26* and *Cyp3a5*), glutathione S-transferases (*Gsta5*, *Gstm5* and *Gstt2*), ATP-binding cassette transporters (e.g., *Abcb1* and *Abcc3*) and aldehyde dehydrogenase (*Aldh1a1*).

Genes coding for enzymes that are necessary for metabolising acyl-CoA to acetyl-CoA, such as acetyl-CoA acyltransferase1 (*Acaa1*), acetyl-CoA oxidase1 (*Acox1*) and enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase (*Ehhadh*), in the peroxisomal fatty acid β-oxidation, were diminished in expression. The expression levels of genes involved in mitochondrial-mediated fatty acid β-oxidation, e.g., carnitine palmitoyltransferases1 and 2 (*Cpt1* and *Cpt2*) and hydroxyacyl-CoA dehydrogenase (*Hadh*) were also decreased.

The expression levels of genes associated with tryptophan metabolism, such as indoleamine-pyrrole 2,3-dioxygenase (*Indo* or *Ido*; catalysing the degradation of L–tryptophan to *N*-formylkynurenine), and tryptophanyl-tRNA synthase (*Wars*; catalysing amino acylation of tRNA by tryptophan) were increased in the colon of OA-fed *Il10*$^{-/-}$ compared to the C57 mice.

5.4.4 Effects of dietary EPA on colon gene expression in *Il10*$^{-/-}$ and C57 mice

After identifying histological and molecular signs of colon inflammation in OA-fed *Il10*$^{-/-}$ mice, the effect of EPA was investigated to define probable mechanisms of action in *Il10*$^{-/-}$ mice. The microarray analysis revealed 321 oligonucleotide probes differentially expressed (FC≥1.3, P<0.01) in the colon of *Il10*$^{-/-}$ mice fed the EPA diet compared with the OA diet (191 up-regulated and 130 down-regulated). The qRT-PCR data confirmed the results obtained by microarrays of pooled RNA for six genes involved in molecular pathways responsive to EPA feeding when comparing EPA- versus OA-fed *Il10*$^{-/-}$ mice (Table 5.2). However, the *Fabp2* gene which was not differentially expressed (P=0.01) in the microarrays for EPA- compared to OA-fed *Il10*$^{-/-}$ mice, showed significant increase in expression by qRT-PCR normalised to both reference genes. The *Ppara* gene did not reach the significance level of 5% by qRT-PCR, but showed a trend (P=0.06) supporting the increased expression level of this gene with microarray.

In IPA, 173 genes were eligible for generating networks and of those, 166 were associated with functions and pathways and for the remaining 7 genes molecular interaction information was lacking. The biological functions that were most significantly affected by the EPA diet in both mouse strains are summarised in Table 5.3. The comparison of EPA versus OA diets in C57 mice showed no change for key genes in the affected pathways observed in EPA-fed *Il10*$^{-/-}$ mice.

The GO analysis of underlying molecular functions and biological processes affected by EPA showed significantly over-represented GO terms linked to genes coding for enzymes with monooxygenase and glucuronosyltransferase activity which are involved in detoxification and biotransformation as seen by IPA analysis. The FUNC results were in agreement with IPA analysis, both indicating lipid metabolism and xenobiotic metabolism as predominant biological processes in *Il10*$^{-/-}$ mice fed the EPA diet (Table 5.6).

IPA analysis revealed the following top pathways linked to genes differentially expressed in the colon of EPA- compared to OA-fed *Il10*$^{-/-}$ mice: xenobiotic metabolism, fatty acid metabolism, inflammatory and immune response and oxidative stress response (Table 5.3, Table 5.4 and summarised in Fig. 5.5). Several genes

involved in the xenobiotic metabolism pathway showed increased mRNA expression in EPA- compared to OA-fed *Il10*$^{-/-}$ mice, including phase I (e.g. *Cyp2c18*, *Cyp2c40*, *Cyp2c44*, *Cyp2d26* and *Cyp3a5*), phase II (*Gsta4*) and phase III (*Abcb1* and *Abcc3*) detoxification enzymes. The expression levels of genes coding for polypeptides of the UDP glucuronosyltransferase-1 and -2 family members, such as *Ugt1a1*, *Ugt2b5* and *Ugt2b37*, were significantly increased in EPA- compared to OA-fed *Il10*$^{-/-}$ mice.

Genes associated with fatty acid metabolism, such as mitochondrial *Acaa2* and peroxisomal *Acaa1b*, were increased in expression in the colon of *Il10*$^{-/-}$ mice in response to dietary EPA. Several genes common to fatty acid and tryptophan metabolism, e.g., *Cyp450* and *Aldh*, were increased in expression levels, but key genes of the tryptophan metabolism pathway *(Indo* and *Wars)* were not affected by the EPA diet compared to the OA-fed *Il10*$^{-/-}$ mice.

The expression levels of genes involved in fibrosis development were increased, while others were decreased, so no clear link can be made about the effect of EPA on fibrosis in the colon tissue of *Il10*$^{-/-}$ mice. Genes involved in the oxidative stress response, e.g., genes for detoxification and antioxidant enzymes *Gsta4* and *Nqo1* were increased in expression levels in EPA-fed *Il10*$^{-/-}$ mice.

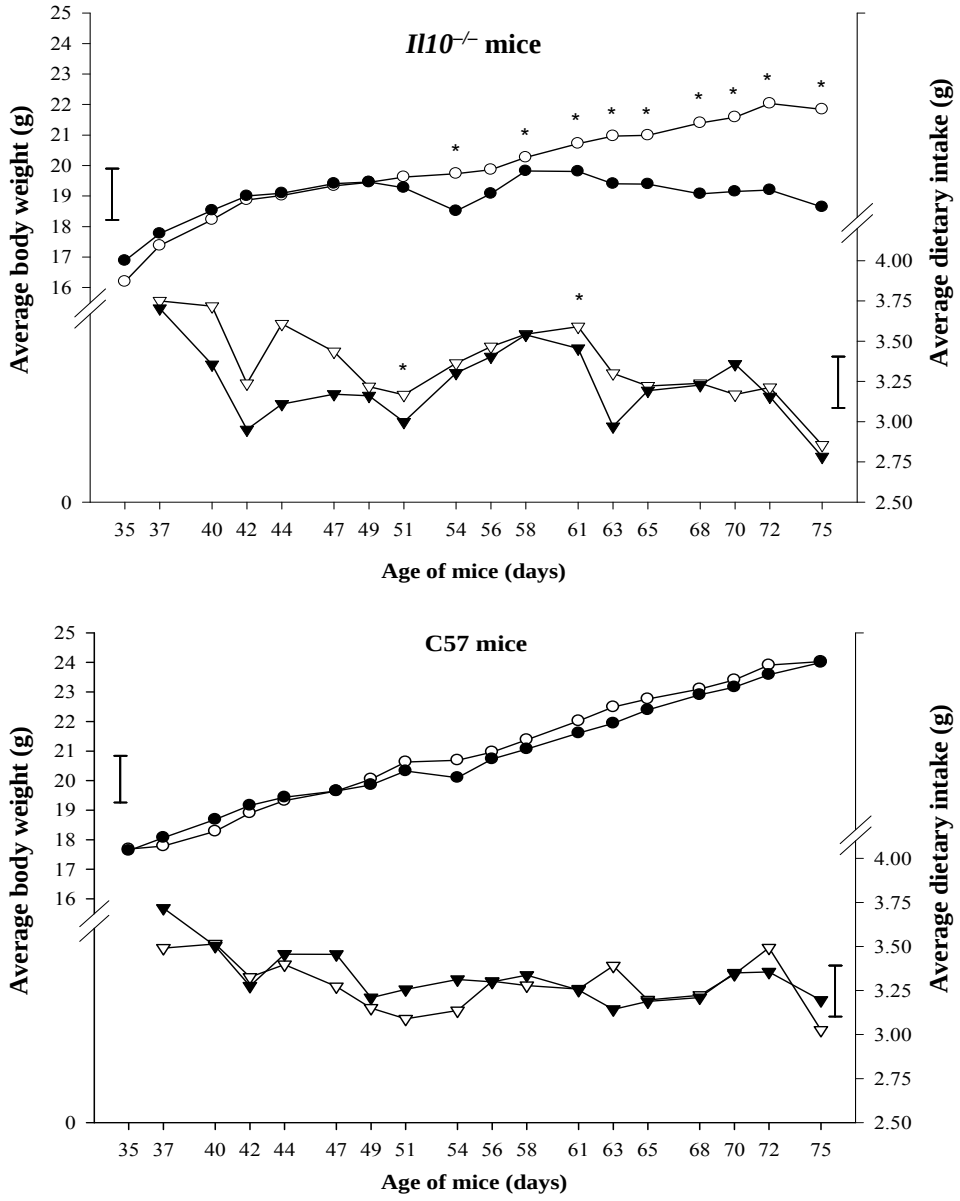


Fig. 5.1 Mean body weight (g) and dietary intake (g) of *Il10*$^{-/-}$ and C57 mice fed OA and EPA diets for the experimental period. Error bars represent the least significant difference (lsd, 5% level) for comparing diets at each time point. For the dietary intake data, these lsd bars represent the average lsd over all time points. ○ = body weight OA diet; ● = body weight EPA diet; Δ = dietary intake OA diet; ▲ = dietary intake EPA diet.

Table 5.1 Inflammation and HIS in colon tissue, and plasma SAA concentrations in C57 and *Il10*$^{-/-}$ *mice* fed OA or EPA diets

	C57 mice		***Il10*$^{-/-}$ *mice***		
	OA diet	**EPA diet**	**OA diet**	**EPA diet**	**lsd**
Number of mice sampled	6	6	5[a]	4[b]	
Number of mice inflamed[c]	0	0	5	3	
Inflammation (%)	0	0	100	75	
Number of colon samples					
Not inflamed	0	0	0	1	
Moderately inflamed	0	0	3	2	
Highly inflamed	0	0	2	1	
Colon HIS	0.13	0.33	7.00	5.38	2.69
Inflammatory cell infiltrates					
Monocytes	0.13	0.17	4.40	3.50	
Neutrophils	0	0.08	0.90	0.38	
Fibrin exudation	0	0	0	0	
Lymphangiectasis	0	0	0	0	
Total	0.13	0.25	5.30	3.88	2.15
Tissue destruction					
Enterocyte loss	0	0	0.80	0.75	
Ballooning	0	0	0	0	
Oedema	0	0.08	0.50	0.63	
Mucosal atrophy	0	0	0	0	
Total	0	0.08	1.30	1.38	1.31
Tissue repair					
Hyperplasia	0	0	0.40	0.13	
Angiogenesis	0	0	0	0	
Granulomas	0	0	0	0	
Total	0	0	0.40	0.13	0.51
SAA (μg/mL)	23.8 (4.28)	82.5 (4.88)	538 (6.38)	1056 (7.01)	1.19

[a] One *Il10*$^{-/-}$ mouse from the OA group died for unknown causes during the experiment. [b] Two *Il10*$^{-/-}$ mice from the EPA group were killed because of weight loss (>20% of pre-trial weight) during the experiment. These three animals have not been scored for histological signs of inflammation and could not be taken into account for statistical analysis. [c] Inflamed was defined as a total histological injury score (HIS) in at least one of the intestinal regions of more than 3.5. The total colon HIS with its scores for the inflammation types and serum amyloid A (SAA) (log-transformed values in parentheses) are shown as mean with least significant difference (lsd). The inflammation type is divided into its subtypes.

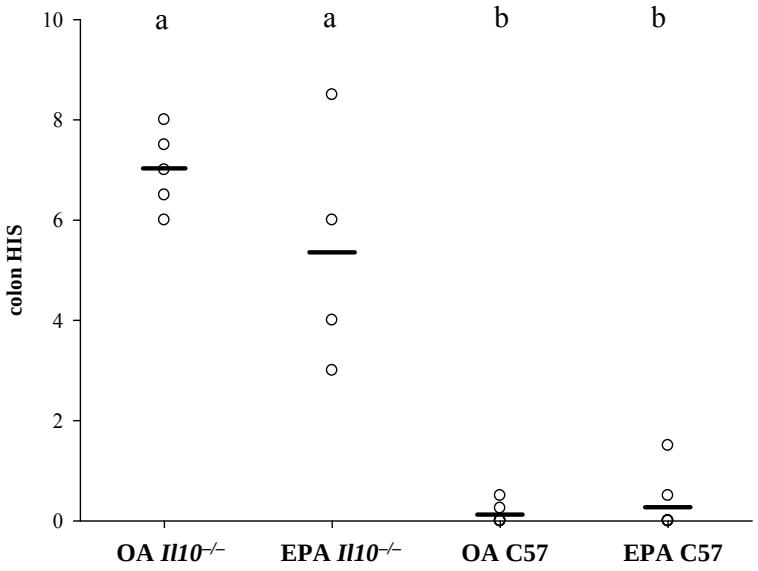
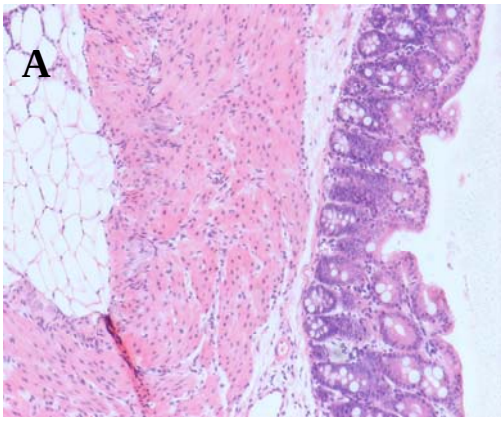
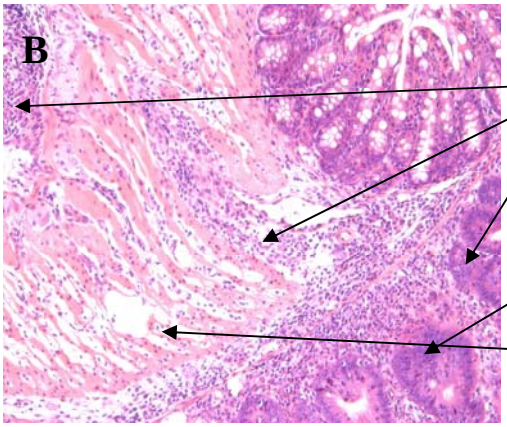


Fig. 5.2 Histological injury score (HIS) of colon tissue for individual *Il10*$^{-/-}$ *mice* on the OA diet (n=5) and the EPA diet (n=4), and C57 mice on the OA diet (n=6) and the EPA diet (n=6). The horizontal line indicates the mean colon HIS within each group. a, b: different letters indicate a significant difference between treatment groups (P<0.05).

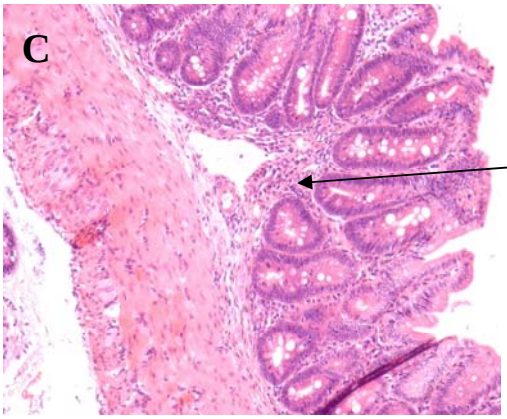




Inflammatory cell infiltration in all layers of the intestinal wall, primarily monocytes, with some neutrophils.

The histological structure of this area is disturbed; the array of crypts is disorganised and much fewer goblet cells appear.

Changes in muscle tissue due to oedema.



Inflammatory cell infiltration into the lamina propria (primarily monocytes).

Fig. 5.3 Haematoxylin-eosin stained colon sections of a C57 and an *Il10*$^{-/-}$ mouse. (A) Colon section (×40) from a C57 mouse fed the EPA diet with a normal, non-inflamed appearance. (B) Colon section (×100) from an *Il10*$^{-/-}$ mouse fed the OA diet with signs of severe colon inflammation. Arrows indicate histological signs of inflammation observed in colon section B and C. Lesions involved areas of the colon section and were mainly caused by inflammatory cell infiltration (monocytes and neutrophils). (C) Colon section (×100) from an *Il10*$^{-/-}$ mouse fed the EPA diet showing signs of moderate colon inflammation.

Table 5.2 Expression levels of selected genes from microarray and qRT-PCR analyses of pooled colon tissue of *Il10*$^{-/-}$ and C57 mice fed OA or EPA diet

Gene	Fold change (microarray)	Fold change (qRT-PCR *Canx*)	Fold change (qRT-PCR *Cyclophilin*)
***Il10*$^{-/-}$ vs. C57 on OA diet**			
Abcb1a	-3.3	-5.9	-5.9
Aldh1a1	-2.1	-4.0	-4.1
Ces2	-3.6	-7.1	-7.0
Fabp2	-2.4	-11.0	-12.3
Igfbp5	2.0*	4.3	4.8
Ppara	-2.5	-4.1	-4.0
Sult1a1	-2.1	-6.8	-6.8
EPA vs. OA in *Il10*$^{-/-}$ mice			
Abcb1a	2.4	4.0	5.0
Aldh1a1	2.1	3.9	5.0
Ces2	1.8*	2.9	3.6
Fabp2	2.0*	2.6	3.5
Igfbp5	-1.8	-2.0	-1.7
Ppara	1.5	2.2$^{\#}$	2.7$^{\#}$
Sult1a1	2.7	4.4	5.6

Canx (calnexin) and *Cyclophilin* (cyclophilinA or peptidylprolyl isomerase A) were used as reference genes to normalise the data; *Microarray result was not significantly different using the moderated t-statistics in limma; genes that satisfied the criterion of FC≥1.3 and P<0.01 were considered to be significantly different; $^{\#}$qRT-PCR result was not significantly different using ANOVA.

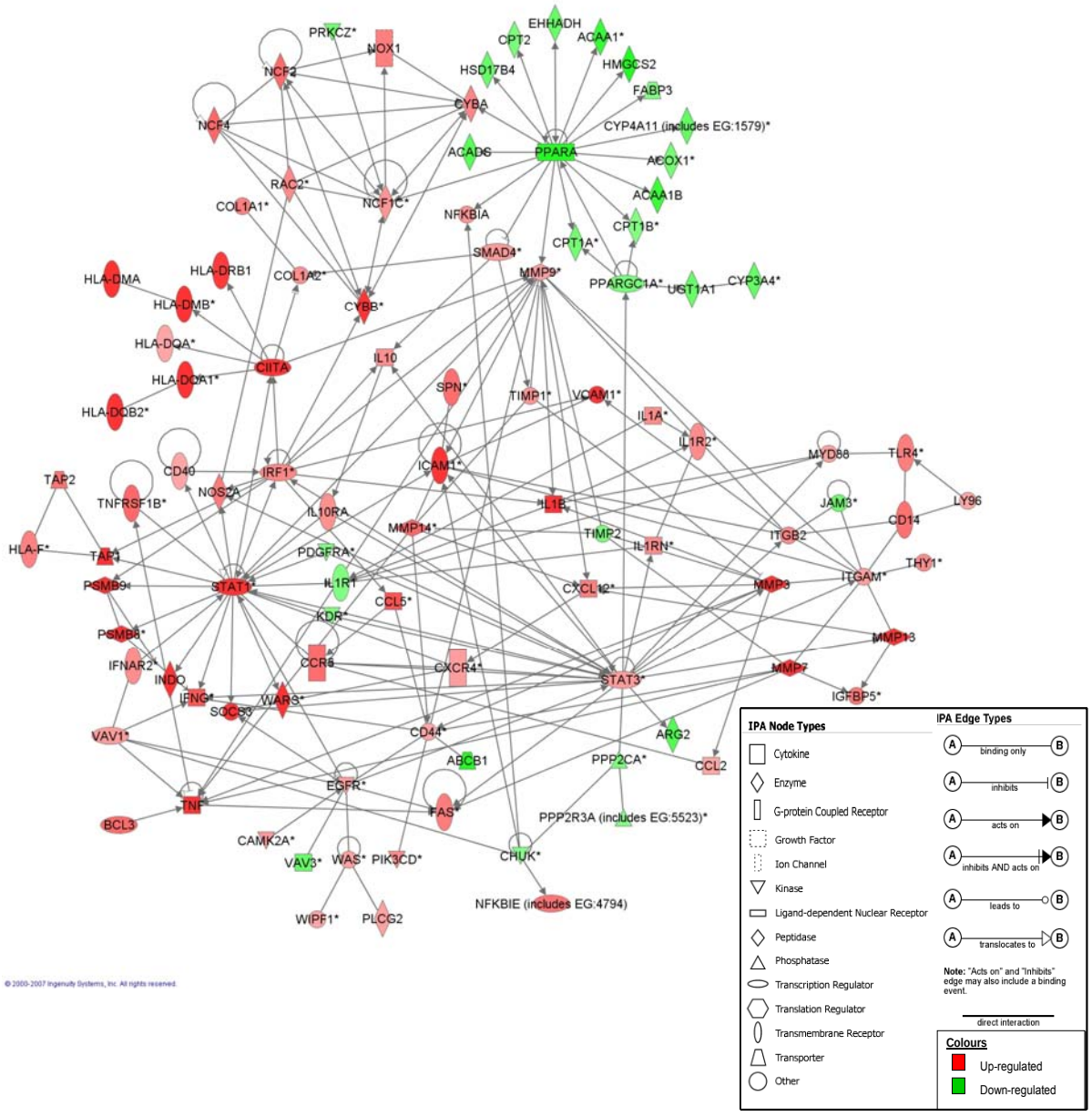


Fig. 5.4 Generation of biological interaction networks of genes of the most significant pathways in *Il10*$^{-/-}$ versus C57 mice fed the OA diet. IPA was used to merge significantly affected pathways (LPS and IL1-mediated inhibition of RXR function, fibrosis/stellate cell activation, IL10 signalling, xenobiotic metabolism signalling, fatty acid metabolism, tryptophan metabolism and leukocyte extravasation signalling). Eight central genes and their direct interactions were identified which were supported by published information. *Genes that are detected two or more times on the array. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as a line (i.e. an edge). All edges are supported by at least one reference from the literature. Red and green coloured nodes indicate genes with increased and decreased expression level, respectively. The intensity of the colours specifies the degree of increase or decrease. Greater intensity represents a higher expression level. Nodes and edges are displayed with various shapes and labels that present the functional class of genes and the nature of the relationships between the nodes.

Table 5.3 Significant biological functions and pathways in the colon of 1) *Il10*$^{-/-}$ versus C57 mice fed OA diet, 2) EPA- versus OA-fed *Il10*$^{-/-}$ mice and 3) EPA- versus OA-fed C57 mice

	***Il10*$^{-/-}$ vs. C57 mice (fed OA diet)**	**EPA vs. OA diet (in *Il10*$^{-/-}$ mice)**	**EPA vs. OA diet (in C57 mice)**
Biological functions			
Diseases and disorders	Immunological disease (238)	Hepatic system disease (17)	Inflammatory disease (33)
	Inflammatory disease (239)	Cancer (57)	Cancer (68)
	Connective tissue disorders (159)	Infectious disease (9)	Connective tissue disorders (21)
Molecular and cellular functions	Cellular movement (275)	Lipid metabolism (45)	Lipid metabolism (45)
	Cell-to-cell signalling and interaction (283)	Vitamin and mineral metabolism (25)	Molecular transport (15)
	Cellular growth and proliferation (393)	Amino acid metabolism (41)	Small molecule biochemistry (29)
Physiological system development / functions	Immune response (300)	Behaviour (6)	Tumour morphology (6)
	Haematological system development and function (264)	Nervous system development and function (15)	Skeletal and muscular system development and function (19)
	Immune and lymphatic system development and function (239)	Organismal survival (12)	Cardiovascular system development and function (12)
Pathways	LPS/IL1-mediated inhibition of RXR function (53/192)	Xenobiotic metabolism signalling (19/251)	Phenylalanine metabolism (5/107)
	Fibrosis/stellate cell activation (41/131)	Metabolism of xenobiotics by cytochrome P450 (12/213)	Complement system (5/37)
	IL10 signalling (22/68)	LPS/ IL1-mediated inhibition of RXR function (13/192)	PI3K/AKT signalling (9/147)
	Metabolism of xenobiotics by cytochrome P450 (27/213)	Fatty acid metabolism (10/187)	Metabolism of xenobiotics by cytochrome P450 (6/213)
	Fatty acid metabolism (33/187)	Tryptophan metabolism (8/237)	Linoleic acid metabolism (5/123)
	Tryptophan metabolism (31/237)	Cholestasis (8/162)	Cholestasis (6/162)
	Leukocyte extravasation signalling (41/188)	IL10 signalling (3/68)	Arachidonic acid metabolism (5/211)

The global functional analysis identified biological functions (molecular and cellular functions; physiological system development and function) and diseases that were most significant to the dataset. The functions and the number of genes differentially expressed in each category are summarised. The number or ratio in parentheses represents the total number of differentially expressed genes associated with each biological function or associated with the total number of genes in that pathway.

Table 5.4 Pathways with genes differentially expressed in the 1) genotype comparison (*Il10*$^{-/-}$ *vs.* C57 mice fed the OA diet), 2) diet comparison (EPA *vs.* OA) in *Il10*$^{-/-}$ mice and 3) diet comparison (EPA *vs.* OA) in C57 mice, as identified by IPA pathway analysis

Gene symbol	Gene description	GeneBank accession	*Il10*$^{-/-}$ *vs.* C57 on OA		EPA *vs.* OA in *Il10*$^{-/-}$		EPA *vs.* OA in C57	
			FC	*P*-value	FC	*P*-value	FC	*P*-value
Fatty acid metabolism								
Acaa1	acetyl-Coenzyme A acyltransferase 1 (peroxisomal 3-oxoacyl-Coenzyme A thiolase)	NM_130864	-2.3	<.00001	1.5	0.03	-1.1	0.4
Acaa2	acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	NM_177470	-2.0	0.001	1.4	0.007	1.1	0.6
Acaa1b	acetyl-Coenzyme A acyltransferase 1B	NM_146230	-2.2	<.00001	1.6	0.005	-1.0	0.9
Acad10	acyl-Coenzyme A dehydrogenase family, member 10	NM_028037	-1.3	0.007	1.1	0.4	-1.1	0.6
Acads	acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	NM_007383	-1.9	0.008	1.1	0.6	-1.3	0.2
Acox1	acyl-Coenzyme A oxidase 1, palmitoyl	NM_015729	-1.5	0.003	1.4	0.02	-1.2	0.2
Acox2	acyl-Coenzyme A oxidase 2, branched chain	NM_053115	-2.0	0.004	1.2	0.4	-1.1	0.8
Acsl3	acyl-CoA synthetase long-chain family member 3	NM_028817	-2.8	<.00001	-1.0	0.9	-1.2	0.1
Cpt2	carnitine palmitoyltransferase II	NM_009949	-1.5	0.002	1.4	0.01	1.1	0.5
Cpt1a	carnitine palmitoyltransferase 1A (liver)	NM_013495	-1.6	0.003	1.3	0.1	-1.2	0.3
Cpt1b	carnitine palmitoyltransferase 1B (muscle)	NM_009948	-1.4	0.003	1.4	0.02	-1.2	0.1
Cyp4a11	cytochrome P450, family 4, subfamily A, polypeptide 11	X71478	-1.8	0.003	1.2	0.05	-1.2	0.4
Ech1	enoyl Coenzyme A hydratase 1, peroxisomal	NM_016772	-2.1	<.00001	1.4	0.06	-1.2	0.2
Ehhadh	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	NM_023737	-1.7	0.009	1.0	0.9	-1.1	0.5
Hadh	hydroxyacyl-Coenzyme A dehydrogenase	AK199677	-1.7	<.00001	1.3	0.04	1.3	0.07
Hsd17b4	hydroxysteroid (17-beta) dehydrogenase 4	NM_008292	-1.7	<.00001	1.2	0.1	-1.1	0.6
Slc27a4	solute carrier family 27 (fatty acid transporter), member 4	NM_011989	-2.0	0.009	1.1	0.6	-1.1	0.6
Xenobiotic metabolism								
Abcb1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	NM_011076	-3.3	<.00001	2.4	0.001	1.1	0.5
Abcc3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	AK006128	-1.9	<.00001	1.6	0.002	-1.5	0.01
Akr1c3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	NM_134066	-1.3	0.2	2.1	<.00001	1.1	0.7
Aldh1a1	aldehyde dehydrogenase 1 family, member A1	NM_013467	-2.1	<.00001	2.1	<.00001	1.1	0.6

Aldh1a7	aldehyde dehydrogenase family 1, subfamily A7	NM_011921	-2.7	<.00001	1.6	0.006	-1.0	0.9
Aldh1l1	aldehyde dehydrogenase 1 family, member L1	NM_027406	2.3	<.00001	-1.2	0.3	1.1	0.7
Aldh2	aldehyde dehydrogenase 2 family (mitochondrial)	NM_009656	-1.8	0.001	1.2	0.3	-1.3	0.07
Ces1	carboxylesterase 1 (monocyte/macrophage serine esterase 1)	NM_053200	-4.8	<.00001	3.0	0.001	-1.1	0.6
Ces2	carboxylesterase 2	NM_145603	-3.6	<.00001	1.8	0.01	-1.7	0.02
Cyp1b1	cytochrome P450, family 1, subfamily B, polypeptide 1	NM_009994	1.5	0.001	-1.2	0.07	1.3	0.03
Cyp2c9	cytochrome P450, family 2, subfamily C, polypeptide 9	NM_007815	-7.7	<.00001	2.0	0.02	-2.2	0.01
Cyp2c18	cytochrome P450, family 2, subfamily C, polypeptide 18	NM_028089	-1.6	0.2	3.1	0.006	1.9	0.02
Cyp2c37	cytochrome P450, family 2. subfamily c, polypeptide 37	NM_010001	-2.6	<.00001	1.4	0.08	-1.8	0.004
Cyp2c40	cytochrome P450, family 2, subfamily c, polypeptide 40	NM_010004	-14.3	<.00001	3.0	0.001	-2.1	0.02
Cyp2c44	cytochrome P450, family 2, subfamily c, polypeptide 44	NM_01001446	-1.2	0.2	1.7	0.008	1.3	0.2
Cyp2c54	cytochrome P450, family 2, subfamily c, polypeptide 54	NM_206537	-1.6	0.002	1.2	0.1	-1.2	0.2
Cyp2d6	cytochrome P450, family 2, subfamily D, polypeptide 6	NM_019823	-1.7	0.005	1.4	0.04	-1.3	0.1
Cyp2d26	cytochrome P450, family 2, subfamily d, polypeptide 26	NM_029562	-2.5	<.00001	1.8	0.003	-1.3	0.2
Cyp2j2	cytochrome P450, family 2, subfamily J, polypeptide 2	NM_010008	-1.3	0.007	1.1	0.6	-1.5	<.00001
Cyp2s1	cytochrome P450, family 2, subfamily S, polypeptide 1	NM_028775	-1.8	<.00001	1.1	0.6	-1.1	0.4
Cyp3a4	cytochrome P450, family 3, subfamily A, polypeptide 4	NM_177380	-1.7	0.006	1.6	0.02	1.2	0.2
Cyp3a5	cytochrome P450, family 3, subfamily A, polypeptide 5	NM_007819	-2.2	<.00001	1.9	0.001	-1.3	0.1
Cyp4b1	cytochrome P450, family 4, subfamily B, polypeptide 1	NM_007823	-8.3	<.00001	2.3	0.06	-1.1	0.7
Cyp51a1	cytochrome P450, family 51, subfamily A, polypeptide 1	NM_020010	1.2	0.1	-1.6	0.001	-1.4	0.006
Fmo1	flavin containing monooxygenase 1	NM_010231	-1.6	0.005	1.3	0.1	1.1	0.7
Fmo2	flavin containing monooxygenase 2 (non-functional)	NM_018881	-1.4	0.003	1.4	0.002	1.1	0.2
Fmo5	flavin containing monooxygenase 5	NM_010232	-4.0	<.00001	1.6	0.1	-1.3	0.3
Gsta4	glutathione S-transferase A4	NM_010357	-3.1	<.00001	1.3	0.005	-1.2	0.09
Gsta5	glutathione S-transferase A5	NM_008182	-3.9	<.00001	1.8	0.01	-1.1	0.6
Gstk1	glutathione S-transferase kappa 1	NM_029555	-2.2	<.00001	1.0	0.9	-1.3	0.04
Gstm1	glutathione S-transferase M1	NM_008183	-2.4	<.00001	1.3	0.04	-1.4	0.006
Gstm4	glutathione S-transferase M4	NM_026764	-1.3	0.005	1.1	0.2	-1.1	0.3
Gstm5	glutathione S-transferase M5	NM_010358	-2.5	0.007	1.1	0.6	-1.5	0.2
Gstm6	glutathione S-transferase, mu 6	NM_008184	-1.4	<.00001	1.1	0.3	-1.4	0.001
Gstt2	glutathione S-transferase theta 2	NM_010361	-1.5	0.003	1.1	0.3	-1.3	0.03
Mgst3	microsomal glutathione S-transferase 3	AK008211	-1.9	<.00001	1.5	0.003	-1.3	0.07
Sult1a1	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	NM_133670	-2.1	0.001	2.7	<.00001	1.4	0.09

Sult1c2	sulfotransferase family, cytosolic, 1C, member 2	NM_026935	-5.6	<.00001	1.9	0.002	-1.2	0.3
Sult2b1	sulfotransferase family, cytosolic, 2B, member 1	NM_017465	-1.7	0.001	1.3	0.1	-1.1	0.4
Ugt8	UDP glycosyltransferase 8 (UDP-galactose ceramide galactosyltransferase)	NM_011674	-1.9	<.00001	-1.0	0.9	-1.3	0.008
Ugt1a1	UDP glucuronosyltransferase 1 family, polypeptide A1	NM_201645	-1.7	0.001	1.6	0.002	-1.1	0.5
Ugt2b5	UDP glucuronosyltransferase 2 family, polypeptide B5	NM_009467	-3.3	<.00001	1.8	<.00001	-1.1	0.3
Ugt2b37	UDP glucuronosyltransferase 2 family, polypeptide B37	NM_053215	-3.0	<.00001	1.8	0.003	-1.2	0.3
Tryptophan metabolism								
Abp1	amiloride binding protein 1 (amine oxidase (copper-containing))	NM_029638	-2.3	<.00001	1.5	0.06	-1.0	0.9
Aoc3	amine oxidase, copper containing 3 (vascular adhesion protein 1)	NM_009675	-2.4	0.001	1.3	0.3	1.1	0.6
Ccbl1	cysteine conjugate-beta lyase; cytoplasmic (glutamine transaminase K, kyneurenine aminotransferase)	NM_172404	-1.6	0.006	1.1	0.5	-1.1	0.4
Ddc	dopa decarboxylase (aromatic L-amino acid decarboxylase)	NM_016672	-2.1	<.00001	1.2	0.06	-1.1	0.2
Indo	indoleamine-pyrrole 2,3 dioxygenase	NM_008324	7.7	<.00001	-	-	-	-
Inmt	indolethylamine N-methyltransferase	NM_009349	-1.8	0.003	1.1	0.6	1.1	0.6
Maob	monoamine oxidase B	NM_172778	-4.0	<.00001	1.4	0.03	-1.2	0.2
Nit1	nitrilase 1	NM_012049	-1.4	0.01	-1.0	0.8	1.1	0.2
Smox	spermine oxidase	NM_145533	1.7	0.001	-1.2	0.2	1.2	0.1
Wars	tryptophanyl-tRNA synthetase	NM_011710	3.5	<.00001	-1.5	0.05	-1.2	0.4
Oxidative stress response								
Gpx4	glutathione peroxidase 4 (phospholipid hydroperoxidase)	NM_008162	-1.8	0.001	1.1	0.5	-1.2	0.2
Gsta4	glutathione S-transferase A4	NM_010357	-3.1	<.00001	1.3	0.005	-1.2	0.09
Gsta5	glutathione S-transferase A5	NM_008182	-3.9	<.00001	1.8	0.01	-1.1	0.6
Gstm1	glutathione S-transferase M1	NM_008183	-2.4	<.00001	1.3	0.04	-1.4	0.006
Gstm5	glutathione S-transferase M5	NM_010358	-2.4	0.007	1.1	0.6	-1.5	0.2
Mpo	myeloperoxidase	NM_010824			1.4	0.001	-1.0	0.7
Nqo1	NAD(P)H dehydrogenase, quinone 1	NM_008706	-2.0	<.00001	1.7	<.00001	-1.2	0.07
Prdx5	peroxiredoxin 5	NM_012021	-1.0	0.8	-1.1	0.2	-1.7	<.00001
Prdx6	peroxiredoxin 6	NM_007453	-1.9	0.01	-1.3	0.2	-2.4	0.002
S100a9	S100 calcium binding protein A9	NM_009114	20.7	<.00001	1.8	0.4	1.6	0.1
Xdh	xanthine dehydrogenase	NM_011723	2.7	<.00001	-1.2	0.3	1.2	0.3
Fibrosis development								

Abcb4	ATP-binding cassette, sub-family B (MDR/TAP), member 4	NM_008830			2.5	<.00001	-1.4	0.1
Acta2	actin, alpha 2, smooth muscle, aorta	NM_007392	-2.1	<.00001	1.5	0.02	1.1	0.5
Adcy7	adenylate cyclase 7	BC057682	-5.3	<.00001	-1.1	0.4	1.4	0.007
Agtr1	angiotensin II receptor, type 1	NM_177322	-1.6	0.007	1.2	0.2	1.1	0.5
Ccl2	chemokine (C-C motif) ligand 2	NM_011331	1.3	0.008	-1.1	0.3	1.1	0.3
Ccl5	chemokine (C-C motif) ligand 5	BC033508	2.6	<.00001	-1.3	0.2	1.2	0.1
Ccr5	chemokine (C-C motif) receptor 5	NM_009917	2.2	<.00001	-1.3	0.005	1.1	0.3
Ccr7	chemokine (C-C motif) receptor 7	NM_007719	1.6	0.001	1.0	0.7	1.3	0.03
Col1a1	collagen, type I, alpha 1	NM_007742	1.9	0.003	-1.7	0.03	1.4	0.1
Col1a2	collagen, type I, alpha 2	NM_007743	1.7	0.006	-1.6	0.01	1.3	0.1
Cxcl3	chemokine (C-X-C motif) ligand 3	NM_009140	1.5	0.004	1.4	0.02	1.1	0.3
Edn1	endothelin 1	NM_010104	-2.1	0.006	1.0	0.9	-1.4	0.1
Ednrb	endothelin receptor type B	NM_007904	-1.6	<.00001	1.2	0.08	1.2	0.1
Egfr	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	NM_207655	1.4	0.001	-1.3	0.005	1.2	0.2
Fas	Fas (TNF receptor superfamily, member 6)	NM_007987	2.0	<.00001	-1.2	0.1	-1.1	0.6
Gcg	glucagon	NM_008100	1.1	0.6	2.0	0.002	1.9	0.004
Icam1	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	BC008626	3.1	<.00001	-1.1	0.1	1.2	0.3
Ifnar2	interferon (alpha, beta and omega) receptor 2	NM_010509	1.7	<.00001	-1.1	0.5	1.2	0.07
Ifng	Interferon, gamma	NM_008337	2.5	<.00001	-1.3	0.08	-1.2	0.2
Igfbp4	insulin-like growth factor binding protein 4	NM_010517	1.7	0.005	-1.4	0.05	-1.0	0.9
Igfbp5	insulin-like growth factor binding protein 5	NM_010518	2.0	0.001	-1.8	0.005	-1.1	0.8
Mmp9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	NM_013599	1.4	0.002	-1.1	0.3	1.2	0.1
Mmp13	matrix metallopeptidase 13 (collagenase 3)	NM_008607	3.2	<.00001	-1.1	0.7	1.5	0.04
Myh11	myosin, heavy chain 11, smooth muscle	NM_013607	-2.1	<.00001	1.6	0.005	-1.1	0.6
Myl7	myosin, light chain 7, regulatory	NM_022879	6.0	<.00001	-1.6	0.02	1.4	0.05
Pdgfra	platelet-derived growth factor receptor, alpha polypeptide	NM_011058	-1.5	0.004	1.3	0.02	1.2	0.1
Slc10a2	solute carrier family 10 (sodium/bile acid cotransporter family), member 2	NM_011388	-3.3	0.003	3.0	0.005	-	-
Smad4	SMAD family member 4	NM_008540	1.5	<.00001	1.0	0.7	-1.1	0.5
Stat1	signal transducer and activator of transcription 1, 91kDa	NM_009283	5.1	<.00001	-1.4	0.05	1.4	0.02
Timp1	TIMP metallopeptidase inhibitor 1	NM_011593	1.6	<.00001	-1.2	0.08	1.2	0.09

Timp2	TIMP metallopeptidase inhibitor 2	NM_011594	-1.5	0.005	1.1	0.5	1.2	0.1
Vcam1	vascular cell adhesion molecule 1	NM_011693	3.1	<.00001	-1.3	0.03	1.2	0.1
Immune and inflammatory response (IL10 signalling, leukocyte extravasation signalling)								
Abcb9	ATP-binding cassette, sub-family B (MDR/TAP), member 9	NM_019875	-1.5	0.003	1.1	0.6	1.0	0.8
Abcg1	ATP-binding cassette, sub-family G (WHITE), member 1	NM_009593	2.2	<.00001	-1.3	0.9	1.2	0.2
Ccr5	chemokine (C-C motif) receptor 5	NM_009917	2.2	<.00001	-1.3	0.005	1.0	0.8
Cd14	CD14 molecule	NM_009841	2.1	<.00001	-1.0	0.9	-1.0	0.9
Cd 44	CD 44 molecule	AK045226	1.3	0.004	-1.2	0.07	1.1	0.3
Cldn8	claudin 8	NM_018778	-3.3	<.00001	1.0	0.8	1.1	0.8
Cxcl12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	NM_021704	1.9	0.001	-1.1	0.3	1.3	0.1
Cxcr4	chemokine (C-X-C motif) receptor 4	NM_009911	1.5	0.002	-1.0	0.7	1.1	0.2
Cyba	cytochrome b-245, alpha polypeptide	NM_007806	1.8	<.00001	-1.1	0.1	-1.0	0.7
Cybb	cytochrome b-245, beta polypeptide (chronic granulomatous disease)	NM_007807	3.1	<.00001	-1.1	0.1	1.4	0.02
Fabp2	fatty acid binding protein 2, intestinal	NM_007980	-2.4	0.003	2.0	0.01	1.1	0.8
Fabp3	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	NM_010174	-1.4	0.006	1.0	0.7	-1.1	0.4
Fabp6	fatty acid binding protein 6, ileal (gastrotropin)	NM_008375	1.0	0.9	-1.1	0.9	4.4	0.006
Icam1	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	NM_010493	1.8	<.00001	-1.1	0.1	1.2	0.3
Il10	interleukin 10	NM_010548	1.7	<.00001	-1.1	0.3	-1.1	0.4
Il18	interleukin 18 (interferon-gamma-inducing factor)	NM_008360	-1.3	0.01	1.6	<.00001	-1.0	0.9
Il10ra	interleukin 10 receptor, alpha	NM_008348	1.7	<.00001	-1.2	0.2	-1.0	0.7
Il1a	interleukin 1, alpha	NM_010554	1.7	<.00001	1.2	0.1	1.2	0.1
Il1b	interleukin 1, beta	NM_008361	8.5	<.00001	-1.1	0.7	1.6	0.07
Il1r1	interleukin 1 receptor, type I	NM_008362	-1.4	0.001	1.1	0.5	-1.2	0.03
Il1r2	interleukin 1 receptor, type II	NM_010555	1.7	<.00001	-1.3	0.01	1.1	0.3
Il1rn	interleukin 1 receptor antagonist	NM_031167	1.7	0.003	-1.2	0.2	1.3	0.1
Itgam	Integrin, alpha M (complement component 3 receptor 3 subunit)	AK040921	1.4	0.005	-1.1	0.4	1.2	0.2
Itgb2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	NM_008404	1.8	<.00001	-1.2	0.03	1.0	0.9
Jam3	junctional adhesion molecule 3	NM_023277	-1.6	0.001	1.2	0.2	-1.1	0.4
Map2k6	mitogen-activated protein kinase kinase 6	NM_011943	-1.4	0.004	1.2	0.09	-1.2	0.3
Mapk9	mitogen-activated protein kinase 9	NM_016961	-1.4	0.004	-1.4	0.007	-1.1	0.3
Mapk12	mitogen-activated protein kinase 12	NM_013871	-1.4	0.001	1.2	0.09	-1.1	0.6
Mmp3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	NM_010809	6.2	<.00001	-1.2	0.4	1.7	0.006

Mmp7	matrix metallopeptidase 7 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	NM_010810	3.2	0.006	-1.4	0.3	2.5	0.02
Mmp9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	NM_013599	1.4	0.002	-1.1	0.3	1.2	0.1
Mmp10	matrix metallopeptidase 10 (stromelysin 2)	NM_019471	5.1	<.00001	-1.3	0.4	2.1	0.01
Mmp13	matrix metallopeptidase 13 (collagenase 3)	NM_008607	3.2	<.00001	-1.1	0.7	1.5	0.04
Mmp14	matrix metallopeptidase 14 (membrane-inserted)	NM_008608	2.3	<.00001	-1.3	0.06	1.3	0.09
Myd88	myeloid differentiation primary response gene (88)	NM_010851	1.4	<.00001	-1.1	0.1	1.0	0.8
Ncf2	neutrophil cytosolic factor 2 (65kDa, chronic granulomatous disease, autosomal 2)	NM_010877	2.2	<.00001	-1.2	0.1	1.0	0.8
Ncf4	neutrophil cytosolic factor 4, 40kDa	NM_008677	2.3	<.00001	-1.2	0.2	1.2	0.1
Ncf1c	neutrophil cytosolic factor 1C pseudogene	NM_010876	1.6	<.00001	-1.2	0.1	1.1	0.6
Nfkbia	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM_010907	1.6	0.001	1.0	0.9	1.1	0.6
Nfkbiε	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	NM_008690	2.1	<.00001	-1.3	0.01	1.2	0.1
Nox1	NADPH oxidase 1	NM_172203	1.9	<.00001	-1.3	0.07	-1.1	0.4
Nr5a2	nuclear receptor subfamily 5, group A, member 2	NM_030676	-1.1	0.8	1.3	0.02	1.5	0.004
Ppara	peroxisome proliferator-activated receptor alpha	NM_011144	-2.5	<.00001	1.5	0.007	1.2	0.2
Selplg	selectin P ligand	NM_009151	2.0	<.00001	-1.4	0.003	1.1	0.5
Socs3	suppressor of cytokine signalling 3	NM_007707	4.4	<.00001	-1.0	0.9	1.5	0.01
Stat3	signal transducer and activator of transcription 3 (acute-phase response factor)	NM_213659	1.5	0.001	1.1	0.6	-1.1	0.3
Thy1	Thy-1 cell surface antigen	NM_009382	1.8	<.00001	-1.1	0.4	-1.1	0.5
Timp1	TIMP metallopeptidase inhibitor 1	NM_011593	1.6	<.00001	-1.2	0.08	1.2	0.09
Tlr4	toll-like receptor 4	NM_021297	2.0	<.00001	-1.2	0.4	1.2	0.2
Tnfa	tumour necrosis factor alpha (TNF superfamily, member 2)	NM_013693	3.3	<.00001	-1.2	0.2	1.0	0.8
Tnfrsf1b	tumour necrosis factor receptor superfamily, member 1B	NM_011610	2.3	<.00001	-1.1	0.2	-1.1	0.1
Vcam1	vascular cell adhesion molecule 1	NM_011693	3.1	<.00001	-1.3	0.03	1.2	0.1

Genes were grouped into one or more of the following IPA pathways: fatty acid metabolism, xenobiotic metabolism, tryptophan metabolism, oxidative stress response, fibrosis development, IL10 signalling and leukocyte extravasation signalling. Genes with fold change (FC)≥1.3 and a $P<0.01$ were considered for pathway analysis. Shaded cells represent genes that were not differentially expressed in the diet or genotype comparison.

Table 5.5 Significantly over-represented gene ontology (GO) terms in the colon of *Il10*$^{-/-}$ *vs*. C57 mice on the OA diet using FUNC analysis

GO ID	GO term	Number of genes	*P* over-representation after data refinement
Molecular function			
GO:0008009	chemokine activity	18	4.81E-10
GO:0003823	antigen binding	12	1.67E-06
GO:0004759	serine esterase activity	13	1.80E-06
GO:0032395	MHC class II receptor activity	7	2.88E-06
GO:0004364	glutathione transferase activity	10	4.48E-06
GO:0048503	GPI anchor binding	16	5.90E-06
GO:0016787	hydrolase activity	275	1.29E-05
GO:0004896	hematopoietin/interferon-class cytokine receptor activity	18	0.00018
GO:0003779	actin binding	47	0.00019
GO:0016641	oxidoreductase activity, oxygen as acceptor	6	0.00037
GO:0016493	C-C chemokine receptor activity	7	0.00042
GO:0004888	transmembrane receptor activity	119	0.00062
GO:0019865	immunoglobulin binding	5	0.00075
GO:0016868	intramolecular transferase activity, phosphotransferases	5	0.00075
GO:0016229	steroid dehydrogenase activity	7	0.00087
GO:0008375	acetylglucosaminyltransferase activity	6	0.00092
GO:0004497	monooxygenase activity	21	0.00216
GO:0016491	oxidoreductase activity	136	0.00216
GO:0016627	oxidoreductase activity	10	0.00258
GO:0005044	scavenger receptor activity	8	0.00343
GO:0004295	trypsin activity	18	0.00395
GO:0005096	GTPase activator activity	31	0.00462
GO:0016860	intramolecular oxidoreductase activity	8	0.00519
GO:0000287	magnesium ion binding	40	0.00581
GO:0008378	galactosyltransferase activity	7	0.00593
GO:0004263	chymotrypsin activity	17	0.00612
GO:0004907	interleukin receptor activity	9	0.00626
GO:0019965	interleukin binding	11	0.00712
GO:0016616	oxidoreductase activity, NAD or NADP as acceptor	21	0.01387
GO:0003824	catalytic activity	679	0.02905
GO:0008289	lipid binding	48	0.03051
Biological process			
GO:0006955	immune response	126	0.00031
GO:0042127	regulation of cell proliferation	36	0.00621

GO terms annotated to at least ten genes and with FDR or $q<0.05$ according to the hypergeometric test were passed to data refinement and those with $P<0.05$ after refinement were considered over-represented in the list of differentially expressed genes, compared with other genes on the array. Only GO terms for molecular function and biological process are shown.

Table 5.6 Significantly over-represented gene ontology (GO) terms in the colon of EPA-fed *Il10*$^{-/-}$ mice compared to OA-fed *Il10*$^{-/-}$ mice using FUNC analysis

GO ID	GO term	Number of genes	*P* over-representation after data refinement
Molecular function			
GO:0004759	serine esterase activity	5	3.26E-05
GO:0016705	oxidoreductase activity, with incorporation or reduction of molecular oxygen	7	0.00025
GO:0015020	glucuronosyltransferase activity	3	0.00027
GO:0004497	monooxygenase activity	7	0.00051
GO:0016229	steroid dehydrogenase activity	3	0.00129
GO:0016491	oxidoreductase activity	26	0.04575
Biological process			
GO:0044255	cellular lipid metabolic process	16	0.00024
GO:0008610	lipid biosynthetic process	8	0.00043

GO terms annotated to at least ten genes and with FDR or $q<0.05$ according to the hypergeometric test were passed to data refinement and those with $P<0.05$ after refinement were considered over-represented in the list of differentially expressed genes, compared with other genes on the array. Only GO terms for molecular function and biological process are shown.

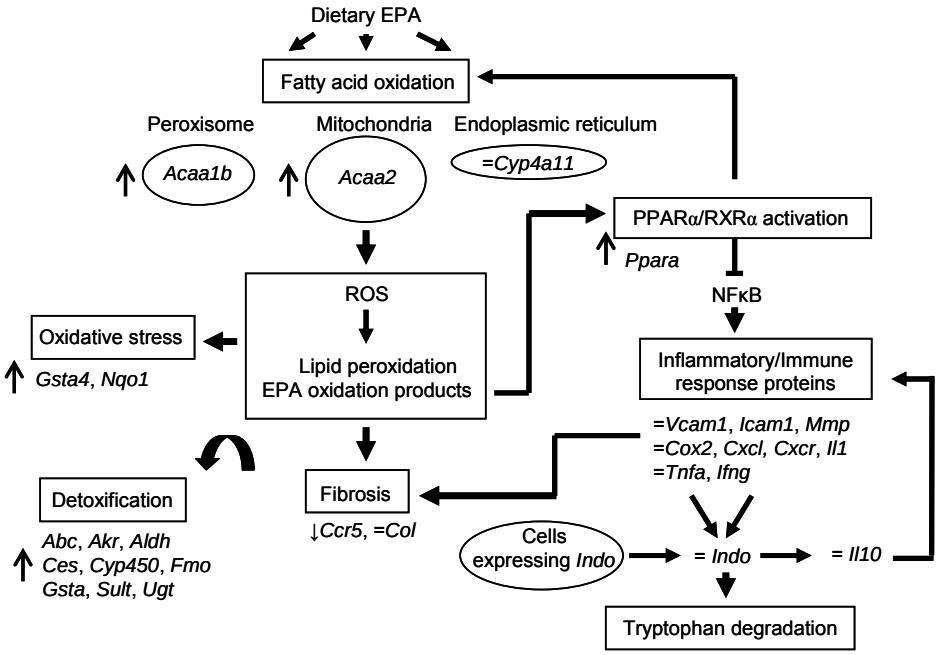


Fig. 5.5 Mechanisms of dietary EPA-induced modulation of colon inflammation in inoculated *Il10*$^{-/-}$ mice (figure adapted from Browning & Horton, 2004). With dietary EPA supplementation, EPA accumulates in the cytosol and increases the expression levels of fatty acid β-oxidation genes in the colon of *Il10*$^{-/-}$ mice, involving genes coding for peroxisomal (*Acaa1b*) and mitochondrial β-oxidation (*Acaa2*) enzymes. Mitochondrial and peroxisomal β-oxidation can also produce ROS. ROS can initiate lipid (e.g. EPA) peroxidation within the cell. Dietary EPA reversed the decrease in colon fatty acid β-oxidation gene expression observed in *Il10*$^{-/-}$ mice fed the OA diet. This might lead to oxidised EPA activating *Ppara* gene expression; *Ppara* is known to inhibit NFκB activation and thus prevent the induction of inflammatory and immune response genes. Detoxification mechanisms and antioxidant enzymes can be activated by dietary EPA to eliminate ROS and lipid peroxidation products, which may be caused by increased ROS formation due to EPA oxidation. Genes associated to fibrosis development (linked by *Vcam*, *Icam* and *Mmp* to inflammatory response) and to tryptophan degradation were mostly unchanged by EPA.

5.5 Discussion

The present study investigated the effects of a high purity n-3 EPA ethyl ester on the expression of genes related to colon inflammation using an inoculated *Il10*$^{-/-}$ mouse model of IBD (Roy *et al.*, 2007). Three comparisons were performed: (1) genotype comparison (inflammation), i.e. *Il10*$^{-/-}$ mice (inflamed) *vs.* C57 mice (not inflamed), all fed the control OA diet; (2) diet comparison (EPA *vs.* OA) in *Il10*$^{-/-}$ mice, and (3) diet comparison (EPA *vs.* OA) in C57 mice. Both IPA and FUNC have proven to be useful tools for the analysis of genome-wide data to detect pathways and significant associations in disease pathophysiology, and in response to EPA to define probable mechanisms of action. Overall, qRT-PCR results verified the reliability of this oligonucleotide microarray approach in identifying key gene changes in *Il10*$^{-/-}$ mice related to inflammation and EPA supplementation.

Il10$^{-/-}$ mice fed the OA diet had higher colon HIS compared to C57 mice fed the same diet. Overall, dietary EPA appears to reduce the severity of colon inflammation compared to the OA diet, although the individual *Il10*$^{-/-}$ mice responded differently to the EPA diet during inflammation (showing low to high colon inflammation). The decreased body weight gain seen in EPA-fed *Il10*$^{-/-}$ mice in the latter part of the study may be due to enhanced thermogenesis and therefore reduced efficiency of body fat deposition. Dietary EPA can affect lipid metabolism by inducing the expression of genes coding for proteins involved in fatty acid oxidation while simultaneously down-regulating lipid synthesis (Clarke, 2000). EPA might regulate its metabolic effects through activation of *Ppara* (Clarke, 2000).

Pathway analysis for the genotype comparison (inflammation) pointed to genes involved in inflammatory and immune response, xenobiotic metabolism, fatty acid metabolism and tryptophan metabolism. The biological network of the genotype comparison showed a connection between genes from these pathways, and a linkage to fibrosis development and antigen presentation pathways. The majority of the genes eligible for pathway analysis were increased in expression levels in the inflamed colon of OA-fed *Il10*$^{-/-}$ mice (compared to OA-fed C57 mice) reflecting an underlying inflammatory condition. When dietary EPA was fed the reverse was often observed in *Il10*$^{-/-}$ mice (compared to OA-fed *Il10*$^{-/-}$ mice). Where the expression of genes was

changed in EPA-fed *Il10*$^{-/-}$ mice, this was generally not the case in C57 mice, strongly suggesting a specific effect of dietary EPA on the expression of genes involved in the inflammation process. The importance of, and the relationship between, the pathways affected during inflammation or dietary EPA in *Il10*$^{-/-}$ mice are discussed below.

Inflammation and inflammatory and immune response pathways

The IL10 signalling pathway is essential in regulating inflammation by blocking the expression of pro-inflammatory genes coding for cytokines, chemokines and cell surface molecules involved in promoting inflammation (Murray, 2006). In the OA-fed *Il10*$^{-/-}$ mice, the expression of pro-inflammatory cytokine (e.g. *Il1a*, *Il1b* and *Tnfa*) and chemokine receptor (e.g. *Ccr5*) genes in IL10 signalling was increased. The expression of the anti-inflammatory *Il10* gene was also increased in *Il10*$^{-/-}$ mice fed the OA diet compared to C57 mice fed the same diet. This change may seem unexpected, but a previous study has shown that a truncated mRNA from the *Il10* knockout allele may be expressed without generating a functional protein (Kühn *et al*., 1993). As the functional IL10 protein is required to suppress pro-inflammatory genes, the over-expression of these genes in the IL10 signalling pathway agrees with the suggestion that the truncated *Il10* gene was not functional.

Intestinal inflammation is usually accompanied by disruption of the epithelial barrier and consistent with the observed down-regulation of the tight junction proteins *Jam3* and *Cldn8*, both required for epithelial barrier maintenance (Wu *et al*., 2007), in the present study. Disruption of the epithelial barrier often leads to inflammatory cell infiltration into the affected tissues (Qi *et al*., 2005). As expected, the expression of leukocyte chemokines and chemokine receptor genes, e.g. *Cxcl12* and *Cxcr4*, was increased in the colon of OA-fed *Il10*$^{-/-}$ mice. These genes are part of the leukocyte extravasation signalling pathway that regulates migration of leukocytes from the circulatory system into sites of tissue inflammation and activation of the inflammatory cells in inflamed tissues, and is an important aspect of the pathogenesis of colitis (Te Velde *et al*., 2007). The expression of the adhesion molecule genes *Icam1* and *Vcam1* associated with leukocyte extravasation signalling was increased in the colon of OA-fed *Il10*$^{-/-}$ mice. Expression of pro-inflammatory genes, particularly adhesion molecules and chemokines, is transcriptionally regulated by NFκB and implicated in early immune, acute phase, and inflammatory responses (Jobin & Sartor, 2000).

A number of genes coding for matrix metalloproteinases (*Mmp*) had also increased expression levels in the colon of OA-fed *Il10*$^{-/-}$ mice. *MMP* are extracellular matrix degrading enzymes secreted by infiltrating and locally activated immune cells present in inflamed tissues (Rieder *et al*., 2007). Genes coding for *MMP* act through their inhibitors (*TIMP*) which control degradation of the extracellular matrix and protect the barrier against inflammatory leukocyte invasion (Rieder *et al*., 2007). Similar to the over-expression of *Mmp* and *Timp1* genes found here in OA-fed *Il10*$^{-/-}$ mice, increased expression levels of these genes have been observed in the inflamed mucosa of IBD patients (Strup-Perrot *et al*., 2004).

The balance of tissue-degrading *MMP* and their inhibitors (*TIMP*) is an important factor in the extent of intestinal wall damage during inflammation (Rieder *et al*., 2007). Continuous inflammation and tissue degradation can lead to fibrosis (Rieder *et al*., 2007), but there is little data linking *MMP* expression to intestinal fibrosis (Strup-Perrot *et al*., 2004). The increased expression level of genes in the leukocyte extravasation pathway (such as *Icam1*, *Vcam1*, *Mmp* and *Timp1*) in OA-fed *Il10*$^{-/-}$ mice is also associated with fibrosis development, which supports the link between fibrosis and intestinal inflammation. Furthermore, the mRNA level for *Il1b* gene was markedly increased in the colon of OA-fed *Il10*$^{-/-}$ mice, agreeing with the finding of Vozenin-Brotons *et al*. (2004) who suggested a possible involvement of *Il1b* in extracellular matrix remodelling in the late phase of radiation fibrosis.

Inflammation and xenobiotic metabolism

Changes in detoxification and biotransformation of luminal antigens are important mechanisms in the initiation and progression of IBD (Te Velde *et al*., 2007). In this study, expression of phase I (*Cyp450*), phase II (conjugating enzymes; *Gst*) and phase III (drug transport proteins; *Abc*) detoxification genes of xenobiotic metabolism was down-regulated in the colon of *Il10*$^{-/-}$ mice fed the OA diet. These data are in agreement with a study done by Dommels *et al*. (2007) who reported a down-regulation of cellular detoxification and defence genes, including *Cyp450*, sulfotransferases, carboxylesterases, *Abc* transporters, aldo-keto reductases and flavin-containing monooxygenases, in the inflamed colon of *mdr1a*$^{-/-}$ mice. Inflamed tissues are known to have down-regulated expression of detoxification enzymes and transporters; the regulation of those enzymes and transporters seems to be complex (Aitken *et al*., 2006)

and might be a multi-level defect in the intestinal epithelial barrier (Langmann & Schmitz, 2006) or could be a secondary effect (Dommels *et al*., 2007).

Inflammation and fatty acid metabolism

In *Il10*$^{-/-}$ mice compared to C57 mice fed OA, there were decreased expression levels of genes involved in fatty acid metabolism. These mainly include genes involved in mitochondrial fatty acid uptake (e.g. *Cpt1a*, *Cpt1b* and *Cpt2*), peroxisomal (e.g., *Acaa1*, *Acaa1b*, *Acox1*, *Acox2* and *Ehhadh*) and mitochondrial fatty acid β-oxidation *(Acaa2* and *Acads)*, fatty acid acetylation (*Acsl3*) and lipid transport (*Slc27a4*). Changes in fatty acid metabolism in DSS-treated hamsters with acute colitis were associated with a decrease in liver *Cyp4a* and *Acox* expression (Karlsson *et al*., 2008). These changes correlated with a similar alteration in colon tissues in that IBD study. Similar changes have been observed in other models of inflammatory conditions (Singh *et al*., 2004; Warren *et al*., 2001) and are likely to result in elevated plasma levels of TG (Karlsson *et al*., 2008). Fatty acids are also an essential energy source and there is a balance between glucose and fatty acid utilisation for energy metabolism under healthy conditions (De Windt *et al*., 2002). It has been found that under various pathological circumstances such as diabetes or heart failure, fatty acid energy metabolism was disrupted (De Windt *et al*., 2002). Fatty acid uptake and oxidation decreased in heart and skeletal muscle during the acute phase response of inflammation, shifting the metabolism from fatty acids as fuel substrate to glucose (Khovidhunkit *et al*., 2004). Development of intestinal inflammation seems to be associated with a suppression of fatty acid oxidation in this IBD mouse model, which might have played a role in disease progression.

Inflammation and tryptophan metabolism

Tryptophan metabolism has been linked to autoimmune disorders associated with inflammation and immune activation (Torres *et al*., 2007). The expression of key genes (*Indo* and *Wars*) in tryptophan oxidation increased in the inflamed colon of *Il10*$^{-/-}$ mice fed the OA diet. This agrees with other studies, one showing over-expression of the *INDO* gene in colon biopsies from CD patients (Wolf *et al*., 2004) and in TNBS-induced colitis in mice (Gurtner *et al*., 2003). CD is primarily mediated by a dysregulated Th1-cell response with concomitant increase in activity of the Th1-like pro-inflammatory cytokines, *TNFA* and *IFNG* (Wolf *et al*., 2004). *TNFA* and *IFNG* are potent inducers of INDO protein expression leading to tryptophan degradation which

can aggravate intestinal inflammation (Torres *et al*., 2007). In the current study, *Tnfa* and *Ifng* expression levels were increased in the colon of OA-fed *Il10*$^{-/-}$ mice. Other studies have described that tryptophan catabolism occurred at sites of tissue inflammation and suggested that increased INDO protein expression may be anti-inflammatory by reducing tissue damage at these sites (Torres *et al*., 2007; Mellor & Munn, 2003). INDO-positive cells (monocytes and macrophages) can produce the immunosuppressive cytokines IL10 and TGFB which can down-regulate T-cell-mediated immune responses in inflammatory diseases, e.g., elevated intestinal IL10 and TGFB protein levels were found in patients with coeliac disease (Torres *et al*., 2007).

Torres *et al*. (2007) also reported that suppressive molecules such as INDO and human leukocyte antigens (HLA-G) were both expressed in coeliac disease to restore tolerance towards dietary antigens. The expression levels of a number of MHC class I and II genes (*Hla-dm*, *Hla-do*, *Hla-dq*, *Hla-dr* and *Hla-f*) of the antigen presentation pathway were increased in the colon of *Il10*$^{-/-}$ mice fed the OA diet. Torres *et al*. (2007) suggested that increased expression of INDO might be a mechanism to control antigen stimulation by down-regulation of the T-cell-mediated autoimmune reaction.

In the OA-fed *Il10*$^{-/-}$ mice, there was an inflammatory response predominantly in the colon that was confirmed histologically and also transcriptionally by changes in expression of genes in fatty acid metabolism, xenobiotic metabolism and inflammatory and immune response. Furthermore, this study, supported by Fig. 5.4 and discussed below, showed how the genes of these pathways are counteracted by EPA in *Il10*$^{-/-}$ mice compared to the OA-fed *Il10*$^{-/-}$ mice.

Dietary EPA effects in Il10*$^{-/-}$ *mice and fatty acid metabolism

Dietary EPA supplementation increased the mRNA levels for fatty acid β-oxidation genes in the colon of *Il10*$^{-/-}$ mice, including those involved in the peroxisomal (*Acaa1b*) and mitochondrial β-oxidation (*Acaa2*) pathways. Generally, accumulation of fatty acids, e.g. EPA, in the cytosol (as result of dietary absorption) can increase fatty acid β-oxidation in peroxisomes, endoplasmatic reticulum and mitochondria (Browning & Horton, 2004). *In vivo* studies have also shown a large increase in tissue and plasma accumulation of fatty acid oxidation products after consuming fish oil even with an adequate level of antioxidants in the diet, indicating extensive endogenous oxidation of

EPA (Wander & Du, 2000; Sethi *et al*., 2002). Mitochondrial fatty acid β-oxidation is the dominant oxidative pathway for the breakdown of fatty acids under normal physiological conditions, but can also produce ROS such as superoxide and hydrogen peroxide (Browning & Horton, 2004). Peroxisomal β- and microsomal ω-oxidation are activated when mitochondrial β-oxidation becomes impaired and results in additional formation of ROS. ROS can initiate lipid (e.g. PUFA) peroxidation within the cell (Browning & Horton, 2004). On the other hand, EPA easily oxidises at room temperature in the absence of exogenous oxidising agents (Sethi *et al*., 2002). In the present study, oxidised and peroxidised lipid products in the diets or tissues were not measured, but the EPA diet contained recommended levels of antioxidants, was kept at −85°C and offered fresh daily; thus this is an unlikely contributor to oxidised EPA. Increased endogenous oxidation of dietary EPA can also occur due to increased oxidative stress during intestinal inflammation (Sethi *et al*., 2002).

Dietary EPA reverse the decrease in colon *Ppara* gene expression observed in the OA-fed *Il10*$^{-/-}$ mice. Most of the genes involved in lipid metabolism which show decreased expression during inflammatory responses are regulated by nuclear hormone receptors (including *PPAR*) that are mediated by the retinoid X receptor *(RXR)* (Khovidhunkit *et al*., 2004; Heimerl *et al*., 2006). Activation of the expression of the *Ppara* gene by EPA in *Il10*$^{-/-}$ mice could induce the expression of key enzymes required for fatty acid β-oxidation. Increased expression levels of the *Ppara*-dependent fatty acid β-oxidation genes were also observed in a study investigating the effects of long chain PUFA on the liver transcriptome of healthy mice (Berger *et al*., 2006). A further study reported the links between *Ppara* and the regulation of fatty acid metabolism, and other lipid-regulated transcription factors in the small intestine (Bünger *et al*., 2007). Sethi *et al*. (2002) have shown that oxidised EPA exerted an inhibitory effect on the inflamed epithelium *in vivo* through a *PPARa*-dependent mechanism to a higher extent than unoxidised EPA. In this study, it is likely that dietary EPA taken up directly by the inflamed colon epithelial cells might have led to increased fatty acid β-oxidation; oxidised EPA then activated the *Ppara* gene which in turn inhibited NFκB activation and thus prevented the induction of its target inflammatory genes.

Dietary EPA effects in Il10$^{-/-}$ mice and xenobiotic metabolism

In contrast to the genotype comparison (inflammation), feeding EPA to *Il10*$^{-/-}$ mice increased the expression of genes involved in the oxidative stress response and microsomal biotransformation of xenobiotics (*Abc*, *Cyp450*, *Gst*, *Sult* and *Ugt*) in the inflamed colon. Most xenobiotic metabolism genes are not substrate specific (*Cyp* enzymes and *Aldh*), so no conclusion can be made for particular lipid peroxidation products as possible triggers of these effects of EPA.

Up-regulation of genes associated with the oxidative stress response (such as *Gsta4* and *Nqo1*) in the EPA-fed *Il10*$^{-/-}$ mice suggested an induction of antioxidant enzyme genes and may be partly caused by increased formation of ROS due to metabolism of EPA by fatty acid β-oxidation enzymes. Partly in support of this, feeding fish oil to healthy C57BL/6 mice increased the expression of antioxidant genes such as uncoupling protein2 and mangan superoxide dismutase (mitochondria) and *Gst* (cytosol) to quench ROS (Takahashi *et al*., 2002).

Dietary EPA effects in Il10$^{-/-}$ mice and inflammatory and immune response

Pathways linked to the inflammatory and immune response were affected by EPA in the colon of *Il10*$^{-/-}$ mice; the expression of genes of the IL10 signalling pathway was generally down-regulated and those of the LPS and IL1–mediated inhibition of RXR activation pathway was generally up-regulated. In contrast to the genotype comparison (inflammation), key genes belonging to the leukocyte extravasation pathway (such as *Cldn8*, *Cxcl*, *Icam1*, *Mmp*, *Timp1* and *Vcam1*) showed no change in expression with EPA feeding to *Il10*$^{-/-}$ mice. The *Ccr5* gene was, however decreased in expression level in EPA-fed *Il10*$^{-/-}$ mice; this receptor is involved in leukocyte activation, adhesion and recruitment. Contrary to the genotype comparison (inflammation), expression of key inflammatory genes (e.g., *Il1a*, *Il1b* and *Tnf*) was not differentially affected by EPA-fed *Il10*$^{-/-}$ mice. The NFκB is an important target of PPAR and its up-regulation induces pro-inflammatory target gene expression. However, the *Nfkb* gene was not differentially expressed in the colon of EPA-fed *Il10*$^{-/-}$ mice which agrees with the findings of Jobin & Sartor (2000) who showed that NFκB activity is regulated at the protein rather than mRNA level.

In this study, EPA fed to *Il10*$^{-/-}$ mice had no clear immunologic effects (from the measurements conducted here) in the colon tissue as the expression of key immune response genes such as *Tnfa* and *Ifng* remained unchanged. In agreement with this study, a study testing pure EPA within the normal fat content of the diet in response to lipopolysaccharide *ex vivo* showed more subtle effects in modulating the immune system and no consistent changes in the cytokine response (Barber *et al*., 2005).

In conclusion, in the *Il10*$^{-/-}$ mouse model of IBD used here, global gene expression profiling and computational pathway analysis provided a detailed knowledge of changes in gene expression induced by EPA in intestinal inflammation. In *Il10*$^{-/-}$ mice fed the OA diet, inflammatory and immune response linked with fibrosis development and key genes from tryptophan metabolism were increased in expression level compared to C57 mice on the same diet. The regulation of fatty acid metabolism, xenobiotic metabolism and oxidative stress response pathways, diminished in OA-fed *Il10*$^{-/-}$ mice compared to OA-fed C57 mice, were affected in *Il10*$^{-/-}$ mice in response to the EPA diet. This diet showed no effect on the pathways that were affected in *Il10*$^{-/-}$ mice, in the C57 mice. Further work is required to investigate the extent of lipid oxidation and the role that *Ppara* may play as a key gene in regulating the expression of pro-inflammatory genes and proteins in response to PUFA.

Chapter 6

Dietary arachidonic acid-mediated effects on colon inflammation using transcriptome analysis

Part of the material presented in this chapter has been published as a paper:

Knoch B, Barnett MPG, McNabb WC, Zhu S, Park ZA, Khan A & Roy NC (2010): Dietary arachidonic acid-mediated effects on colon inflammation using transcriptome analysis. *Mol Nutr Food Res* **54**, S62-S74.

6.1 Abstract

Increased levels of n-6 AA, a precursor of pro-inflammatory eicosanoids, have been found in the colon mucosa of IBD patients when compared with healthy subjects. The hypothesis was that dietary AA would aggravate colon inflammation by changing expression of genes in inflammatory signalling pathways. AA-enriched diet was fed to *Il10*$^{-/-}$ mice, an IBD model, and compared with *Il10*$^{-/-}$ mice fed the OA control diet. Effects of AA on gene expression profiles during colitis were examined using whole genome microarray analysis. Dietary AA decreased the expression levels of some colonic genes in ER stress, complement system, nuclear respiratory factor 2-mediated oxidative stress and positive acute phase response pathways compared with *Il10*$^{-/-}$ mice fed the OA diet. AA increased the expression levels of fatty acid catabolism genes, but decreased that of lipid synthesis genes during colitis, likely by *Srebf1* and target gene regulation. A link has been suggested between AA and reduction of intestinal fibrosis by down-regulating the expression levels of pro-inflammatory and fibrotic marker genes. Contrary to the hypothesis, these findings suggest that dietary AA, in the present experimental conditions, is not pro-inflammatory, reduces ER stress and protects colonocytes from oxidative stress in *Il10*$^{-/-}$ mice.

6.2 Introduction

Long chain PUFA are involved in cellular processes such as membrane biosynthesis, energy metabolism, eicosanoid production, signal transduction and regulating the expression of genes that modulate inflammatory processes by altering fatty acid metabolism in intestinal epithelial cells (Mutch *et al*., 2005; Belluzzi, 2002). Metabolism of n-3 and n-6 PUFA is interlinked. For example, the n-3 PUFA EPA competes with AA, the most abundant n-6 PUFA in animal cell membranes, for incorporation into membrane phospholipids, replacing it and blocking the production of pro-inflammatory eicosanoids (Serhan & Chiang, 2008). Higher levels of AA are also found in the colon mucosa of IBD patients compared with that of healthy subjects (Pacheco *et al*., 1987).

Epidemiological studies have associated increased intake of dietary n-6 PUFA and decreased intake of n-3 PUFA with a higher incidence of IBD (Kroman & Green, 1980; Shoda *et al*., 1996). This is supported by a study with *Il10*$^{-/-}$ mice (a model for a T-

helper type 1-mediated colitis), which found that n-6 PUFA-rich corn oil increased colon inflammation compared with n-3 PUFA-rich fish oil (Chapkin *et al*., 2007). Although AA has been postulated to have pro-inflammatory effects compared with EPA (Belluzzi, 2002), another study has reported that feeding an AA ethyl ester oil-enriched diet to mice with DSS-induced colitis increased AA content in colon tissue without increasing colon inflammation. It also reduced weight loss and diarrhoea scores compared with the diets containing n-3 PUFA-rich fish oil and n-9 OA-rich sunflower seed oil (Ramakers *et al*., 2008).

Dietary PUFA can regulate the expression levels of several genes crucial for immuno-regulatory reactions (Camandola *et al*., 1996) by interacting with transcription factors that play an important role in IBD (Heimerl *et al*., 2006; Salter & Tarling, 2007). Both n-6 and n-3 PUFA and their metabolites are natural ligands for PPAR such as PPARα (Sampath & Ntambi, 2005; Salter & Tarling, 2007). PPARα induces changes in the expression levels of genes involved in fatty acid oxidation and lipoprotein metabolism and inhibits that of genes coding for pro-inflammatory proteins. PUFA can inhibit lipogenic (cholesterol, TG and fatty acid synthesis) gene expression by suppression of the transcription factor SREBP (Sampath & Ntambi, 2005; Salter & Tarling, 2007). PUFA also regulate another transcription factor, NFkB, which controls the expression of pro-inflammatory genes (e.g. COX2 and IL6). AA can induce NFkB translocation and subsequent activation of target gene transcription leading to inflammation, whereas EPA inhibits NFkB translocation by PPARα-mediated activation, resulting in decreased pro-inflammatory and immuno-regulatory genes and proteins (Sampath & Ntambi, 2005). The interaction of PUFA with the transcription factors SREBP and NFkB in the regulation of fatty acid metabolism and immune and inflammatory responses is still poorly defined. Rodent studies in which AA-enriched diets were fed showed AA present at inflammatory sites, and acting as an important regulator of inflammatory responses within tissues (Mariotto *et al*., 2007), but AA also has protective effects in mice with experimental colitis (Ramakers *et al*., 2008).

The present study was designed to test whether dietary AA aggravates colon inflammation in *Il10*$^{-/-}$ mice, particularly the immune and inflammatory pathways. *Il10*$^{-/-}$ and C57 mice were inoculated with a mix of "complex intestinal microflora" and pure cultures of *E. faecalis* (Roy *et al*., 2007), and genome-wide expression profiling of

colon tissues was used to identify gene regulatory networks by which dietary AA affected colon inflammation. Dietary AA supplementation was also studied in non-inflamed colon tissue of C57 mice to determine levels of gene expression compared with inflamed colon. The genotype comparison (*Il10*$^{-/-}$ versus C57 mice) on the OA diet reflects the changes in gene expression levels due to colon inflammation alone.

6.3. Materials and methods

6.3.1 Animals, treatment, and tissue collection

Housing and induction of colitis are described in Materials and Methods chapter 2.1.1 and 2.1.2. Twelve male *Il10*$^{-/-}$ (C57 background, formal designation B6.129P2-*Il10*<tm1Cgn>/J) and 12 male C57 wildtype (C57BL/6J) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). All mice were 35 days or 5 weeks of age at the start of the experiment.

Experimental design, diet compositions and fatty acid analysis are described in Materials and Methods chapter 2.1.3 and 2.2. All mice were fed for 6 weeks an AIN-76A-based diet enriched with OA or an AIN-76A-based diet enriched with AA. This was part of the PUFA intervention study described in Chapters 4 and 5. The OA and AA diets were formulated to contain AIN-76A (fat-free) + 1% corn oil, supplemented with 0.2% purified LA and 0.06% ALA ethyl esters and 3.4% OA or AA ethyl ester. The diets were isocaloric and varied only in lipid composition, both diets containing 5% fat (w/w), the usual fat content of the AIN-76A diet. The killing of the mice at the end of the PUFA feeding study was done in randomised order and the OA and AA group used here for more detailed analysis.

Note: *The lipid and fatty acid analysis of the diets was performed by Michael Agnew at AgResearch and Fliss Jackson at Massey University, respectively, before the study began.*

Tissue collection and fast-feed period prior to sampling are described in Materials and Methods chapter 2.1.4. Plasma was separated from the blood, frozen in liquid nitrogen and stored at −85°C for analysis of SAA. One colon section was stored in phosphate-buffered 10% formaldehyde at room temperature for histopathological assessment, and another was immediately frozen in liquid nitrogen and stored at −85°C for gene profiling.

6.3.2 Histology and SAA

Histopathological assessment of the colon and SAA measurement in the plasma are described in Materials and Methods chapter 2.3.1 to 2.3.3.

Note: *Tissue processing and histopathological assessment was conducted by Dr Shuotun Zhu at The University of Auckland. The SAA analysis was performed by Kim Oden at AgResearch.*

6.3.3 RNA isolation

RNA isolation and reference RNA preparation are described in Materials and Methods chapter 2.4.2. Equimolar pools of colon RNA extracts (2-3 samples/pool) were used in the reference microarray design.

6.3.4 Microarrays

Microarray reference design, synthesis of labelled cRNA, hybridisation and scanning are described in Materials and Methods chapter 2.4.3. Array data have been submitted to the Gene Expression Omnibus, accession number GSE12028.

6.3.5 Analysis of microarray data

Statistical analysis of the microarray data is described in Materials and Methods chapter 2.4.4. Three comparisons were performed: (1) diet comparison (AA *vs.* OA) in *Il10*$^{-/-}$ mice; (2) diet comparison (AA *vs.* OA) in C57 mice and (3) genotype comparison of inflammation (*Il10*$^{-/-}$ mice (inflamed) *vs.* C57 mice (not inflamed)) on the OA control diet. For each comparison, a list of differentially expressed probe sets was generated.

Note: *The microarray analysis in Bioconductor (written in R) was conducted with assistance from Zaneta Park, a statistician at AgResearch.*

6.3.6 Quantitative real-time PCR

qRT-PCR is described in Materials and Methods chapter 2.5. Ten genes (*Abcb1a*, *Aldh1a1*, *Ces2*, *Fabp2*, *Igfbp5*, *Il1b*, *Mmp13*, *Ppara, Srebf1* and *Sult1a1*) were quantified to verify microarray data and the results expressed as mRNA level normalised to Calnexin (*Canx*) as reference gene. The primer sequences for target and reference genes are listed in Materials and Methods chapter Table 2.2.

6.3.7 Bioinformatics analysis of pathways, networks and functions

IPA was used for pathway and network analyses of differentially expressed probes in the microarray dataset. FUNC GO enrichment analysis was applied as a complementary method to IPA to relate changes in gene expression levels to specific biological functions and pathways. More details on these bioinformatics methods can be found in Materials and Methods chapter 2.7.1 and 2.7.2. Probe sets that satisfied the criteria of FC≥1.3 and a moderated $P<0.01$ were selected for IPA and FUNC analysis. This FC was chosen because a recent study showed that a FC>1.6 could underestimate the number of differentially expressed genes affected by dietary treatment (Sülzle *et al*., 2004).

Note: *FUNC analysis was performed with assistance from Anar Khan, a bioinformatician at AgResearch.*

6.3.8 Statistics

SAS (9.1.3 Service Pack 4, SAS Institute Inc., Cary, NC, USA) was used for statistical analyses of body weight and dietary intake, and GenStat (10^{th} edition, VSN International, Hemel Hempstead, UK) used to analyse HIS, SAA and qRT-PCR data. Analysis of repeated measurements with ante-dependence and auto-regressive variance-covariance structures was used to fit the body weight and dietary intake data and to test for treatment differences in each strain over time. Linear regression and ANOVA was applied to estimate whether the rate of body weight gain differed between AA and OA diets for *Il10*$^{-/-}$ and C57 mice. For ANOVA analyses, log-transformations of raw data were performed in cases of unequal variances. Differences in HIS between AA and OA diets for each mouse strain were analysed using an ANOVA with pooled variance. A constant was added to the SAA data prior to log-transformation to avoid negative values found in C57 mice. Differences were considered significant at $P<0.05$. A trend was declared when $0.05<P<0.10$. One *Il10*$^{-/-}$ mouse from the OA diet group died during the experiment (due to unknown causes) and no samples could be obtained from this animal for histological examination, scoring and statistics.

6.4 Results

6.4.1 Body weight and dietary intake

The average body weight and dietary intake of mice from 35 to 75 days of age are shown in Fig. 6.1. Average body weight of *Il10*$^{-/-}$ and C57 mice increased over time in both diet groups. From days 47 to 75 of age, C57 mice fed AA gained less weight than C57 mice fed OA (P<0.05). The body weight of *Il10*$^{-/-}$ mice fed the AA diet compared with *Il10*$^{-/-}$ mice fed the OA diet was reduced only at day 54 (P=0.02). There was a significant decrease (P<0.05) in dietary intake from day 61 to 65 and from day 70 to 72 between AA- and OA-treated *Il10*$^{-/-}$ mice. The analysed levels of OA and AA ethyl esters were 3.6% and 3.0% resulting in an intake of 0.14 g/d of OA and 0.12 g/d of AA when offered approximately 4 g of diet.

6.4.2 Histology and SAA

There was a trend (0.05<P<0.10) to reduce colon HIS in AA-fed *Il10*$^{-/-}$ mice compared with OA-fed *Il10*$^{-/-}$ mice, but no difference between C57 mice fed the OA and AA diets (Table 6.1). *Il10*$^{-/-}$ mice on the OA diet showed severe (HIS≥7) colon inflammation, AA-fed *Il10*$^{-/-}$ mice showed moderate (HIS 4–6) inflammation, and C57 animals no sign of colon inflammation (Fig. 6.2). Figure 6.2 shows that colon HIS was mainly characterised by inflammatory cell infiltration (mostly monocytes) and to a lesser extent by tissue destruction and repair. The inflammatory lesions only occurred in some areas of the colon, but involved most layers of the colon. There was no difference in inflammatory cell infiltration or tissue destruction and repair in the colon of *Il10*$^{-/-}$ mice in the AA compared with the OA group. There was a 22-fold difference in plasma SAA concentration between *Il10*$^{-/-}$ and C57 mice on the OA diet (P<0.05) (Table 6.1). There was also a trend (0.05<P<0.10) to reduced plasma SAA in AA-fed *Il10*$^{-/-}$ mice compared with OA-fed *Il10*$^{-/-}$ mice.

6.4.3 Effects of dietary AA on gene expression during colon inflammation

The number of shared and unique differentially expressed genes for each comparison is summarised in Fig. 6.3. The following comparisons were made: (i) diet comparison (AA versus OA in *Il10*$^{-/-}$ mice); (ii) diet comparison (AA versus OA in C57 mice) and (iii) genotype comparison (inflammation; *Il10*$^{-/-}$ versus C57 mice on the OA diet). In total, 426 genes were differentially expressed (FC ≥ 1.3; P<0.01) in the colon of *Il10*$^{-/-}$ mice fed the AA compared with the OA diet (213 up- and 213 down-regulated). Of these, 208 of the 426 differentially expressed genes were common to *Il10*$^{-/-}$ mice fed

OA (compared with C57 on the same diet) and *Il10*$^{-/-}$ mice fed AA (compared with *Il10*$^{-/-}$ mice fed OA), and 202 genes were related to the effect of dietary AA on colon inflammation. In C57 mice, the AA diet resulted in differential expression of only 217 genes, and of those, 7 genes were common to those found in AA-fed *Il10*$^{-/-}$ mice.

Microarray results were confirmed for selected genes by qRT-PCR for the pooled colon samples (Table 6.2). The *Srebf1* gene showed a tendency but not a significant expression (P=0.06) by qRT-PCR in *Il10*$^{-/-}$ mice (unlike the array result) comparing AA with the OA diet, due to higher variability in the AA-fed *Il10*$^{-/-}$ mice.

6.4.4 Pathway analysis of the dietary AA effects on colon inflammation

The metabolic and regulatory pathways most affected by dietary AA during colon inflammation in *Il10*$^{-/-}$ mice (compared with OA-fed *Il10*$^{-/-}$ mice), in which at least 10% of the genes were differentially expressed according to IPA, are discussed here (Table 6.3). These pathways involved stress and immune/inflammatory response, RXR activation pathways, lipid metabolism and fibrosis development.

The expression levels of genes involved in stress and immune/inflammatory response pathways (ER stress, complement system, nuclear respiratory factor2 (NRF2)-mediated oxidative stress response and acute phase response signalling) were down-regulated in the colon of AA-fed *Il10*$^{-/-}$ mice. This includes genes coding for complement component 1q or s subcomponent (*C1qa*, *C1qb*, *C1s*), eukaryotic translation initiation factor2 alpha kinase3 (*Eif2ak3*), *Il1b*, IL1 receptor type2 (*Il1r2*), Kelch-like ECH associated protein 1 (*Keap1*), *Tnf*) and TNF receptor associated factor2 (*Traf2*).

Genes from pathways related to RXR activation were also affected in the colon of AA- compared with OA-fed *Il10*$^{-/-}$ mice. These include constitutive androstane receptor (CAR)/RXR activation, LXR/RXR activation, LPS/IL1-mediated inhibition of RXR function and co-factor metabolism. RXR, as a major heterodimeric partner for nuclear receptors such as CAR and LXR, regulates the expression of several genes of xenobiotic metabolism and immune/inflammatory response (*Il1a*, *Il1b*, *Il1r2* and *Tnf*). Analysis of biological networks by IPA showed interaction of genes involved in xenobiotic metabolism with those involved in stress and immune/inflammatory response (Fig. 6.4).

The expression levels of xenobiotic metabolism genes up-regulated in AA-fed *Il10*$^{-/-}$ mice included *Aldh1a1*, cytochrome P450 family members (CYP, e.g. *Cyp3a5*), glutathione S-transferase A4 (*Gsta4*), flavin containing monooxygenase2 (*Fmo2*), sulfotransferase family1 members (*Sult1a1*, *Sult1c2*) and uridine diphosphate glucuronosyltransferase family1 member (*Ugt1a1*). ABC transporter (Phase III) enzyme (e.g. *Abcb1*), and carboxylesterase (e.g. *Ces2*) gene expression levels, were significantly increased in the colon of AA-fed *Il10*$^{-/-}$ mice when examined with the more sensitive qRT-PCR. The mRNA abundance of retinol dehydrogenase5 (*Rdh5*), a gene involved in retinoic acid synthesis, was increased in the colon of AA-fed *Il10*$^{-/-}$ mice.

The AA-fed *Il10*$^{-/-}$ mice had decreased colonic expression levels of the *Srebf1* gene, which is also regulated by RXR. Gene networks show that the decrease in *Srebf1* gene expression levels might be due to a reduction in *Tnf* mRNA abundance and indirectly due to a reduction in *Il1b* gene expression levels. Expression of the *Srebf1* gene is further linked to decreased expression levels of the cholesterol biosynthesis genes 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*) and farnesyl diphosphate synthase (*Fdps*). The expression levels of other lipogenic transcription factors such as *Ppara* and members of the nuclear receptor superfamily *Nr1h3* (LXRa), *Nr1i3* (CAR) and *Rxra* (RXRa) were unchanged in the AA-fed *Il10*$^{-/-}$ mice. Dietary AA up-regulated the expression levels of genes of fatty acid catabolism including acetyl-CoA acyltransferase2 (*Acaa2*) and acyl-CoA thioesterase5 (*Acot5*), whereas that of genes of fatty acid synthesis, e.g. acetyl-CoA carboxylase alpha (*Acaca*) were down-regulated in the colon of *Il10*$^{-/-}$ mice.

Genes of the fibrosis pathway (such as collagen type1 alpha2 (*Col1a2*), fibroblast growth factor7 (*Fgf7*) and *Igfbp5*), had decreased mRNA levels in the colon of AA- compared with OA-fed *Il10*$^{-/-}$ mice. Decreased expression levels of fibrosis development genes was also associated with decreased expression levels of genes involved in tissue degradation (e.g. *Mmp13*) and immune/inflammatory response in the colon of AA-compared with OA-fed *Il10*$^{-/-}$ mice. In contrast, the levels of these genes were more highly expressed in *Il10*$^{-/-}$ mice on the OA diet versus C57 mice on the OA diet.

6.4.5 Functional analysis of dietary AA effects on colon inflammation

GO enrichment analysis was performed on the differentially expressed gene list to

identify biological processes and functions affected by dietary AA (compared with OA-fed *Il10*$^{-/-}$ mice). Table 6.4 shows 15 terms most strongly over-represented in the colon of *Il10*$^{-/-}$ mice fed AA diet compared with *Il10*$^{-/-}$ mice fed the OA diet. These included GO terms such as extracellular space (complement component, insulin-like growth factor binding protein, and immunoglobulin-complexes) and collagen (fibrillar and transmembrane collagen), which can be linked to immune response and fibrosis processes, respectively. Other over-represented GO terms were antigen binding and monooxygenase activity, which are descriptors for immune response and lipid metabolism. The FUNC analysis agreed well with the IPA analysis, so dietary AA can be confidently linked with those processes involved in colon inflammation.

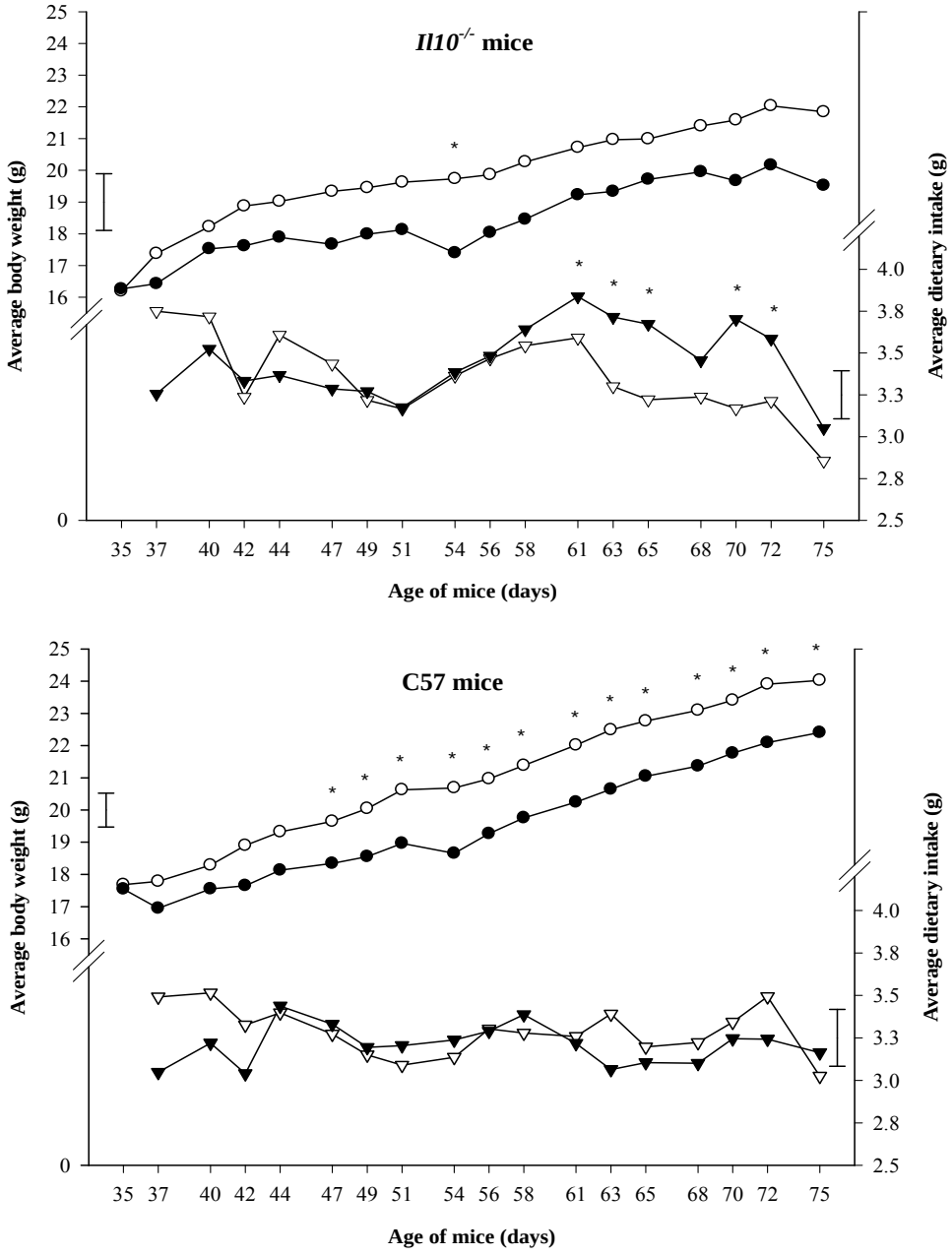


Fig. 6.1 Mean body weight (g) and dietary intake (g) of *Il10*$^{-/-}$ and C57 mice fed OA and AA diets for the experimental period. Error bars represent the least significant difference (lsd, 5% level) over the time points within each strain. Asterisks indicate significant daily difference (P<0.05). ○ = body weight OA diet; ● = body weight AA diet; Δ = dietary intake OA diet; ▲ = dietary intake AA diet.

Table 6.1 Inflammation and HIS in colon tissue, and plasma SAA concentrations in C57 and *Il10*$^{-/-}$ mice fed OA or AA diets

	C57 mice		***Il10*$^{-/-}$ mice**		
	OA diet	**AA diet**	**OA diet**[a]	**AA diet**	**lsd**
Colon HIS	0.13	0.34	7.00	5.00	3.00
Inflammatory cell infiltration	0.13	0.34	5.30	3.58	1.95
Tissue destruction	0	0	1.30	1.17	1.17
Tissue repair	0	0	0.40	0.25	0.47
SAA (μg/mL)	23.8 (4.28)	82.2 (4.87)	538.0 (6.38)	161.0 (5.35)	1.26

[a]One *Il10*$^{-/-}$ mouse from the OA group died during the experiment and could not be taken into account for statistical analysis. The total colon histological injury scores (HIS) and inflammation type (inflammatory cell infiltration, tissue destruction and repair) are shown as average of all mice in the treatment group and least significant difference (lsd), statistical analysis within *Il10*$^{-/-}$ mice, mostly 0 scores for C57 mice). Serum amyloid A (SAA) is shown as mean with log-transformed values in parentheses, and lsd of the log-transformed data. A trend ($0.05<P<0.10$) has been observed for colon HIS and plasma SAA.

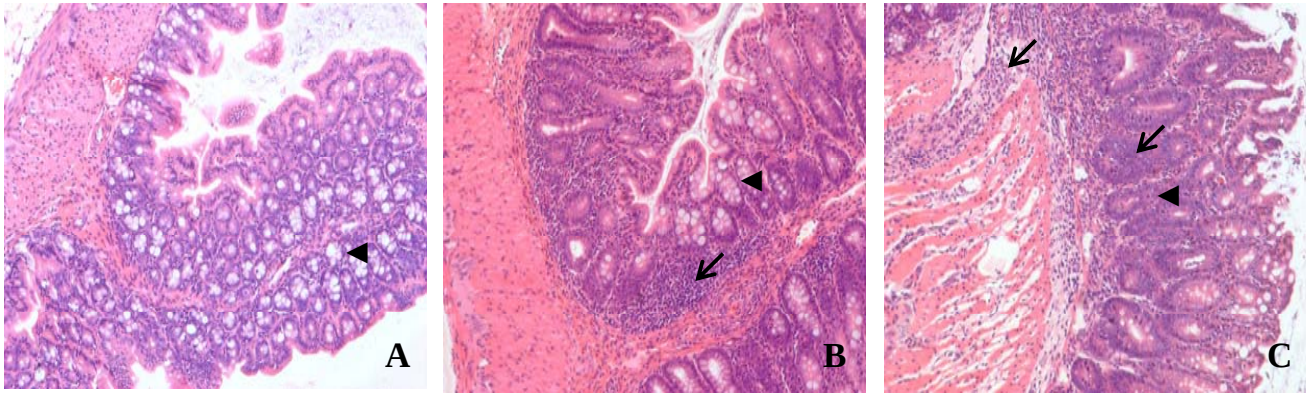


Fig. 6.2 Haematoxylin-eosin stained colon sections of a C57 and an *Il10*$^{-/-}$ mouse. (A) Colon section (×40) from a normal, non-inflamed C57 mouse fed the AA diet. (B) Moderate inflamed colon section (×100) from an *Il10*$^{-/-}$ mouse fed the AA diet. (C) Severe inflamed colon section (×100) from an *Il10*$^{-/-}$ mouse fed the OA diet. Lesions are mainly caused by inflammatory cell infiltration with a high number of monocytes and neutrophils (←), loss of crypt cells through crypt abscesses and loss of goblet cells (◄) in the lamina propria.

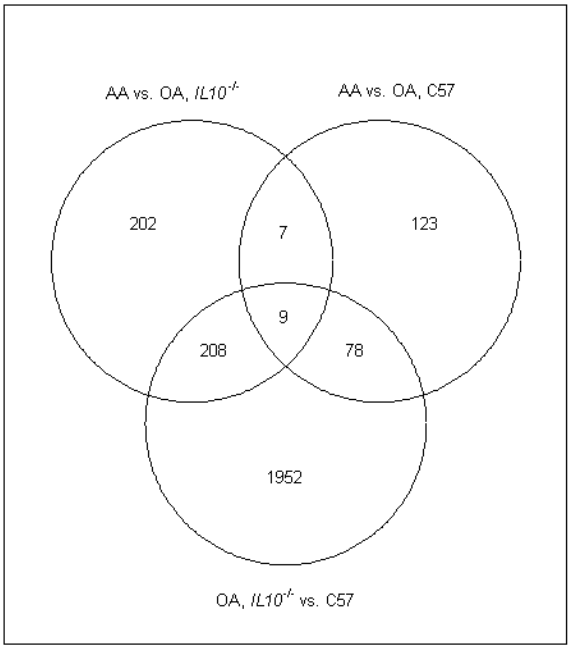


Fig. 6.3 Venn diagram showing the numbers of differentially expressed genes unique to or in common in the genotype comparison (*Il10*$^{-/-}$ *vs.* C57 mice on the OA diet) and the two diet comparisons (AA *vs.* OA in *Il10*$^{-/-}$ mice and AA *vs.* OA in C57 mice). Numbers represent genes that satisfied the criteria of FC≥1.3 and P<0.01 in each comparison.

Table 6.2 Validation of gene expression results from microarray analysis using qRT-PCR

Gene symbol	**Fold change microarray**		**Fold change qRT-PCR *Canx***	
	***Il10*$^{-/-}$ *vs.* C57 OA diet**	**AA *vs.* OA *Il10*$^{-/-}$ mice**	***Il10*$^{-/-}$ *vs.* C57 OA diet**	**AA *vs.* OA *Il10*$^{-/-}$ mice**
Abcb1a	-3.3	1.6*	-9.1	2.2
Aldh1a1	-2.1	1.6	-5.3	2.7
Ces2	-3.6	1.6*	-7.7	3.7
Fabp2	-2.4	1.4*	-14.3	2.2
Igfbp5	2.0	-2.3	3.0	-1.8
Il1b	8.5	-2.6	16.9	-3.3
Mmp13	3.2	-2.2	9.0	-3.6
Ppara	-2.5	1.4*	-5.0	2.5$^{\#}$
Srebf1	1.1*	-1.7	1.8$^{\#}$	-1.4$^{\#}$
Sult1a1	-2.1	2.2	-8.1	2.9

Canx (calnexin) reference gene used to normalise the data; *Microarray result was not significantly different using the moderated t-statistics in limma; genes that satisfied the criteria of FC≥1.3 and $P<0.01$ were considered to be significantly different; $^{\#}$qRT-PCR result was not significantly different using ANOVA.

Table 6.3 Pathways associated with gene expression changes in the colon of *Il10*$^{-/-}$ and C57 mice fed AA compared to the OA diet using IPA analysis

Gene symbol	Gene name	GeneBank accession	AA/OA *Il10*$^{-/-}$ FC	*P*-value	AA/OA C57 FC	*P*-value	*Il10*$^{-/-}$/C57 OA FC	*P*-value
Stress and immune/inflammatory response (ER stress, complement system, positive acute phase response, Nrf2-mediated oxidative stress response)								
Acta2	actin, alpha 2, smooth muscle, aorta	NM_007392	1.8	0.002	1.0	0.954	-2.1	<0.0001
C1qa	complement component 1, q subcomponent, A chain	NM_007572	-1.4	0.002	1.2	0.022	2.2	<0.0001
C1qb	complement component 1, q subcomponent, B chain	NM_009777	-1.3	0.01	1.0	0.686	1.6	<0.0001
Cyp2c40	cytochrome P450, family 2, subfamily c, polypeptide 40	NM_010004	1.7	0.07	-1.7	0.07	-14.3	<0.0001
Cyp3a5	cytochrome P450, family 3, subfamily A, polypeptide 5	NM_007819	1.8	0.002	-1.0	0.896	-2.2	<0.0001
Cyp4a11	cytochrome P450, family 4, subfamily A, polypeptide 11	NM_010011	1.4	0.005	-1.2	0.09	-1.8	0.003
Eif2ak3	eukaryotic translation initiation factor 2-alpha kinase 3 (Perk)	NM_010121	-1.4	0.002	1.1	0.512	1.3	0.011
Fkbp5	FK506 binding protein 5	NM_010220	2.0	<0.0001	1.2	0.056	-1.3	0.281
Gsta4	glutathione S-transferase A4	NM_010357	1.3	0.004	-1.5	0.001	-3.1	<0.0001
Il1b	interleukin 1, beta	NM_008361	-2.6	0.001	-1.0	0.894	8.5	<0.0001
Il1r2	interleukin 1 receptor, type II	NM_010555	-1.5	0.001	-1.1	0.315	1.7	<0.0001
Keap1	Kelch-like ECH associated protein 1	NM_016679	-1.4	0.001	-1.1	0.308	1.2	0.001
Nfe2l2	nuclear factor (erythroid-derived 2)-like 2 (Nrf2)	NM_010902	-1.1	0.359	1.0	0.077	-1.1	0.027
Ptgs2	Prostaglandin-endoperoxide synthase 2	NM_011198	-1.8	<0.0001	-1.3	0.023	1.4	0.008
Tnf	tumor necrosis factor	NM_013693	-1.5	0.002	1.0	0.675	3.3	<0.0001
Traf2	TNF receptor-associated factor 2	NM_009422	-1.4	0.009	-1.2	0.089	1.1	0.488
RXR activation pathways (CAR/RXR activation, LXR/RXR activation, LPS/IL-1mediated inhibition of RXR function) and co-factor metabolism (retinol metabolism)								
Alas1	aminolevulinate, delta-, synthase 1	NM_020559	-1.5	0.01	-1.1	0.42	1.4	0.025
Abcb1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	NM_011076	1.6	0.028	1.2	0.311	-3.3	<0.0001
Acaca	acetyl-Coenzyme A carboxylase alpha	NM_133360	-1.4	0.000	1.1	0.656	1.5	<0.0001
Aldh1a1	aldehyde dehydrogenase 1 family, member A1	NM_013467	1.6	0.003	1.0	0.99	-2.1	<0.0001
Ccnd1	cyclin D1	NM_007631	-1.4	0.002	1.1	0.481	2.2	<0.0001
Ces2	carboxylesterase 2	NM_145603	1.6	0.069	-1.3	0.2	-3.6	<0.0001
Cyp3a5	cytochrome P450, family 3, subfamily A, polypeptide 5	NM_007819	1.8	0.002	-1.0	0.896	-2.2	<0.0001
Cyp4a11	cytochrome P450, family 4, subfamily A, polypeptide 11	NM_010011	1.4	0.005	-1.2	0.09	-1.8	0.003
Fabp2	fatty acid binding protein 2, intestinal	NM_007980	1.4	0.159	1.0	0.995	-2.4	0.003
Fmo2	flavin containing monooxygenase 2	AK009224	1.9	0.008	-1.3	0.17	-1.4	0.003
Gsta4	glutathione S-transferase A4	NM_010357	1.3	0.004	-1.5	0.001	-3.1	<0.0001

Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	NM_008255	-1.5	0.002	-1.1	0.244	1.7	0.001
Hs3st5	heparan sulfate (glucosamine) 3-O-sulfotransferase 5	AK086098	-1.8	0.004	-1.1	0.439	1.6	0.012
Il1a	interleukin 1, alpha	NM_010554	-1.4	0.003	1.1	0.241	1.7	<0.0001
Il1b	interleukin 1, beta	NM_008361	-2.6	0.001	-1.0	0.894	8.5	<0.0001
Il1r2	interleukin 1 receptor, type II	NM_010555	-1.5	0.001	-1.1	0.315	1.7	<0.0001
Maob	monoamine oxidase B	NM_172778	1.7	0.005	-1.0	0.88	-4.0	<0.0001
Nos2	nitric oxide synthase 2, inducible (iNOS)	NM_010927	-1.3	0.149	-1.1	0.637	2.0	<0.0001
Nr5a2	nuclear receptor subfamily 5, group A, member 2	NM_030676	1.5	0.004	1.1	0.316	-1.0	0.761
Ppara	peroxisome proliferator-activated receptor alpha	NM_011144	1.4	0.013	-1.1	0.412	-2.5	<0.0001
Rdh5	retinol dehydrogenase 5 (11-cis/9-cis)	NM_134006	1.4	0.002	1.0	0.623	-1.3	0.012
Slc27a2	solute carrier family 27 (fatty acid transporter), member 2 (Fatp2)	NM_011978	1.3	0.004	1.3	0.168	-1.1	0.222
Smox	spermine oxidase	NM_145533	-1.5	0.007	1.3	0.113	1.7	0.001
Srebf1	sterol regulatory element binding transcription factor 1	NM_011480	-1.7	0.001	-1.2	0.23	1.1	0.402
Sult1a1	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	NM_133670	2.2	0.001	1.2	0.434	-2.1	0.001
Sult1c2	sulfotransferase family, cytosolic, 1C, member 2	NM_026935	1.9	0.002	-1.1	0.74	-5.6	<0.0001
Tnf	tumor necrosis factor	NM_013693	-1.5	0.002	1.0	0.675	3.3	<0.0001
Traf2	TNF receptor-associated factor 2	NM_009422	-1.4	0.009	-1.2	0.089	1.1	0.488
Ugt1a1	UDP glucuronosyltransferase 1 family, polypeptide A1	NM_201645	1.6	0.002	-	-	-1.7	0.001
Lipid metabolism (cholesterol biosynthesis, fatty acid synthesis/oxidation)								
Acaa2	acetyl-Coenzyme A acyltransferase 2	NM_177470	1.5	0.004	1.0	0.888	-2.0	0.001
Acaca	acetyl-Coenzyme A carboxylase alpha	NM_133360	-1.4	0.000	1.1	0.656	1.5	<0.0001
Acot5	acyl-CoA thioesterase	NM_145444	1.5	0.002	-	-	-1.5	0.003
Fdps	farnesyl diphosphate synthase	AK077979	-1.3	0.007	-	-	1.8	<0.0001
Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	NM_008255	-1.5	0.002	-1.1	0.244	1.7	0.001
Fibrosis development								
Col1a2	collagen, type I, alpha 2	NM_007743	-1.8	0.004	1.2	0.228	1.7	0.006
Fgf7	fibroblast growth factor 7 (keratinocyte growth factor)	NM_008008	-1.4	0.006	-1.3	0.017	-1.2	0.074
Ifng	interferon, gamma	NM_008337	-1.2	0.245	-1.1	0.315	2.5	<0.0001
Igfbp5	insulin-like growth factor binding protein 5	NM_010518	-2.3	0.000	1.4	0.107	2.0	0.001
Il1a	interleukin 1, alpha	NM_010554	-1.4	0.003	1.1	0.241	1.7	<0.0001
Il1b	interleukin 1, beta	NM_008361	-2.6	0.001	-1.0	0.894	8.5	<0.0001

Il1r2	interleukin 1 receptor, type II	NM_010555	-1.5	0.001	-1.1	0.315	1.7	<0.0001
Ltbp2	latent transforming growth factor beta binding protein 2	NM_013589	-1.5	0.002	1.2	0.2	2.0	<0.0001
Mmp13	matrix metallopeptidase 13	NM_008607	-2.2	0.000	-1.2	0.382	3.2	<0.0001
Myl7	Myosin, light chain 7, regulatory	NM_022879	-1.9	0.002	1.1	0.66	6.0	<0.0001
Tnf	tumor necrosis factor	NM_013693	-1.4	0.002	1.0	0.675	3.3	<0.0001

Genes were grouped into one or more of the following IPA pathways: ER stress, complement system, positive acute phase response, Nrf2-mediated oxidative stress response, CAR/RXR activation, LXR/RXR activation, LPS/IL1mediated inhibition of RXR function, retinol metabolism, cholesterol biosynthesis, fatty acid synthesis/oxidation and fibrosis development. Genes with FC≥1.3 and a $P<0.01$ were considered for pathway analysis. Shaded cells represent genes that were not differentially expressed in the diet or genotype comparison.

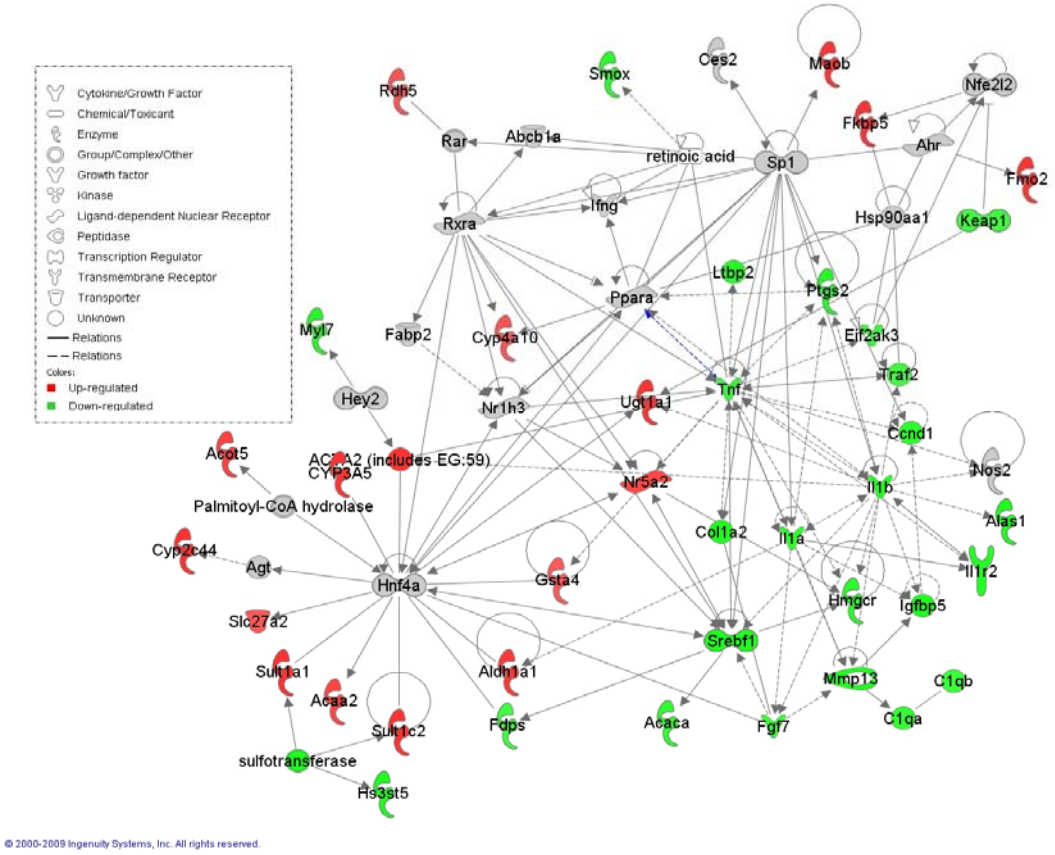


Fig. 6.4 Biological interaction network of genes of the most significantly regulated pathways generated for the AA *vs.* OA *Il10*$^{-/-}$ mice comparison using IPA analysis. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge. All edges are supported by at least one reference from the literature. Red and green coloured nodes indicate genes with up- and down-regulated expression, respectively, in *Il10*$^{-/-}$ mice fed the AA diet compared to *Il10*$^{-/-}$ mice fed the OA diet. The intensity of the colours specifies the degree of increase or decrease. Greater intensity represents a higher gene expression level. Nodes and edges are displayed with various shapes and labels that present the functional class of genes and the nature of the relationships between the nodes.

Table 6.4 Significantly over-represented gene ontology (GO) terms in the colon of *Il10*$^{-/-}$ mice fed the AA diet compared to OA-fed *Il10*$^{-/-}$ mice using FUNC analysis

GO ID	GO term	Number of genes	*P* over-representation after data refinement
Molecular function			
GO:0016616	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	15	6.13E-05
GO:0030020	extracellular matrix structural constituent conferring tensile strength	8	1.28E-04
GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	14	3.99E-04
GO:0003823	antigen binding	6	1.35E-03
GO:0003924	GTPase activity	16	2.07E-03
GO:0003774	motor activity	18	2.24E-03
GO:0004497	monooxygenase activity	13	3.64E-03
GO:0008483	transaminase activity	6	3.70E-03
GO:0017111	nucleoside-triphosphatase activity	42	2.26E-02
Cellular component			
GO:0005615	extracellular space	185	2.48E-12
GO:0005792	microsome	23	8.68E-07
GO:0005581	collagen	8	3.74E-04
GO:0031672	A band	5	1.04E-03
GO:0005859	muscle myosin complex	5	1.34E-03
GO:0005737	cytoplasm	221	8.59E-03

GO terms annotated to at least ten genes and with FDR or $q<0.05$ according to the hypergeometric test were passed to data refinement and those with $P<0.05$ after refinement were considered over-represented in the list of differentially expressed genes, compared with other genes on the array. Only GO terms for molecular function and cellular component are shown.

6.5 Discussion

Contrary to the hypothesis, this study showed beneficial effects of dietary AA on colon inflammation in *Il10*$^{-/-}$ mice, using a combination of statistical, functional, pathway and network analysis of the whole murine transcriptome. Dietary AA tended to decrease colon HIS and plasma SAA in *Il10*$^{-/-}$ mice when compared to OA-fed *Il10*$^{-/-}$ mice. Genome-wide expression analysis revealed a complex network of transcript changes distinctive to the AA-fed *Il10*$^{-/-}$ mice and characterised by changes in stress and immune/inflammatory responses, fibrosis development, lipid metabolism, RXR activation pathways and LPS/IL1mediated inhibition of RXR function. These pathways were shown to be affected by inflammation (*Il10*$^{-/-}$ versus C57 mice, both fed OA, reported by Knoch *et al*., 2009; Chapter 5); dietary AA counteracted some of these effects in *Il10*$^{-/-}$ mice (compared with OA-fed *Il10*$^{-/-}$ mice). In most cases, dietary AA fed to C57 mice did not affect these pathways (compared with OA-fed C57 mice). The following paragraphs discuss the pathways most significantly affected by dietary AA, and explain how the various transcript changes are interconnected and modulate adaptive cellular responses at the transcriptional level during the course of colon inflammation.

Dietary AA reduced colonocyte stress

Compared to dietary OA, dietary AA reduced the expression levels of several genes of ER stress (*Eif2ak3* and *Traf2*), the complement system (*C1qa* and *C1qb*) and positive acute phase response (*C1s*, *Il1b* and *Tnfa*) pathways in the colon of *Il10*$^{-/-}$ mice. These pathways are associated with cellular stress, humoral immune responses and cytokine signalling suggesting reduced inflammatory responses with dietary AA. Inhibition of complement components (e.g. C5) has been shown to attenuate the activity of several mediators of intestinal tissue injury including neutrophil trafficking, cytokine production and adhesion molecule expression (Wada *et al*., 2001). Activation of the complement system has been linked with mediation of tissue damage, and increase in intestinal inducible nitric oxide synthase (iNOS) protein activity seems to play a role in this activation (Montalto *et al*., 2003). iNOS is induced by the cytokines TNF, IL1, IFNγ, and by LPS in a variety of cell types (Dijkstra *et al*., 2002). The gene network built here using IPA indicates that the reduced *Tnfa* and *Il1b* cytokine gene expression

levels observed in the colon of AA-fed *Il10*$^{-/-}$ mice were not linked to a change in mRNA abundance of the *Nos2* gene.

Dietary AA reduced the abundance of the *Eif2ak3 (Perk*) gene, which codes for an ER transmembrane protein kinase that phosphorylates the eukaryotic translation initiation factor eIF2α and suppresses protein synthesis in response to ER stress (Harding et al., 1999). Decreased *Eif2ak3* gene expression levels suggest reduced ER stress in response to dietary AA, which is supported by reduced mRNA abundance of *Keap1*. Upon ER stress, NRF2 is released from its repressor KEAP1, which leads to accumulation of NRF2 in the nucleus. NRF2 binds to the antioxidant response element and activates transcription of genes coding for detoxifying and antioxidant enzymes, GSTs and UGTs (Itoh *et al*., 2003). Reduced ER stress in the colon of *Il10*$^{-/-}$ mice fed the AA diet was not associated with change in expression levels of NRF2 (*Nfe2l2*) gene, but linked to increased mRNA abundance of its target enzyme genes, e.g. *Gsta4* and *Ugt1a1*. This implies an increase in detoxification activities in the colon of those mice and likely an effect of dietary AA on NRF2 protein phosphorylation, which was not measured in this study, leading to increased GST and UGT activities. Dietary AA also increased expression levels of the *Aldh1a1* gene (which is involved in oxidative stress and prevents lipid damage by destruction of reactive aldehydes (Berger *et al*., 2006)) in *Il10*$^{-/-}$ mice, and decreased expression levels of the *Traf2* gene, which codes for a protein that can elicit (when activated during stress response) c-Jun N-terminal kinase phosphorylation and activation (Malhotra & Kaufman, 2007). The findings suggest mechanisms by which dietary AA could reduce ER stress responses and protect colonocytes from oxidative stress in *Il10*$^{-/-}$ mice.

Dietary AA and lipid metabolism genes

As expected, dietary supplementation with AA ethyl ester affected several genes critical to lipid metabolism in the inflamed colon. Dietary AA increased fatty acid transport (*Slc27a2* or *Fatp2*), mitochondrial (*Acaa2*), peroxisomal (*Acot5*) and microsomal (*Cyp4a11*) fatty acid oxidation genes in the colon of *Il10*$^{-/-}$ mice. It decreased the expression levels of cholesterol and fatty acid (*Acaca*) synthesis genes likely via reduced down-regulation of *Srebf1* and its target genes, for example, the rate-limiting enzyme of sterol biosynthesis *Hmgcr* and signalling co-factors such as *Tnf* in the colon of *Il10*$^{-/-}$ mice. These transcript changes are in agreement with existing knowledge

about fatty acid transport, synthesis and metabolism; fatty acids can be ß-oxidised in mitochondria or peroxisomes or ω-oxidised in microsomes for energy production and cell signalling (Dulpus *et al*., 2000; Salter & Tarling, 2007).

In this study, COX-dependent AA metabolism was reduced in the AA-fed *Il10*$^{-/-}$ mouse colon, because expression levels of the *Cox2* (*Ptgs2*) gene, and subsequently inflammatory response genes, were decreased, suggesting a protective effect of dietary AA in the colon of *Il10*$^{-/-}$ mice. This finding does not agree with a study where supplementation of purified AA (1.5% AA ethyl ester) to an EPA diet (10% w/w fat total) was tumorigenic by reducing the anti-tumorigenic effect of purified EPA on colorectal cancer using the *Apc*$^{Min/+}$ mouse model (Hansen Petrik *et al*., 2000). AA can be metabolised by different enzyme systems: COX, LOX and CYP (Zeldin *et al*., 1997). COX (e.g., prostaglandins, thromboxane and prostacyclin) and LOX (e.g., leukotrienes and hydroxyeicosatetraenoic acids) products are differentially distributed in the intestine with effects on intestinal motility, secretion and blood flow (Zeldin *et al*., 1997). CYP in intestinal microsomal fractions metabolise AA to several oxygenated metabolites (epoxyeicosatrienoic acids or hydroxyeicosa-tetraenoic acids, HETE) (Tsao *et al*., 2001). Expression levels of the *Cyp2c40* gene were highly reduced in inflamed colon of *Il10*$^{-/-}$ mice, and showed a tendency to be increased by dietary AA. CYP2C40, the main CYP2C in the intestinal tract, has also been identified in murine colon and metabolises AA to 16-HETE (Tsao *et al*., 2001). The anti-inflammatory mediator 16-HETE can inhibit adhesion and aggregation of neutrophils, suggesting a role in resolution of inflammation (Tsao *et al*., 2001). The exact roles of the CYP2C differentially expressed in various extrahepatic tissues remains unknown, but CYP2C40 may play an important role in intestinal inflammation and in metabolising AA, and may contribute to specific AA metabolite production in those tissues to reduce or promote inflammation.

However, similar to the present study, Ramakers *et al*. (2008) reported a protective effect of dietary AA in DSS-induced colitis mice (colon mostly affected) fed AA-enriched diet (1.1% AA ethyl ester oil, 6% w/w total fat; approximately one-third of the AA amount used here); the animals showed decreased weight loss and less diarrhoea compared with the OA-enriched diet. Sustained increased delivery of dietary AA during the onset of colon inflammation might have resulted in increased transport of AA from the peripheral circulation into the colonocytes, increased storage in the colonocyte

membrane and esterification of AA with glycerol in membrane phospholipids. Therefore, the concentration of free AA in the intracellular compartment might have remained at a level insufficient to initiate inflammation.

Dietary AA reduced colonocyte fibrosis

Several genes implicated in the development of intestinal fibrosis were expressed at lower levels in the colon of AA-fed *Il10*$^{-/-}$ mice. Supporting this observation, colonic shortening and deformation appeared to be reduced in the colon tissue of *Il10*$^{-/-}$ mice fed AA diet compared with that of OA-fed *Il10*$^{-/-}$ mice (visual observation noted at tissue sampling, data not shown), although fibrotic features were not scored in detail in the histological examination. CD-associated fibrosis is the result of a complex interplay among fibroblasts, smooth muscle cells, cytokines, inflammatory cells and chronic transmural inflammation.

Increase in collagen synthesis by smooth muscle cells, fibroblasts, and an increase in muscle layer thickness have also been observed in CD patients with intestinal fibrosis (Martinez-Augustin *et al*., 2008). In AA-fed *Il10*$^{-/-}$ mice, decreased expression levels of pro-inflammatory cytokines were linked to lower *Mmp13* gene expression levels. MMP can degrade extracellular proteins (e.g. collagen, laminin and fibronectin) and their expression is activated by pro-inflammatory cytokines (e.g. TNF and IL1A), with over-expression leading to deregulated extracellular matrix turnover and intestinal inflammation (Burke *et al*., 2007).

Reduction of intestinal fibrosis by dietary AA is also suggested by down-regulation of the expression levels of *Col1a2* gene involved in collagen synthesis. Reduced mRNA abundance of *Igfbp5* gene by dietary AA in the colon of *Il10*$^{-/-}$ mice is in agreement with observations that higher expression levels of *Igfbp5* are related to extracellular matrix remodelling by increasing collagen synthesis and cell proliferation, and thus, to fibrosis development in inflamed colon (Martinez-Augustin *et al*., 2008). The colonic expression levels of *Fgf7* gene, which is involved in epithelial tissue homeostasis, repair and disease and exerts some of its cytoprotective effects through activation of NRF2 under stress conditions (Beer *et al*., 2005), was also decreased in AA-treated *Il10*$^{-/-}$ mice. It has been reported that an AA-enriched oil can decrease the expression levels of

genes involved in collagen synthesis and cell adhesion in mice (Berger et al., 2006), but the link between dietary AA and intestinal fibrosis has not been made previously.

While the changes in immune and inflammatory responses to dietary AA may be specific to the present experimental conditions, the impact of the IL10 mutation on colitis development varies as a function of the inbred strain background when maintained under the same conditions. The colitis in *Il10*$^{-/-}$ mice on the C57BL/6J background for example is mild (Berg *et al*., 1996), compared with when the *Il10*^{tm1Cgn} allele is bred into the 129/SvEv, C3H/HeJBir and C3H.SW background strains, where colitis is severe and progressive (Takeda *et al*., 1999; Bristol *et al*., 2000; Mahler & Leiter, 2002). This suggests that other genes or gene interactions particular to the genetic background of each strain modify the development of colitis and probably the response to a dietary intervention. For example, the study by Andrikopoulos *et al*. (2005) has shown that the effect of a high fat diet on insulin secretory function was dependent on the mouse strain. Future investigations of the effects of dietary AA on whole genome gene expression profiles of *Il10*$^{-/-}$ mice on different genetic backgrounds may unravel gene interactions that lead to phenotypic differences.

In conclusion, it was found that in *Il10*$^{-/-}$ mice, dietary AA reduced the expression levels of some genes associated with cellular stress, inflammatory and immune response and intestinal fibrosis development, compared with dietary OA. This suggests that the colonocytes were able to deal with a sustained exposure of AA as used here, a recognised pro-inflammatory fatty acid during colitis development. Dietary AA could reach the colonocytes from the peripheral circulation and remain at a level that did not initiate inflammatory processes resulting from reduced COX-dependent metabolism. Since sampling was performed at only one time point in this disease model, further investigation is warranted to study the effect of AA on changes in the gene expression profile at several time points, including postprandial intervention and after clinical manifestation of colitis. The different bioavailability of AA and OA ethyl esters and TG containing these fatty acids is also important to explain their bioactive role in the process of inflammation.

Chapter 7

Diversity of caecal bacteria is altered in interleukin-10 gene-deficient mice with colitis when fed polyunsaturated fatty acids

Part of the material presented in this chapter has been submitted as a paper:

Knoch B, Nones K, Barnett MPG, McNabb WC & Roy NC: Diversity of caecal bacteria is altered in interleukin-10 gene-deficient mice before and after colitis onset and when fed polyunsaturated fatty acids. Submitted to *Microbiology*

7.1 Abstract

Il10$^{-/-}$ mice show a hyper-reaction to normal bacteria and develop spontaneous human CD-like colitis unlike their wildtype counterparts or germ-free *Il10*$^{-/-}$ mice. This suggests that the IL10 cytokine is important in the tolerance towards commensal bacteria in these mice. Therefore, this study aimed to test the hypothesis that the lack of IL10 protein causes changes in intestinal metabolic and regulatory processes before and after onset of colitis in *Il10*$^{-/-}$ mice, and thus may alter the intestinal bacteria community. It was also tested whether n-3 and n-6 PUFA alter large intestine bacteria profiles in colitic *Il10*$^{-/-}$ mice. Denaturing gradient gel electrophoresis and quantitative real-time PCR were used to identify differences in caecal bacteria diversity 1) between *Il10*$^{-/-}$ mice and C57 wildtype mice before (7 weeks of age) and after onset (12 weeks of age) of colitis, and 2) by dietary n-3 EPA and n-6 AA during colitis development. The reduced caecal bacteria diversity in *Il10*$^{-/-}$ compared to C57 mice at 7 and 12 weeks of age is at least partially associated with the lack of IL10 as differences occurred before colitis onset in *Il10*$^{-/-}$ mice. In contrast, an increased number and diversity of caecal bacteria was observed in *Il10*$^{-/-}$ mice during the course of colitis possibly by increased intestinal permeability. Dietary AA and EPA affected caecal bacteria diversity in colitic *Il10*$^{-/-}$ mice, particularly increasing *E. coli* cell numbers compared to the OA fatty acid control.

7.2 Introduction

The interactions between intestinal bacteria and the mucosal immune system of a genetically-susceptible host are important in the development of IBD such as CD (Strober *et al.*, 2007). There is evidence that an abnormal mucosal immune response to normal intestinal bacteria underlies mucosal inflammation in human disease (Strober *et al.*, 2007). The induction of mucosal inflammation in animal models of IBD involves innate immune factors such as pattern-recognition receptors, e.g. TLR, which recognise molecular patterns of bacteria at intestinal mucosal surfaces in order to mount antigen-specific adaptive immune responses (Strober *et al.*, 2007; Honda & Takeda, 2009).

Both human clinical and animal studies have shown differences in bacterial communities between disease and control subjects, which suggest the involvement of luminal bacteria and bacterial products in initiation and progression of intestinal

inflammation (Mangin *et al.*, 2004; Bibiloni *et al.*, 2005). Antibiotic treatment decreased intestinal bacteria concentrations and attenuated colitis in CD patients (Sutherland *et al.*, 1991; Colombel *et al.*, 1997; Gui *et al.*, 1997) and in the *Il10*$^{-/-}$ mouse model of IBD (Madsen *et al.*, 2000). Colonisation studies with germ-free rodent models have also indicated that enteric bacteria vary in their ability to induce colitis and specifically interact with the genetically susceptible host (Rath *et al.*, 1999; Waidmann *et al.*, 2003; Kim *et al.*, 2005). Probiotic bacteria, such as *Lactobacillus* spp., were able to prevent colitis in *Il10*$^{-/-}$ mice (Madsen *et al.*, 2000). *Enterococcus* spp. are common intestinal bacteria of healthy humans and animals (Jett *et al.*, 1994; Sellon *et al.*, 1998), but *E. faecalis* has been shown to induce IBD in germ-free *Il10*$^{-/-}$ mice (Balish & Warner, 2002). *Il10*$^{-/-}$ mice carrying a null mutation in the gene that normally codes for the anti-inflammatory cytokine IL10 show a hyper-reaction to normal intestinal bacteria and develop intestinal inflammation, unlike their wildtype counterparts (Kim *et al.*, 2005). Intestinal inflammation does not occur when *Il10*$^{-/-}$ mice are born and maintained in germ-free conditions (Kim *et al.*, 2005), but develops from 20 weeks of age in *Il10*$^{-/-}$ mice housed in specific pathogen-free conditions (McCarthy *et al.*, 2003). These *in vivo* studies suggest that a lack of IL10 protein as in *Il10*$^{-/-}$ mice leads to a loss of tolerance towards commensal bacteria. This is supported by *in vitro* evidence that IL10 produced by T regulatory lymphocytes is effective in controlling tolerance to commensal bacteria (Izcue *et al.*, 2006).

Effects of dietary lipids on gastrointestinal bacteria have only been reported by a few human studies investigating faecal bacteria (Cummings *et al.*, 1978; Endo *et al.*, 1991; Eastwood *et al.*, 1995; Farnworth *et al.*, 2007). There have been no studies on potential effects of dietary lipids such as PUFA on intestinal bacteria profiles in colitic *Il10*$^{-/-}$ mice. Anti-inflammatory actions of the n-3 PUFA EPA include the regulation of pro-inflammatory cytokines from several cell types and have mainly been attributed to the substitution of AA in cell membranes. This results in decreased production of AA-derived pro-inflammatory eicosanoids (Calder, 2006). Das (2002) reported that long-chain PUFA also show antibiotic-like and growth inhibitory actions against pathogenic bacteria and can enhance beneficial actions of probiotics, e.g. *Lactobacillus* spp., on the mucosal surface and aid probiotics in colonising the gastrointestinal tract.

Since the lack of IL10 protein lead to significant changes in metabolic and signalling processes in the intestine of *Il10*$^{-/-}$ mice (study 1; Chapter 3), it was hypothesised that this *Il10* gene-deficiency alters the intestinal bacteria community before and consequently after colitis onset. We further showed that dietary n-3 and n-6 PUFA had anti-inflammatory effects on colonic gene expression profiles in colitic *Il10*$^{-/-}$ mice (study 2; Chapter 5 and 6), thus it was also hypothesised that these PUFA alter the large intestine bacteria profiles in those mice. To test these hypotheses caecal samples from these previous studies were used to identify differences in bacterial diversity between *Il10*$^{-/-}$ and C57 mice before (7 weeks of age) and after (12 weeks of age) onset of colitis in *Il10*$^{-/-}$ mice, and to identify changes in bacterial diversity in response to dietary n-3 EPA and n-6 AA compared to the OA control fatty acid in colitic *Il10*$^{-/-}$ mice. The caecal bacteria in both studies were analysed qualitatively using DGGE analysis and qPCR.

7.3. Materials and methods

7.3.1 Experimental design of the animal experiments

The experimental design of study 1 (time-course) and study 2 (PUFA intervention), housing, induction of colitis, tissue collection and fast-feed period prior to sampling are described in Materials and Methods chapter 2.1.1 to 2.1.4. All male *Il10*$^{-/-}$ (C57BL/6J background, formal designation B6.129P2-*Il10*<tm1Cgn>/J) and C57 wildtype (C57BL/6J) mice purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) were raised in specific pathogen-free conditions and maintained individually in conventional conditions. The mice were 35 days or 5 weeks of age at the start of the trial. All *Il10*$^{-/-}$ and C57 mice were randomly divided into experimental groups for study 1 and study 2. To increase the likelihood of bacteria colonising and inducing intestinal inflammation, all mice were inoculated with the same bacterial mixture (*E. faecalis* and *E. faecium* strains combined with complex intestinal microflora from healthy, age-matched C57 mice) as described previously (Roy *et al*., 2007). This was to ensure that any difference in the bacterial community between the mouse genotypes was because of the intestinal inflammation in *Il10*$^{-/-}$ mice. Certain *E. faecalis* strains are known to induce IBD in *Il10*$^{-/-}$ mice (Balish & Warner, 2002). Therefore a number of *E. faecalis* and *E. faecium* strains from a few sources were combined in this inoculum.

In study 1, five to six mice were sacrificed at 7, 8.5, 10, 12 and 14 weeks of age. The following time points as used for gene and protein expression profiling (Chapter 3) were selected for caecal bacterial community profiling: *Il10*$^{-/-}$ mice 7 weeks of age (three mice), *Il10*$^{-/-}$ mice 12 weeks of age (four mice), C57 mice 7 weeks of age (four mice) and C57 mice 12 weeks of age (four mice) which was based on the histopathological assessment to ensure a group of individuals with no (7 weeks of age) or severe (12 weeks of age) colitis. One 7-week-old *Il10*$^{-/-}$ mouse died of unknown causes and two others showed moderate colitis, thus were excluded from the analysis.

In study 2, four to six *Il10*$^{-/-}$ and C57 mice were sacrificed at 11 weeks of age. The following dietary groups as used for gene expression profiling (Chapters 5 and 6) were selected for caecal bacterial community profiling: AIN-76A (5% corn oil), AIN-76A (fat-free) + 1% corn oil + 3.4% OA, AIN-76A (fat-free) + 1% corn oil + 3.4% EPA, and AIN-76A (fat-free) + 1% corn oil + 3.4% AA. The 1% corn oil was supplemented with 0.2% purified LA and 0.06% ALA ethyl esters. One *Il10*$^{-/-}$ mouse in the OA diet group and two *Il10*$^{-/-}$ mice in the EPA diet group died for unknown causes. One caecal sample of C57 mice on the AIN-76A, OA, EPA or AA diet and one sample of an *Il10*$^{-/-}$ mouse on the AA diet were excluded because of degraded DNA. From each group caeca including digesta were snap-frozen in liquid nitrogen and stored −85°C until analyses were carried out.

7.3.2 DNA extraction and PCR amplification

Isolation of bacterial genomic DNA and PCR amplification of the bacterial 16S rRNA gene using universal primers are described in Materials and Methods chapter 2.8.1 and 2.8.2. DNA from individual or pooled samples was used for DGGE analysis. Pooled DNA (consensus profiles) was prepared with equal amounts of DNA from each experimental group, generating four pools for study 1 and eight pools for study 2.

7.3.3 Denaturing gradient gel electrophoresis

Generation of DGGE profiles is described in Materials and Methods chapter 2.8.3.

7.3.4 Analysis of DGGE profiles

Analysis of DGGE profiles using the BioNumerics software package is described in Materials and Methods chapter 2.8.4.

7.3.5 Cloning and sequencing DNA from DGGE fragments

Cloning using the TOPO-TA cloning system and sequencing of isolated DNA fragments from the DGGE profiles are described in Materials and Methods chapter 2.8.5.

Note: *The DNA fragments were sequenced by the Allan Wilson Centre Genome Service at Massey University in Palmerston North.*

7.3.6 Real-time PCR quantification of total bacteria and selected bacterial groups

The real-time PCR quantification of detected bacteria is described in Materials and Methods chapter 2.8.6. The primers used in the real-time quantification of total bacteria, *Bacteroides-Prevotella-Porphyromonas, Enterococcus* spp., *Clostridium perfringens* (*C. perfringens*) group, *E. coli* group, and *B. vulgatus* of the caecal DNA from *Il10*$^{-/-}$ and C57 mice are listed in Table 7.1. The presence of *Helicobacter* spp. was tested by conventional PCR using the primers H267f 5'-CTATGACGGGTATCCGGC-3' and H676r 5'-ATTCCACCTACCTCTCCCA-3' (Riley *et al*., 1996).

The bacterial strains used for preparing the standard curves were: *E. coli* ATCC 35150, *B. fragilis* NZRM 964, *B. vulgatus* DSM 1447, *E. faecalis* AGR 991, *Clostridium perfringens* ATCC 13124 and *Lactobacillus reuteri* DPC 16 (*L. reuteri*). All bacterial strains were grown anaerobically overnight at 37°C. Tryptone Soy Broth (Oxoid, SA, Australia) was used for *E. coli*, Man-Rogosa-Sharpe (MRS) broth (Oxoid) for *L. reuteri*, Todd-Hewitt broth (Oxoid) for *E. facecalis*; and Brain Heart Infusion broth (Oxoid) for *B. fragilis*, *B. vulgatus* and *C. perfringens*. Bacterial cells were counted using a Neubauer haemocytometer after 18-h incubation.

Note: *The standards for E. coli, B. fragilis, L. reuteri and C. perfringens were provided by Dr Gunaranjan Paturi from Plant & Food Research, NZ.*

The standard curves were prepared using a 10-fold dilution series (1×10^4 to 1×10^9 cfu/mL) from genomic DNA extracted of each bacterial reference strain. The interpretations of the bacterial quantifications were based on the assumption that the analysed community represented a similar mean number of 16S copies per bacterial cell. The qPCR results (number of cells/gram of wet caecum content) were log-transformed and statistical analysis was performed using two-way ANOVA, followed by the least significant difference *post hoc* test in GenStat (11th edition, VSN International Ltd, Hertfordshire, UK). A $P<0.05$ was considered significant.

7.4 Results

7.4.1 DGGE profiles of murine caecum before and after onset of colitis

Global bacterial changes in the caecum of 7- and 12-week-old mice were monitored with DGGE. The consensus DGGE fingerprints of the caecal bacteria from *Il10*$^{-/-}$ and C57 mice aged 7 (no colitis in both mouse genotypes) and 12 weeks (severe colitis in *Il10*$^{-/-}$ mice) showed clear differences in the profiles between mouse genotypes at both times (Fig. 7.1). The consensus fingerprints from C57 mice at 7 and 12 weeks of age were 84.4% similar and from *Il10*$^{-/-}$ mice 83.9% similar at 7 and 12 weeks of age. The DGGE profiles from non-inflamed C57 and inflamed *Il10*$^{-/-}$ mice were 72.4% similar, regardless of the age. There was a reduction in bacterial diversity in colitic *Il10*$^{-/-}$ mice (Fig.7.1).

Individual *Il10*$^{-/-}$ or C57 mice in both age groups showed similar profiles for the predominant DNA fragments, while more variation was seen for the less dominant DNA fragments. To support these observations, similarity matrix analysis using the Dice similarity coefficient (D_{sc}) for the DGGE profiles of individual mice at 7 and 12 weeks of age was conducted (Fig. 7.2, Table 7.2). This analysis showed similarities (81.4-91.0%) of DGGE profiles between individual mice within each age group and also within each mouse genotype.

When excised, purified and sequenced, the best BLAST match of bands 1-6 and 9-16 from DGGE profiles of 7- and 12-week-old *Il10*$^{-/-}$ and C57 mice was to uncultured strains (Fig. 7.2, Table 7.3). Bands 7 and 8 from DGGE profiles of C57 mice in the 7 and 12 week age group, were identified as *Helicobacter pullorum* (*H. pullorum*)

(GenBank accession FJ236465, 99-100% identity); this particular species appeared to be absent in *Il10*$^{-/-}$ mice. Bacterial DNA isolated from the caecum of all *Il10*$^{-/-}$ and C57 mice tested positive for this bacteria using a *Helicobacter* genus-specific PCR (data not shown). This means that *Helicobacter* spp. were present in all mice which were not shown in the DGGE profile.

7.4.2 Real-time PCR quantification of bacterial groups found in murine caecum before and after onset of colitis

Total bacteria, *Bacteroides-Prevotella-Porphyromonas* and *Enterococcus* spp. were quantified by real-time PCR in 7- and 12-week-old *Il10*$^{-/-}$ and C57 mice (Fig. 7.3). The total number of bacteria was higher in the caecum of 12-week-old *Il10*$^{-/-}$ mice compared to 7-week-old *Il10*$^{-/-}$ mice (P=0.008), and was lower in 7-week-old *Il10*$^{-/-}$ mice compared to 7-week-old C57 mice (P<0.001). At 12 weeks of age, the total bacterial number was higher in the caecum of C57 compared to *Il10*$^{-/-}$ mice (P=0.02) and remained within the variance shown (Fig. 7.3a). The cell number from the *Bacteroides-Prevotella-Porphyromonas* group differed between *Il10*$^{-/-}$ and C57 mice at 7 weeks of age with higher numbers in 7-week-old C57 mice (P=0.003). An increase with age in the number of *Bacteroides-Prevotella-Porphyromonas* spp. was also observed in the caecum of *Il10*$^{-/-}$ mice (P=0.05) (Fig. 7.3b). While there was an increase in total bacterial cell number with age in *Il10*$^{-/-}$ mice, the cell number of *Enterococcus* spp. decreased from 7 to 12 weeks of age in the caecum of *Il10*$^{-/-}$ mice (P=0.001). More *Enterococcus* spp. cells were present in *Il10*$^{-/-}$ mice compared to C57 mice at 7 weeks (P=0.0005) and 12 weeks (P=0.03) of age (Fig. 7.3c).

7.4.3 DGGE profiles of murine caecum in response to dietary PUFA

Global bacterial changes in the caecum of mice fed n-3 and n-6 PUFA were monitored with DGGE. The consensus DGGE fingerprints of the caecal bacteria from *Il10*$^{-/-}$ and C57 mice fed AA, EPA, or control diets (OA and AIN-76A) are shown in Fig. 7.4. The DGGE profiles from *Il10*$^{-/-}$ mice fed the AA and EPA diets were 95.0% similar and more similar to the profile of AIN-76A-fed (92.5%) than to OA-fed (83.6%) *Il10*$^{-/-}$ mice. The consensus fingerprint from AA-fed C57 mice clustered with the profiles of AA, EPA and AIN-76A-fed *Il10*$^{-/-}$ mice (84·7 %) instead of with the other C57 groups. The DGGE profiles from C57 mice fed OA, EPA and AIN-76A were 88.2% similar.

The DGGE fingerprint showed a similar effect of AA and EPA diets on the diversity of the caecal bacteria in colitic *Il10*$^{-/-}$ mice but showed a different effect on the bacterial diversity in non-inflamed C57 mice (Fig. 7.4).

DGGE fingerprints of individual mice are shown in Fig. 7.5 and the D_{sc} values of the similarity analysis of the DGGE profiles within dietary groups are listed in Table 7.2. The similarity analysis showed that caecal profiles of individual *Il10*$^{-/-}$ mice fed the EPA diet were less similar (67.5%) to each other than the profiles between individual mice of the other dietary groups (79.3 to 87.0%). There were changes observed in the individual DGGE profiles which seem to be associated not only with the mouse genotype differences but also with dietary PUFA. The DGGE profiles of *Il10*$^{-/-}$ mice on the AIN-76A, EPA and OA diets showed a lower number and diversity of caecal bacteria than those of C57 mice on the same diets, which may be related to the IL10 genotype. In contrast, AA diet caused a significant shift in the bacterial pattern of C57 mice whose profiles appeared of similar complexity than those of the AA-fed *Il10*$^{-/-}$ mice (Fig. 7.4).

Bands A and B (Table 7.4) identified as an uncultured member of the order *Bacteroidales* (closest relative *B. vulgatus*, 99-100% identity) were present in DGGE profiles of *Il10*$^{-/-}$ mice fed PUFA or control diets, but not in those of C57 mice. The DNA fragments excised from bands G and H were identified as *E. coli* (EU555536, 100% identity) and were present in profiles of AA-fed C57 mice and in EPA-, AA- and AIN-76A-fed *Il10*$^{-/-}$ mice. This band seemed to be absent in the profile of *Il10*$^{-/-}$ mice fed the OA diet. DNA fragments (bands K and L) identified as *C. perfringens* (FN356962, 100% identity) were present in DGGE profiles of *Il10*$^{-/-}$ mice fed the control diets, whereas an uncultured *Clostridium* spp. (band M and N, EF710221, 98 and 100% identities) was identified in profiles of *Il10*$^{-/-}$ mice fed the PUFA diets. Other DNA fragments (bands C-F, I, J, O-R) were identified as uncultured bacteria and their closest relatives are listed in Table 7.4.

7.4.4 Real-time PCR quantification of bacterial groups found in murine caecum in response to dietary PUFA

The quantitative analysis of the number of cells of total bacteria, *Bacteroides-Prevotella-Porphyromonas* spp., *E. coli*, *C. perfringens*, *Enterococcus* spp. and *B. vulgatus* is shown in Fig. 7.6. The qPCR data suggest an effect of diet (total bacteria, *E. coli*, *B. vulgatus*, $P<0.05$), mouse genotype (for total bacteria, *C. perfringens*, *B. vulgatus*, $P<0.01$) and a combined mouse genotype-diet effect (*E. coli*, $P<0{\cdot}01$) based on the total copy number of caecal bacteria in *Il10*$^{-/-}$ and C57 mice. There was a lower total cell number of bacteria in the *Il10*$^{-/-}$ OA group than in OA-fed C57 mice ($P=0.008$). The reduction in total bacterial cell numbers in AA-fed C57 mice relative to OA- or EPA-fed C57 mice (Fig. 7.6a) tends to support the apparent reduced abundance of DGGE bands by dietary AA.

Cell numbers of the *C. perfringens* group were increased in the caecum of *Il10*$^{-/-}$ mice on all diets compared to C57 mice fed the same diets, suggesting a mouse genotype effect. The only dietary effect on *C. perfringens* spp. was observed in AA-fed compared to OA-fed C57 mice (Fig. 7.6b). The qPCR results showed a clear diet effect on *E. coli* in the caecum of *Il10*$^{-/-}$ mice. The number of *E. coli* cells was higher in AA-fed compared to OA-fed *Il10*$^{-/-}$ mice ($P<0.001$) and higher in AA-fed compared to OA-fed C57 mice ($P=0.01$). The EPA diet also increased the number of *E. coli* cells in the caecum of *Il10*$^{-/-}$ mice compared to the OA diet. The number of *E. coli* cells was lower in C57 mice fed the EPA compared to AA-fed C57 mice (Fig. 7.6c). There was no significant effect of diet or mouse genotype on the number of cells of *Enterococcus* spp. in the caecum of these *Il10*$^{-/-}$ and C57 mice (Fig. 7.6d).

There was a significant effect of mouse genotype ($P<0.001$) and diet ($P=0.04$) was observed for *B. vulgatus*. Numbers of *B. vulgatus* (a member of the *Bacteroides-Prevotella-Porphyromonas* group) were significantly higher in the caecum of *Il10*$^{-/-}$ mice fed AIN-76A ($P=0.05$) and OA ($P=0.003$) control diets or the AA ($P=0.02$) diet compared to C57 mice on the same diets. The EPA diet increased the number of *B. vulgatus* in the caecum of C57 mice compared to OA-fed C57 mice, which showed a lower number of *B. vulgatus* than AIN-76A-fed C57 mice (Fig. 7.6f).

Table 7.1 Primers used for bacterial quantification by real-time PCR

Bacterial group	**Primers**	**Reference**
Total bacteria	5'-ACTCCTACGGGAGGCAGCAGT-3' 5'-GTATTACCGCGGCTGCTGGCAC-3'	Tannock *et al.* (2000)
Bacteroides-Prevotella-Porphyromonas	5'-GGTGTCGGCTTAAGTGCCAT-3' 5'-CGGA(C/T)GTAAGGGCCGTGC-3'	Rinttila *et al.* (2004)
Enterococcus spp.	5'-CCCTTATTGTTAGTTGCCATCATT-3' 5'-ACTCGTTGTACTTCCCATTGT-3'	Rinttila *et al.* (2004)
Clostridium perfringens group	5'-ATGCAAGTCGAGCGA(G/T)G-3' 5'-TATGCGGTATTAATCT(C/T)CCTTT-3'	Rinttila *et al.* (2004)
Escherichia coli group	5'-CATGCCGCGTGTATGAAGAA-3' 5'-CGGGTAACGTCAATGAGCAAA-3'	Huijsdens *et al.* (2002)
Bacteroides vulgatus	5'-GCATCATGAGTCCGCATGTTC-3' 5'-TCCATACCCGACTTTATTCCTT-3'	Wang *et al.* (1996)

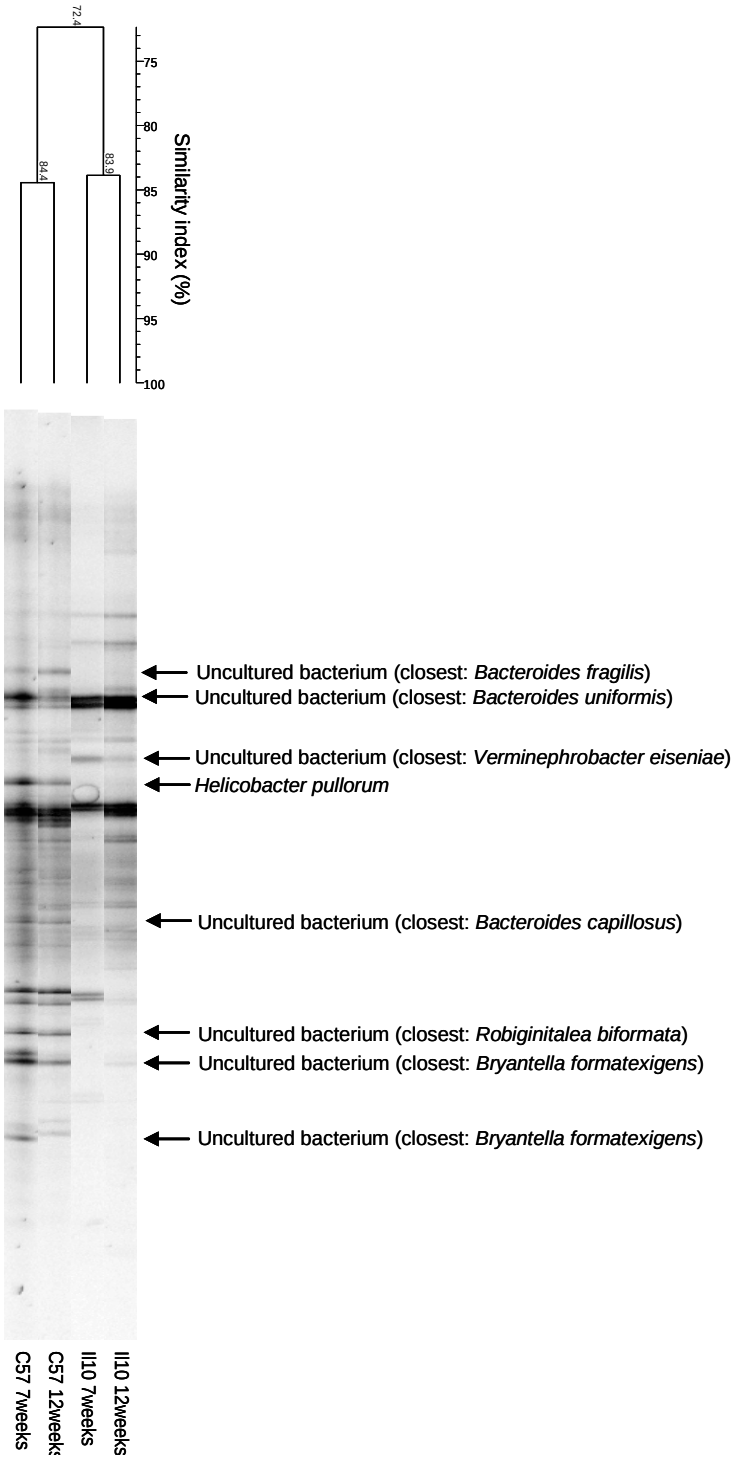


Fig. 7.1 Consensus DGGE profiles of caecal bacteria from *Il10*$^{-/-}$ and C57 mice at 7 and 12 weeks of age. Similarity analysis was performed by using the Dice coefficient. Arrows indicate bacterial species (including the closest known cultured relative) identified by sequencing that were different in the profiles.

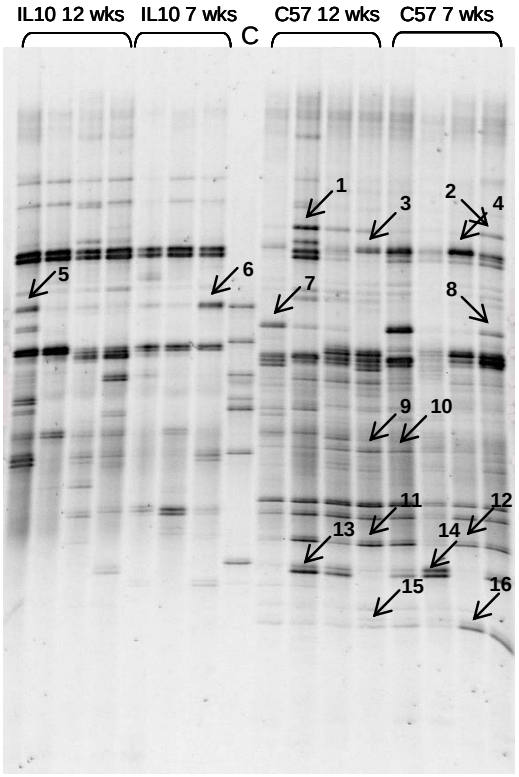


Fig. 7.2 DGGE profiles of caecal bacteria from individual *Il10*$^{-/-}$ and C57 mice at 7 and 12 weeks of age. C, control sample, used to compare profiles between gels. Arrows indicate the positions in the gel where bands were excised, DNA recovered and sequenced. Numbers match the sequencing results in Table 7.3.

Table 7.2 Similarity analysis of DGGE profiles between mice within experimental groups (weeks of age or diets)

Experimental groups	**No. mice per group**	**Dice similarity coefficient (%) between mice within experimental groups**
Study 1		
C57 7 weeks of age	4	86.0
C57 12 weeks of age	4	91.0
Il10$^{-/-}$ 7 weeks of age	3	81.4
Il10$^{-/-}$ 12 weeks of age	4	82.0
Study 2		
C57 AIN-76A diet	5	82.5
C57 OA diet	5	87.0
C57 EPA diet	5	86.7
C57 AA diet	5	81.0
Il10$^{-/-}$ AIN-76A	6	79.3
Il10$^{-/-}$ OA diet	5	82.8
Il10$^{-/-}$ EPA diet	4	67.5
Il10$^{-/-}$ AA diet	5	81.9

Table 7.3 Species identified from excised bands in study 1

Band excised	No. clones sequenced per band	Species identified by sequencing	GeneBank ID (Identity)
1, 2	3	Uncultured bacterium clone H83N4_89h02, isolated from mouse caecum	EU458036 (99-100%)
		Closest known cultured relative - *Bacteroides fragilis*	ABZX01000086 (95%)
3, 4	3	Uncultured bacterium clone R-8325, isolated from faeces of rats fed wheat bran	FJ881325 (100%)
		Closest known cultured relative - *Bacteroides uniformis* ATCC 8492	NZ_AAYH02000029 (95%)
5, 6	3	Uncultured bacterium clone MMP7++82, isolated from MMP7++ mouse distal small intestine	EU791198 (98-100%)
		Closest known cultured relative - *Verminephrobacter eiseniae* EF01-2	NC_008786 (87-88%)
7, 8	3	*Helicobacter pullorum* NCTC 12824	FJ236465 (99-100%)
9, 10	3	Uncultured bacterium clone OF2A10, isolated from stool sample	GQ285980 (99-100%)
		Closest known cultured relative - *Bacteroides capillosus* ATCC 29799	NZ_AAXG02000037 (96%)
11, 12	3	Uncultured bacterium clone 5.14F, isolated from faeces of BALB/c mice	EU656022 (100%)
		Closest known cultured relative - *Robiginitalea biformata* HTCC2501	NZ_AAOI01000001 (85%)
13, 14	3	Uncultured bacterium clone YO00363F09, isolated from *Salmonella*-infected mouse caecum	FJ834844 (100%)
		Closest known cultured relative - *Bryantella formatexigens* DSM 14469	ACCL02000018 (93%)
15, 16	2	Uncultured bacterium clone YO00278D01, isolated from mouse caecum	FJ837861 (100%)
		Closest known cultured relative - *Bryantella formatexigens* DSM 14469	ACCL02000018 (92%)

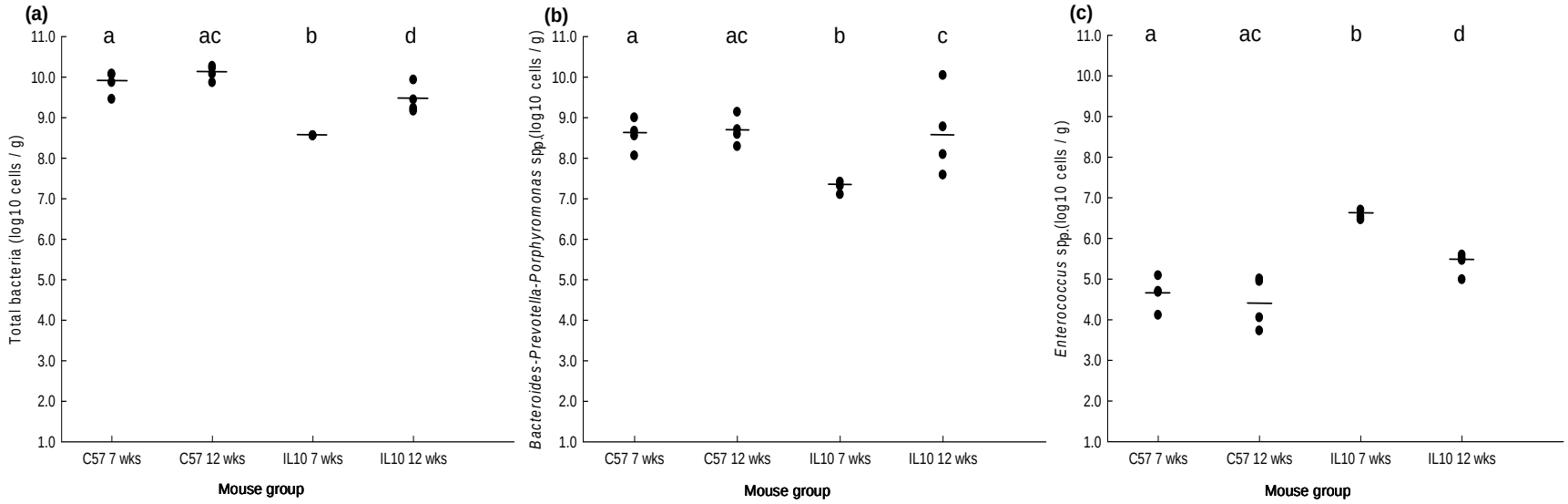


Fig. 7.3 Real-time PCR quantification of cells of (a) total bacteria, (b) *Bacteroides-Prevotella-Porphyromonas* spp. and (c) *Enterococcus* spp. in the caecum of *Il10*$^{-/-}$ and C57 mice at 7 and 12 weeks of age. The horizontal line represents the mean bacterial cell number of the individual mice; a-d: different letters mean that cell numbers of bacteria differ significantly between groups (P<0.05) when comparing between mouse genotypes at the same age or within mouse genotypes at different age.

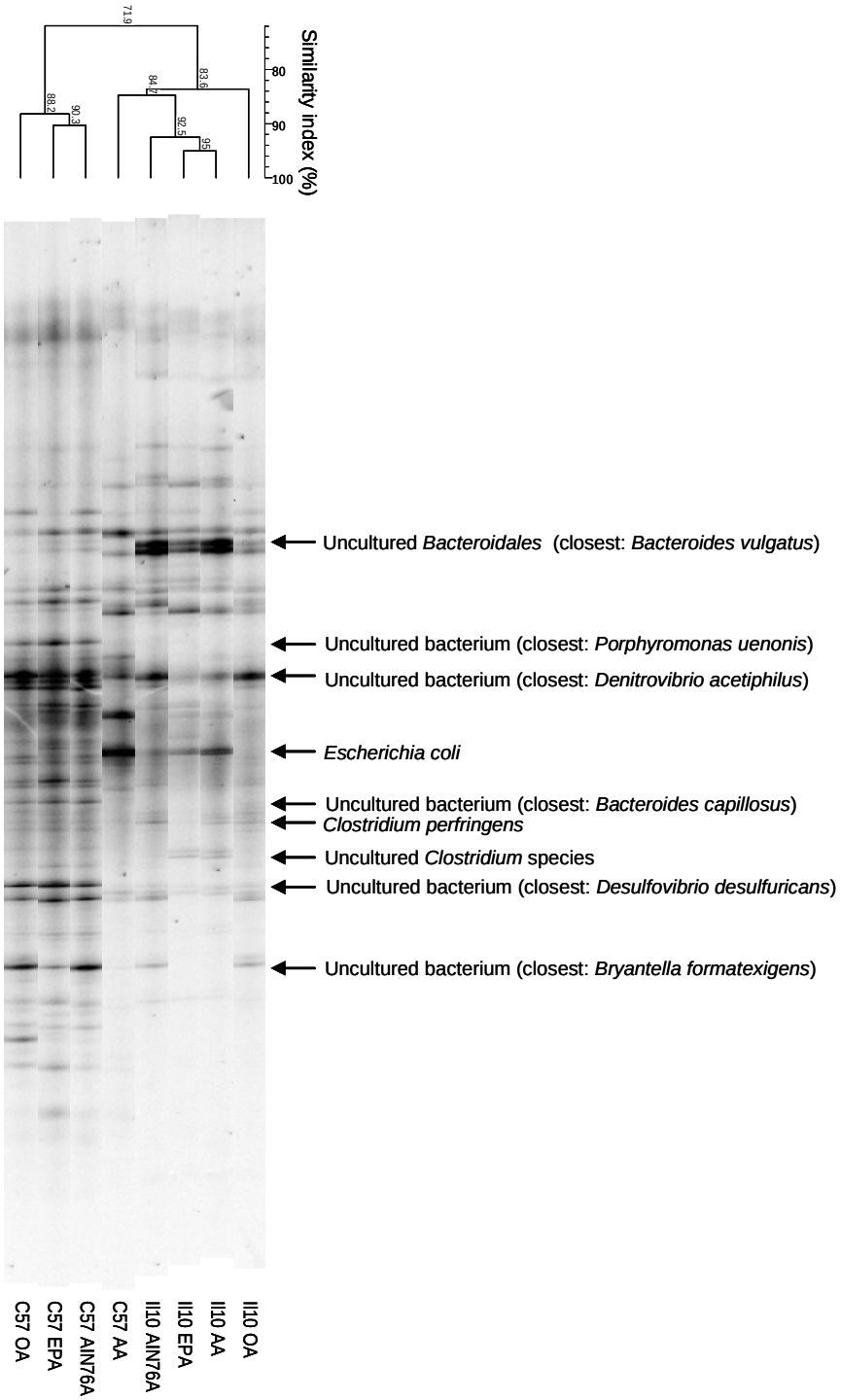


Fig. 7.4 Consensus DGGE profiles of caecal bacteria from *Il10*$^{-/-}$ and C57 mice fed EPA, AA, OA or AIN-76A diets. Similarity analysis was performed by using the Dice coefficient. Arrows indicate bacterial species (including the closest known cultured relative) identified by sequencing that were different in the profiles.

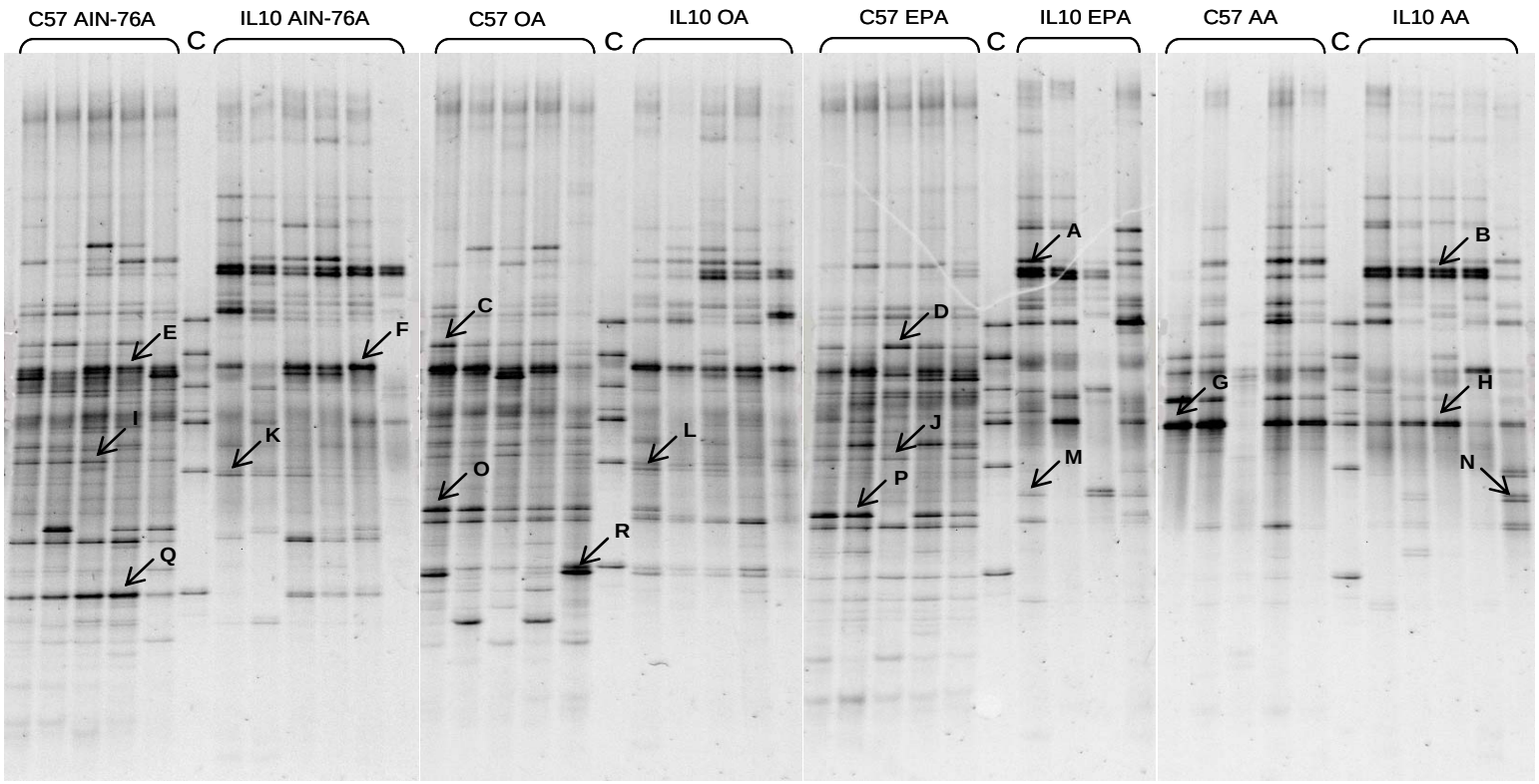


Fig. 7.5 DGGE profiles of caecal bacteria from individual *Il10*$^{-/-}$ and C57 mice fed PUFA diets. C, control sample, used to compare profiles between gels. Arrows indicate the positions in the gel where bands were excised, DNA recovered and sequenced. Letters match the sequencing results in Table 7.4.

Table 7.4 Species identified from excised bands in study 2

Band excised	No. clones sequenced per band	Species identified by sequencing	GeneBank ID (Identity)
A, B	3	Uncultured *Bacteroidales* bacterium clone Fhc31, isolated from human and animal faeces	EU913590 (99-100%)
		Closest known cultured relative - *Bacteroides vulgatus* ATCC 8482	NC_009614 (99-100%)
C, D	3	Uncultured bacterium clone 2.77F, isolated from mouse faeces	EU655985 (100%)
		Closest known cultured relative - *Porphyromonas uenonis* 60-3 deg1118640599623	NZ_ACLR01000152 (89%)
E, F	2	Uncultured bacterium clone YO00272E02, isolated from streptomycin-treated *Salmonella*-infected murine ileum	FJ838603 (100%)
		Closest known cultured relative - *Denitrovibrio acetiphilus* DSM 12809	NZ_ABTN01000006 (87%)
G, H	3	*Escherichia coli* strain CNM 477-02	EU555536 (100%)
I, J	3	Uncultured bacterium clone OF2A10, isolated from stool of healthy human	GQ285980 (100%)
		Closest known cultured relative - *Bacteroides capillosus* ATCC 29799	NZ_AAXG02000037 (96%)
K, L	2	*Clostridium perfringens* partial, isolate colony 3, isolated from canine faeces	FN356962 (100%)
M, N	2	Uncultured *Clostridium* sp. clone MS213A1_H11, isolated after gastrointestinal resection of IBD patient	EF710221 (98 and 100%)
		Closest known cultured relative - *Clostridium* species	ACDK01000101 (98 and 100%)
O, P	3	Uncultured bacterium clone mcbc120, isolated from caecum of TLR2-deficient mice	AM932646 (99-100%)
		Closest known cultured relative - *Desulfovibrio desulfuricans* subsp. desulfuricans str. ATCC 27774	NC_011883 (95-96%)
Q, R	3	Uncultured bacterium clone YO00360E03, isolated from streptomycin-treated *Salmonella*-infected murine ileum	FJ837456 (100%)
		Closest known cultured relative - *Bryantella formatexigens* DSM 14469	ACCL02000018 (93%)

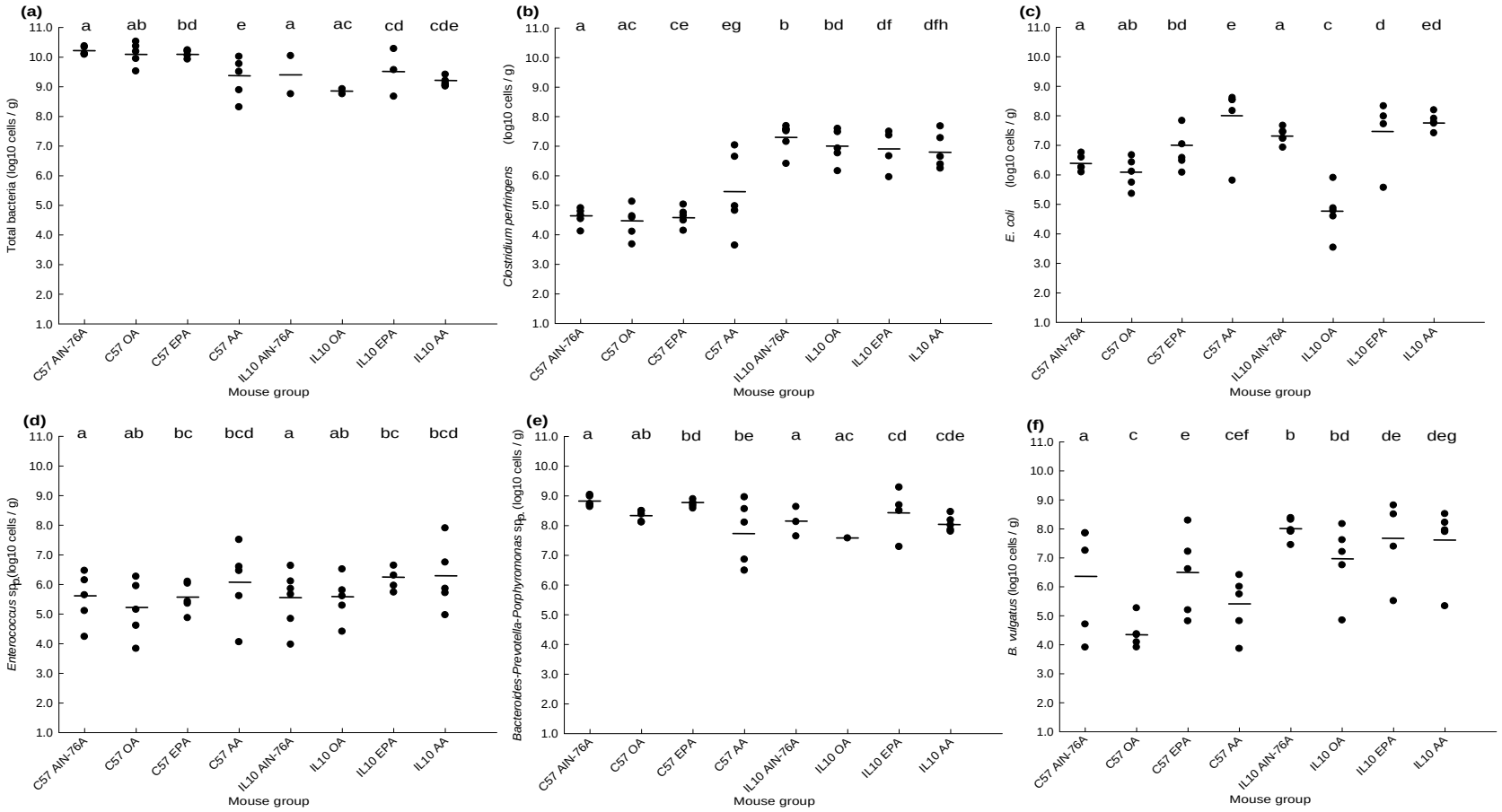


Fig. 7.6 Real-time PCR quantification of cells of (a) total bacteria, (b) *Clostridium perfringens*, (c) *E. coli*, (d) *Enterococcus* spp., (e) *Bacteroides-Prevotella-Porphyromonas* spp. and (f) *B. vulgatus* in the caecum of *Il10*$^{-/-}$ and C57 mice fed dietary PUFA (AA, EPA) or control (AIN-76A, OA) diets. The horizontal line represents the mean bacterial cell number of the individual mice; a-h: different letters mean that cell numbers of bacteria differ significantly between groups (P<0.05) when comparing between mouse genotypes on the same diet or within mouse genotypes on different diets. The AIN-76A diet has only been compared with the OA diet, and the PUFA diets with the OA fatty acid control.

7.5 Discussion

Bacterial changes and colitis

This study showed a reduced number and diversity of caecal bacteria in *Il10*$^{-/-}$ mice before colitis onset at 7 weeks of age. This is likely associated with the *Il10* gene knockout and lack of IL10 protein in *Il10*$^{-/-}$ mice for which changes in metabolism were observed in the transcriptome and proteome profiles (described in Chapter 3). A reduction in bacterial diversity has also been observed in the large intestine of colitic *Il10*$^{-/-}$ mice on the 129/SvEv background compared to their wildtype counterpart (Wohlgemuth *et al*., 2009). Changes in caecal bacteria community preceding the onset of intestinal inflammation have been reported in conventional *mdr1a*$^{-/-}$ mice (another IBD mouse model) carrying a disruption of the multi-drug resistance gene compared to wildtype mice (Nones *et al.*, 2009). An increase in the number and diversity of caecal bacteria in 12-week-old compared to 7-week-old *Il10*$^{-/-}$ mice was observed in parallel with colitis development in these mice possibly due to impaired epithelial barrier function. The differences observed in the large intestine bacterial community between the two mouse genotypes are in line with findings from others showing that the host genotype affects the composition of intestinal bacteria (Vaahtovuo *et al*., 2001, 2003; Zoetendal *et al*., 2001). Differences were also shown between intestinal bacteria of IBD patients and healthy subjects (Frank *et al.*, 2007).

Bacterial changes and dietary PUFA

This study showed for the first time that AA- and EPA-enriched diets changed the bacterial community in the caecum of colitic *Il10*$^{-/-}$ mice. The bacterial profiling represent complementary data to the colonic mucosal responses observed by those PUFA in colitis as discussed in Chapters 5 and 6 suggesting that these changes might be associated with prevention of colitis in *Il10*$^{-/-}$ mice. Effects of dietary lipids on gastrointestinal bacteria have only been reported by a few studies investigating faecal bacteria (Cummings *et al*., 1978; Endo *et al*., 1991; Eastwood *et al*., 1995; Farnworth *et al*., 2007). Cummings *et al*. (1978) showed that the faecal bacteria were unaltered by the dietary changes (low and high animal fat diet). The study by Endo *et al*. (1991) reported an increased incidence of faecal *C. perfringens* with high cholesterol diet compared to low cholesterol diets consumed by healthy subjects. They also suggested a change in activity of particular bacterial species rather than different bacteria genera in the

intestinal metabolism with these diets. Eastwood *et al.* (1995) showed that olestra, a calorie-free fat substitute which was used as substitute to replace conventional fat and increased intake of dietary fibre, did not change faecal bacteria counts and intestinal fermentation in healthy humans.

Farnworth *et al*. (2007) studied the effect of milk containing CLA on faecal bacteria in humans and found that the differences might be attributed to increased milk consumption, but not to the different CLA. Kajiura *et al*. (2009) investigated the effect of elemental diet on changes in caecal bacteria and its influence on therapeutic effects in colitis of *Il10*$^{-/-}$ transfer mice. Elemental diet used as a primary therapy for CD patients, contains only small quantity of fat (soybean oil 0.6 % by weight). They suggested that reduction in the number and diversity of intestinal bacteria and suppression of inflammatory responses via a change in composition of lactic acid bacteria by elemental diet seem to contribute to the reduction of colitis in their model. The present results indicate that changes in dietary fatty acid composition, i.e. AA- or EPA-enriched diets, can alter the number and diversity of bacteria in healthy and colitic mice, especially observed by the effect of dietary AA on *E. coli*. It was reported that dietary AA tended to decrease local (colon tissue) and systemic (plasma serum amyloid A) inflammation levels and reduced the expression levels of some genes associated with cellular stress, inflammatory and immune responses in *Il10*$^{-/-}$ mice when compared with OA-fed *Il10*$^{-/-}$ mice (Chapter 6).

Enterococci

In *Il10*$^{-/-}$ mice on the C57BL/6J background, a more consistent colitis was achieved using a mixture of *E. faecalis* and *E. faecium* isolates combined with commensal bacteria from healthy C57 mice (Roy *et al*., 2007). *E. faecalis* is the only known bacterial species in this inoculum to be associated with IBD development in *Il10*$^{-/-}$ mice (Balish & Warner, 2002). Whether the quantified enterococci in the present studies were the specific inoculation strains, was not investigated. Nevertheless, the decreased cell number of enterococci in the caecum of 12- week-old colitic *Il10*$^{-/-}$ mice suggested that other bacteria in the complex bacterial environment might have affected the presence of *Enterococcus* spp. by competing with or displacing them compared to *E. faecalis* monocolonization of *Il10*$^{-/-}$ mice. Also a recent study investigating global bacterial changes using DGGE and FISH in colitic *Il10*$^{-/-}$ mice (129/SvEv background) and the

respective wildtype mice conventionally housed, did not detect any enterococci in the colon or caecum of these mice (Wohlgemuth *et al.*, 2009). They showed that the inflammation in their *Il10*$^{-/-}$ mice was more severe in the caecum than in the colon. In the present study, the presence of caecal enterococci could not be correlated with caecal inflammation in conventional C57BL/6J *Il10*$^{-/-}$ mice as histopathology was not assessed in caecum. Dietary AA and EPA showed no effects on the number of enterococci in the caecum of *Il10*$^{-/-}$ and C57 mice.

E. coli

Interestingly, both dietary AA and EPA increased the cell number of *E. coli* in *Il10*$^{-/-}$ mice compared to OA-fed *Il10*$^{-/-}$ mice. The increase in *E. coli* in C57 mice by AA but not EPA, compared to the OA diet was associated with reduced number and diversity of total caecal bacteria in AA-fed C57 mice. The present study suggests that the *E. coli* isolated from caecal profiles of colitic *Il10*$^{-/-}$ and C57 mice fed with AA may thus not be involved in colitis development. In the study by Wohlgemuth *et al*. (2009), no correlation between increasing *E. coli* cell numbers in *Il10*$^{-/-}$ mice fed a standard diet and colitis severity could be established, and the authors suggested that *E. coli* may not be cause of the disease in conventional *Il10*$^{-/-}$ mice. Therefore, the putative differences in the presence of *E. coli* and actual differences in total *E. coli* cell number might be attributed to the PUFA diets in the caecum of *Il10*$^{-/-}$ and C57 mice. Further support for the differences in *E. coli* cell number been attributed to the PUFA diets instead of aggravating the colitis was that no increase in *E. coli* number was seen in germ-free *Il10*$^{-/-}$ mice colonised with faecal bacteria from healthy wildtype mice (Bibiloni *et al.*, 2005). This indicates that *E. coli* may not be necessary for the onset of colitis in this host.

Some *E. coli* strains are non-pathogenic (e.g. *E. coli* strain EM0 isolated from a healthy humans) and may exert a protective effect against other *Enterobacteriaceae* by producing anti-bacterial substances (Hudault *et al.*, 2001). Conversely, accumulation of commensal *E. coli* as a result of epithelial barrier damage in the colon, likely by impaired mucosal physiology, has been reported in murine DSS-induced colitis (Heimesaat *et al.*, 2007) and in IBD patients (Swidsinski *et al.*, 2005). Population shifts towards towards Gram-negative bacteria such as *E. coli* have the potential to trigger

TLR-dependent accumulation of neutrophils and T cells leading to acute intestinal inflammation (Heimesaat *et al.*, 2007).

Helicobacter

Qualitative analysis showed the presence of *Helicobacter* spp. in 7- and 12-week-old *Il10*$^{-/-}$ and C57 mice and in those fed PUFA-enriched diets. The 16S rRNA gene of *H. pullorum* was identified in caecal profiles of 7- and 12-week-old immunocompetent C57 mice with no signs of colitis. As such, the present result suggests that this species could belong to the resident murine microflora in this host and its environment and does not suggest a correlation between the extent of colitis and the presence of *H. pullorum*. *H. pullorum*, a bacterium of poultry origin (Laharie *et al.*, 2009), was found in patients with gastroenteritis (Burnens *et al.*, 1994), but also in stools of healthy subjects (Ceelen *et al.*, 2006). There is *in vitro* evidence of direct pro-inflammatory properties of *H. pullorum* strains from human and poultry origin on human intestinal epithelial cells, suggesting a role in IBD pathology (Varon *et al.*, 2009). So whether *H. pullorum* or other *Helicobacter* spp. play a role in inducing colitis in the conventional *Il10*$^{-/-}$mice or not requires further investigation as certain hosts may respond differently to particular *Helicobacter* spp.

Other bacteria in the caecum of conventional Il10$^{-/-}$ mice

There was primarily a mouse genotype effect observed for *C. perfringens* and *B. vulgatus* with an increase in caecal cell numbers in *Il10*$^{-/-}$ compared to C57 mice. An involvement of bacteroides and clostridia, which include species considered as undesirable members of the intestinal microflora (McBain & Macfarlane, 1998), in the development of colitis in *Il10*$^{-/-}$ mice could thus not be excluded. As there were quantitative differences in *Bacteroides-Prevotella-Porphyromonas* spp., not only between *Il10*$^{-/-}$ and C57 mice before, but also an increase in *Il10*$^{-/-}$ mice after colitis onset, it seems that some *Bacteroides* spp. may be associated with progression of colitis. *Bacteroides-Prevotella-Porphyromonas* spp. have been detected in experimental colitis models (Rath *et al.*, 1999) and in IBD patients (Swidsinski *et al.*, 2005). Although *B. vulgatus* is known to be associated with colitis in IBD rodent models (Rath *et al.*, 1999; Kishi *et al.*, 2000), monocolonisation of germ-free *Il10*$^{-/-}$ mice with *B. vulgatus* did not lead to colitis in this host (Kim *et al.*, 2007). Likewise, colonisation of germ-free *Il10*$^{-/-}$ mice with *B. vulgatus* and commensal bacteria such as *E. faecium*, *E. coli*,

Peptostreptococcus productus, *Eubacterium contortium*, and *E. avium* did not induce colitis (Sellon *et al*., 1998). This indicates that differences in colitis induction may be due to the composition of the commensal bacteria community in a genetically susceptible host.

Caecal bacteria changes associated with gene expression changes in colitis and in response to PUFA

Differences in the caecal bacteria community of 7-week-old *Il10*$^{-/-}$ and C57 mice might be linked to increased expression levels of extracellular (e.g. complement components, *C3*) and membrane bound (e.g. TLR9) pattern recognition receptor genes in *Il10*$^{-/-}$ mice, but not to changes in pro-inflammatory cytokine gene expression levels because colitis was not developed. At 12 weeks of age, the mRNA levels of pro-inflammatory cytokine genes (e.g. *Tnfa*, *Il1b* and chemokine ligand5, *Ccl5* or *Rantes*; Chapter 3) were increased likely as a consequence of increased expression of bacterial recognition receptor genes (*Tlr2*, *Tlr9*) in colitic *Il10*$^{-/-}$ mice. TLR2, the main receptor for Gram-positive bacteria such as enterococci and clostridia, bind to the specific bacterial components to induce signalling processes that activate NFκB and MAPK pathways which in turn lead to pro-inflammatory cytokine production and recruitment of neutrophils and regulatory T cells to sites of inflammation (Heimesaat *et al*., 2007).

The marked increase of *E. coli* cell numbers in *Il10*$^{-/-}$ mice in response to dietary AA may be associated with the decreased expression levels of pro-inflammatory cytokine genes *Tnfa* and *Il1b* compared to colitic *Il10*$^{-/-}$ mice fed the OA diet (Chapter 6). This potential link could not be made for EPA-fed *Il10*$^{-/-}$ mice. For dietary AA, it may imply a protective effect on colitis in this host by the presence of *E. coli*. The study by Hudault *et al.* (2001) reported that non-virulent (EM0, a human faecal strain) and virulent *E. coli* strains (JM105 K-12) protected germ-free C3H/He/Oujco mice against *Salmonella* infection. This effect of the *E. coli* strains was based on the formation of a biofilm of bacteria protecting the intestinal epithelium against attachment and thus invasion by the pathogen. Investigations on adhesion properties of probiotics using *in vitro* adhesion models showed that low concentrations of free AA in growth medium can promote growth and mucus adhesion of *L. casei* Shirota (Kankaanpaa *et al*., 2001). Dietary AA at the concentration used in the present study may be able to alter the bacterial adhesion sites by changing the fatty acid composition of intestinal epithelial cell membranes in

favour of adhesion by *E. coli* which then can produce a biofilm. This may reduce the adherence to intestinal mucus by certain commensal bacteria that can trigger colitis in conventional *Il10*$^{-/-}$ mice, and as such may aid in the colonisation of probiotics. Kankaanpaa *et al*. (2001) suggested a beneficial effect of PUFA on the presence of probiotics and the importance of these interactions in designing novel functional foods.

In conclusion, this study suggests that the differences in caecal bacteria composition between the mouse strains could be associated with the *Il10*$^{-/-}$ genotype and with subsequent colitis development. Further investigation should determine specific *E. coli*-associated activities that might be involved in the PUFA-mediated effects on caecal bacteria community in *Il10*$^{-/-}$ mice and further define their anti-inflammatory mode of action of these PUFA. Associations of particular bacterial species with colitis could not be made due to the complexity and heterogeneity of bacteria present in the large intestine. Those associations may only be deduced from colonisation of germ-free mice with an individual bacterium or a defined mix of commensal bacteria.

Chapter 8

Putative PPARα target gene regulation in the murine colon by unsaturated fatty acids

Part of the material presented in this chapter will be submitted as a review to PPAR Res

8.1 Abstract

The peroxisome proliferator-activated receptor alpha (PPARα) regulates the expression of numerous genes involved in lipid metabolism and inflammation in liver. Much less is known about PPARα and target gene regulation in the colon where it is specifically expressed in immune and epithelial cells lining the lumen. Interestingly, PPARα arose as a potential key mediator of the response to dietary EPA in genome-wide analysis in the colon of *Il10*$^{-/-}$ mice. Accordingly, the aim of the present study was to identify new PPARα target genes in response to dietary unsaturated fatty acids in the colon and to better understand the regulatory role of PPARα during colon inflammation. Nine genes (*Adra2a*, *Ca2*, *Cbr1*, *Cbr3*, *Ces1*, *Chga*, *Chgb*, *Sgk1* and *Vnn1*) for which a direct link with PPARα has not yet been established were tested in colon samples of PPARα$^{-/-}$ and wildtype mice fed OA, EPA or DHA or synthetic PPARα ligand WY14643. These genes were selected from the list of colonic genes increased in expression in *Il10*$^{-/-}$ mice fed the EPA relative to those fed the OA diet. qRT-PCR data indicated that WY144643 and DHA increased the expression level of vanin1 (*Vnn1*) gene in the colon of PPARα wildtype but not PPARα$^{-/-}$ mice. Vanin1 is a pantetheinase that can act as a sensor for oxidative stress through cysteamine. A direct regulation of the *Vnn1* promoter by PPARα was shown by transactivation assays. These findings suggest that *Vnn1* along with PPARα is involved in lipid metabolism. Further work is required to investigate a possible involvement of PPARβ/δ in the physiological regulation of *Vnn1* in order to distinguish the role between PPARs in the colon and under inflammatory conditions.

8.2 Introduction

PPARs are transcription factors of the nuclear hormone receptor superfamily that regulate the transcription of target genes in response to natural and synthetic ligands. The PPARα isoform was found to be expressed at high levels in tissues and organs with high fatty acid catabolism such as liver, brown adipose tissue, heart, intestine and kidney as reviewed by Mandard *et al.*, (2004). Human PPARα showed an inhibitory role in colorectal cancer (Jackson *et al*., 2003) and has a lower baseline expression in non-malignant colonic mucosa (Auboeuf *et al*., 1997). PPARα was shown to be involved in inflammation (Devchand *et al*., 1996) and exerts its effects in inflammation mainly by decreased expression levels of genes such as those involved in the acute phase response, but also by increased expression levels of PPARα target genes, e.g.

interleukin-1 receptor antagonist in liver (Stienstra *et al*., 2007). PPARα target genes in rodents and humans are mainly involved in pathways of lipid metabolism, e.g. peroxisomal and mitochondrial fatty acid β-oxidation, cellular fatty acid uptake and intracellular fatty acid transport, ketogenesis and gluconeogenesis (Mandard *et al*., 2004).

Native and oxidised PUFA and AA-derived inflammatory eicosanoids such as LTB_4, hydroxyeicosatetraenoic acid, PGD_1 and PGD_2 are natural agonists with varying affinity for all PPAR isoforms (Mandard *et al*., 2004; Desvergne *et al*., 2004; Bordoni *et al*., 2006; Salter & Tarling, 2007). n-3 PUFA such as EPA were shown to be a more potent inducer of PPARα in primary hepatocytes than n-6 PUFA AA (Sampath & Ntambi, 2007). Increased expression levels of the *Pparα* gene and of some of its target genes in the colon was shown in EPA-fed *Il10*$^{-/-}$ mice relative to *Il10*$^{-/-}$ mice fed the OA diet (Chapter 5; Knoch *et al*., 2009). These findings suggested that inflammatory conditions and dietary EPA enrichment initiated fatty acid oxidation and that oxidised EPA-activated PPARα might inhibit NFκB activity and down-stream pro-inflammatory pathways. Recent studies indicated that in contrast to plasma free fatty acids elevated upon fasting, dietary fatty acids and *de novo* synthesised fatty acids are able to activate hepatic PPARα (Chakravarthy *et al*., 2005; Sanderson *et al*., 2009).

As PPARα has arisen as a key gene from the genome-wide expression analysis in the colon of *Il10*$^{-/-}$ mice in response to dietary EPA, it was hypothesised that there were new PPARα-target genes among the up-regulated colonic genes in EPA-fed *Il10*$^{-/-}$ mice. The aim of the present study was to identify new PPARα target genes related to lipid metabolism in the colon and to better understand the regulatory role of PPARα during colon inflammation. To achieve that, PPARα-dependent gene expression levels were studied in the colon of normal (study 1) and dinitrobenzene sulfonic acid (DNBS)-treated PPARα$^{-/-}$ and wildtype mice (study 2) treated with PPARα ligands. The following genes were hypothesised to be PPARα-dependent and were selected from the list of colonic genes increased in expression in EPA-fed *Il10*$^{-/-}$ mice relative to those fed the OA diet (microarray analysis PUFA intervention study): adrenergic receptor alpha 2a (*Adra2a*), carbonic anhydrase 2 (*Ca2*), carbonyl reductase1 (*Cbr1*), *Cbr3*, carboxylesterase1 (*Ces1*), chromograninA (*Chga*), *Chgb*, serum/glucocorticoid regulated kinase1 (*Sgk1*) and vanin1 (*Vnn1*). PPARα-dependency was investigated in

the colon of PPARα$^{-/-}$ and wildtype mice using qRT-PCR and the putative PPARα target gene validated with a cell-based transactivation assay.

8.3 Materials and methods

8.3.1 Animal experiments

Study 1. Colon samples obtained from PPARα$^{-/-}$ and wildtype mice (Sanderson *et al.*, 2008) were used to investigate PPARα-dependent gene expression. The study of Sanderson *et al.* (2008) looked at the effects of unsaturated fatty acids on hepatic gene expression. The experiment had been approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University. Four month old male PPARα$^{-/-}$ (129S4/SvJae) and wildtype (129S1/SvImJ) mice (n=4 to 5 per group) were used. The mice were started on a background diet, which was a modified AIN-76A diet (Research Diet Services, Wijk bij Duurstede, The Netherlands) for 2 weeks. The AIN-76A diet contained 5% fat in the form of corn oil (w/w) (Bieri, 1980). The corn oil was replaced by the same amount of olive oil (predominantly oleic acid) because Ren *et al*. (1997) showed that an olive oil-rich diet did not regulate PPARα target genes. The mice were fasted for 4 hours before being dosed by oral gavage with 400 µL of different synthetic TG; triolein, trieicosapentaenoin or tridocosahexaenoin, or the fatty acid ethyl ester (EE) of OA, EPA or DHA or 400 µL of a 0.1% WY14643 (synthetic PPARα agonist) suspension in 0.5% carboxymethyl cellulose (CMC, Sigma-Aldrich, Zwijndrecht, The Netherlands), the latter CMC vehicle served as a control treatment. The synthetic TG contained 3 identical acyl moieties, namely OA, EPA or DHA. After 6 h mice were anaesthetised and killed by cervical dislocation. The 6-h time point was chosen because of an initial fat load experiment which indicated that at that point in time the fat bolus had been taken up by liver, heart, muscle and white adipose tissue (Sanderson *et al*., 2008). Full-thickness colon sections were snap-frozen in liquid nitrogen and used for RNA isolation.

Note: *Colon samples were obtained from a previous experiment carried out by the Nutrition, Metabolism and Genomics Group at Wageningen University in The Netherlands.*

Study 2. In order to investigate the expression of the putative PPARα target gene identified in study 1 under inflammatory conditions, colitis was induced in PPARα$^{-/-}$ and wildtype mice and the colon samples used. This study was first described by

Cuzzocrea *et al.* (2004) who generously repeated their experiment with fewer animals. The mouse experiment was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes. PPARα$^{-/-}$ and wildtype mice (n=5 per group), 4-5 week old, were used and fed with a standard rodent chow diet. Colitis was induced by intrarectal injection of DNBS (4 mg in 100 µL of 50% ethanol). The following experimental groups were studied: 1) PPARα$^{-/-}$ DNBS vehicle (saline); 2) PPARα wildtype DNBS vehicle; 3) PPARα$^{-/-}$ DNBS WY14643 (1 mg/kg dissolved in 10% ethanol given daily by intraperitoneal injection 30 min after DNBS induction) and 4) PPARα wildtype DNBS WY14643. On day 4, mice were anaesthetised and the colons sampled for histology (fixed for 1 week in Dietrich solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) at room temperature) and for RNA isolation (snap-frozen in liquid nitrogen and stored and sent in dry-ice to Wageningen University).

Note: *Colon samples were obtained from the Department of Clinical and Experimental Medicine and Pharmacology at the University of Messina in Italy.*

8.3.2 RNA isolation and qRT-PCR

Total RNA was isolated from colon samples collected in studies 1 and 2 using TRIzol reagent (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions and as described in Materials and Methods chapter 2.4.2. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Isogen, Maarssen, The Netherlands). Total RNA (1 µg) was reverse transcribed to single-stranded cDNA using iScript kit (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturer's instructions. cDNA was amplified on a MyIQ thermal cycler (Bio-Rad laboratories BV, Veenendaal, The Netherlands) using Platinum Taq DNA polymerase (Invitrogen, Breda, The Netherlands) and SYBR green. All PCR primer sequences were chosen from PrimerBank (Table 8.1) and ordered from Eurogentec (Seraing, Belgium). The following thermal cycling conditions were used: 8 min at 94°C, followed by 45 cycles of 94°C for 15s and 60°C for 1 min. Standards and samples were performed in duplicate and normalised to cyclophilin (study 1) or to 18S (study 2) expression.

Note: *qRT-PCR on the colon samples from study 2 was carried out by Shohreh Keshtkar at Wageningen University.*

8.3.3 Histology

The colon tissues of the PPARα$^{-/-}$ and wildtype mice were fixed for 1 week in Dietrich solution at the University of Messina and sent in that solution to Wageningen University. The next steps in this tissue processing were dehydration and clearing under vigorous stirring. The water was removed from the wet fixed colon tissues by dehydration with a series of ethanols (70% to 90% to 100%, Merck, Prolabo BDH). The next step involved the removal or clearing of dehydrant with xylene (Merck) being mixable with the embedding medium (paraffin). Last, the colon tissues were infiltrated with paraffin. The tissues were removed from each cassette, placed into a metal tray and embedded with molten paraffin. Thereafter, 5 μm-thin sections were cut from the paraffin blocks using a microtome (sharp knife with mechanism to advance the block to the knife). The cut sections were floated on a warm water bath to remove any wrinkles and placed on a glass microscopic slide. The slides were dried in an oven overnight to help the tissue adherence to the slide.

For the staining with haematoxylin and eosin, the colon sections were deparaffinised with xylene, ethanol and water and then placed into the stain until the desired tint was achieved. The stained sections were placed again in ethanol, xylene, then rinsed with water, enclosed with a mounting medium (Depax) and covered with a thin plastic to preserve the tissue and for viewing under the microscope.

The researcher blinded to the experimental protocol scored 15 features in total, 10 features pertaining to the tissue and characteristics of the tissue (crypt hyperplasia, injury, loss and abscess, aberrant crypts, goblet cell loss, lymphoid aggregates, sub-mucosal thickening, hyperchromatic staining and surface loss) and five features for the inflammatory cells (monocytes and macrophages, and plasma cells and lymphocytes are grouped together). Each of the 15 aspects was scored from 0 to 10 for a total maximum score of 150. This scoring system has more features than the traditional HIS and has considered inflammatory and architectural features. The detection of inflammatory cells are just as important as any changes to the tissue landscape and/or architecture, so the values for these components have been multiplied by 2 in order to give them the same

numerical weighting as the tissue features. The total score for tissue characteristics and inflammatory cell features has been combined to give the percentage inflammation in the overall slide.

Note: *Tissue processing was carried out by the PhD candidate, cutting of the paraffin block and staining by Shohreh Keshtkar at Wageningen University and histopathological assessment by Kelly Armstrong at AgResearch.*

8.3.4 Plasmids and DNA constructs

Plasmids and DNA construction for the transactivation assay and the *in silico* analysis are described in Materials and Methods chapter 2.9.1 and 2.9.2. *In silico* analysis of the mouse *Vnn1* gene using a published algorithm (Heinaniemi *et al*., 2007) identified conserved PPRE for PPARα at 4175 and 4182 bp down-stream of the TSS. Mouse genomic DNA (C57BL6/J strain) was used to PCR-amplify 191 bp of the mouse *Vnn1* sequence containing the 2 overlapping putative PPRE and subcloned into the KpnI and BglII sites of the pGL3-promoter vector. Two site-directed mutations of the PPRE were introduced into the mouse *Vnn1* sequence using two separate partially overlapping PCR fragments obtained using the mVnn1 fragment as a template. The fragments were sequenced to confirm the integrity of the PPRE in the construct. The mPPARα expression plasmid, the (PPRE)3-LUC reporter plasmid containing three copies of acyl-CoA oxidase PPRE and the β-galactosidase reporter plasmid were kindly provided by Wageningen University.

Note: *The in silico analysis for PPRE locations and PPAR binding strength prediction was carried out by Dr Linda Sanderson at Wageningen University.*

8.3.5 Cell culture and transfections

Cell culture and transfections are described in Materials and Methods chapter 2.9.3. Reporter plasmids ((PPRE)3-LUC as positive control or PPRE-Vnn1-LUC or PPRE-Vnn1mut-LUC) were transfected into human hepatoma HepG2 cells together with the PPARα expression plasmid and co-transfected with a β-galactosidase reporter plasmid to normalise for differences in transfection efficiency, in the presence or absence of PPARα ligand WY14643 (50 μM). The Promega luciferase assay and standard β-

galactosidase assay with 2-nitrophenyl-BD galactopyranoside were used to measure the relative activity of the promoter. Experiments were performed in triplicate.

8.3.6 Statistical analysis

ANOVA, followed by the least significant difference *post hoc* test in GenStat (11th edition, VSN International Ltd, Herforshire, UK) was used for statistical analyses and differences were considered significant when $P<0.05$. In the PPARα$^{-/-}$ DNBS groups, two mice on the WY14643 and three mice on the vehicle treatment died because of the DNBS administration and subsequent colitis development. These mice were not included in the analyses.

8.4 Results

8.4.1 PPARα-dependent gene regulation by dietary unsaturated fatty acids or synthetic agonist in the colon of PPARα$^{-/-}$ and wildtype mice

Previously, *Ppara* was identified as a potential key mediator of the effects of dietary EPA on colonic gene expression profiles in the *Il10*$^{-/-}$ colitis mouse model (Knoch *et al*., 2009; Chapter 5). *Adra2a*, *Ca2*, *Cbr1*, *Cbr3*, *Ces1*, *Chga*, *Chgb*, *Sgk1* and *Vnn1* gene expression levels were significantly increased (FC≥1.3, $P<0.01$) by EPA compared to OA in the colon of *Il10*$^{-/-}$ mice, suggesting they might be PPARα-regulated genes. The expression levels of the selected genes in the liver and small intestine of WY14643-treated PPARα wildtype mice and in the colon of EPA-fed *Il10*$^{-/-}$ mice are shown in Table 8.2. To study direct regulation of gene expression by fatty acids and PPARα, wildtype and PPARα$^{-/-}$ mice were given a single oral dose of synthetic TG consisting of one single fatty acid ranging from OA to DHA, and colons were harvested 6 h thereafter. TG composed of long chain saturated fatty acids could not be included as they are solid at room temperature. Additionally, wildtype and PPARα$^{-/-}$ mice were given a single oral dose of the ethyl esters of the fatty acids OA, EPA and DHA, as well as the synthetic PPARα agonist WY14643. Throughout the various treatments, *Vnn1* expression was significantly lower ($P<0.05$) in the colon of PPARα$^{-/-}$ mice compared to the wildtype (Fig. 8.1). There was no significant difference between agonist-treated wildtype and PPARα$^{-/-}$ mice for the other selected genes, the qRT-PCR result of *Ca2* shown in Fig. 8.1 is representative of those genes. All fatty acids except OA significantly induced *Vnn1* expression, which was partially blunted in PPARα$^{-/-}$ mice.

For EPA and DHA, ethyl esters and TG were about equally effective at inducing *Vnn1* expression. WY14643 was the most potent inducer of *Vnn1* expression, causing an increase of about 4-fold. These results indicate that *Vnn1* is a PPARα-regulated gene in mouse colon and indicate that the effect of dietary fatty acids on colonic *Vnn1* expression is at least partially mediated by PPARα.

8.4.2 *In silico* analysis of PPARα-responsive genomic regions of mouse *Vnn1*

To establish whether *Vnn1* might represent a direct PPARα target gene, the mouse *Vnn1* gene was screened for the presence of putative PPRE (Heinäniemi *et al.*, 2007). Several strong and medium PPRE for PPARα were identified within 50 kb up- and down-stream of the TSS of *Vnn1* (Fig. 8.2). Two strong adjacent and partially overlapping PPRE located around 4 kb down-stream of the TSS were chosen because of their proximity to the TSS and conservation between species.

8.4.3 PPARα-mediated effect on mouse *Vnn1* expression *in vitro*

To determine whether the two PPRE identified are functional, a 200 bp genomic region of the mouse *Vnn1* gene that includes both PPRE was amplified by PCR and subcloned in front of the *thymidine kinase* promoter driving the *luciferase* reporter gene. The activity of the construct in the absence or presence of a PPARα expression vector in response to the PPARα-specific agonist WY14643 was tested by promoter reporter gene assay in HepG2 cells (Fig. 8.3C). Use of the (PPRE)3-LUC reporter construct showed effective transactivation by co-transfection of PPARα and further enhancement by WY14643. Similarly, PPARα transfection and WY14643 each increased activity of the mouse *Vnn1* promotor reporter. However, no synergistic effect of PPARα and WY14643 on the reporter activity was observed.

To investigate whether the two overlapping PPRE may be responsible for the PPARα-mediated transactivation of the mouse *Vnn1* gene, the response element was disabled by two site-directed mutations (Fig. 8.3B). After mutating the PPRE, the response to PPARα and WY14346 was abolished, suggesting that the PPRE identified mediate the effect of PPARα on *Vnn1* expression (Fig. 8.3A).

8.4.4 PPARα-dependent effect on mouse *Vnn1* gene regulation during colon inflammation

As DNBS treatment was lethal for a number of PPARα$^{-/-}$ mice, the number of mice left for histopathological and gene expression analysis was limited. To investigate whether PPARα is involved in the regulation of mouse *Vnn1* gene during colon inflammation, the expression levels of *Vnn1* gene and pro-inflammatory cytokine (*Il1b, Il6*, *Cox2* and *Tnfa*) genes was determined in colitic PPARα$^{-/-}$ mice and wildtype mice (Fig. 8.4). After four days of DNBS-induced colitis, *Vnn1* gene expression level remained unchanged when comparing between or within genotype. Increased mRNA abundance of *Il1b, Il6* and *Cox2* gene in the colon of vehicle-treated PPARα$^{-/-}$ mice relative to vehicle-treated wildtype mice indicated a state of colon inflammation in absence of PPARα (P<0.05). The expression levels of the *Il1b, Il6*, *Cox2 and Tnfa* genes were diminished in WY14643-treated PPARα$^{-/-}$ mice relative to vehicle-treated PPARα$^{-/-}$ mice (P<0.05) showing that WY14643 can decrease pro-inflammatory gene expression and potentially improve DNBS-induced colitis in the absence of PPARα.

The histopathological assessment of the DNBS-treated colon tissues of PPARα$^{-/-}$ and wildtype mice did not confirm their pro-inflammatory gene expression profile. This seems to be due to the low number of PPARα$^{-/-}$ mice in the vehicle and WY14643 groups (Fig. 8.5 and 8.6). All mice showed severe colon inflammation. The PPARα$^{-/-}$ vehicle group had a lower colon HIS score than the WY14643-treated PPARα$^{-/-}$ mice (P<0.05) which is in contrast to the increased expression levels of pro-inflammatory cytokine genes observed in the vehicle-treated compared to WY14643-treated PPARα$^{-/-}$ mice. The results obtained before by Cuzzocrea *et al.* (2004) indicated decreased colon injury after WY14643 treatment of the PPARα wildtype mice.

Table 8.1 Primers used for qRT-PCR

Gene symbol	GenBank accession [PrimerBank ID]	Forward primer	Reverse primer
Adra2a	NM_007417.4 [6680662a2]	5'-CGAGGTTATGGGTTACTGGTACT-3'	5'-CGGTGACAATGATGGCCTTGA-3'
Ca2	NM_009801.4 [31981657a1]	5'-TCCCACCACTGGGGATACAG-3'	5'-TGGACGCAGCTTTATCATA-3'
Cbr1	NM_007620.2 [6671690a1]	5'-TCAATGACGACACCCCCTTC-3'	5'-CCTCTGTGATGGTCTCGCTTC-3'
Cbr3	NM_173047.2 [27413160a2]	5'-GGGCATCGCCTTTAGAATGGA-3'	5'-GCAGTAACTCAGTGCAGACAT-3'
Ces1	NM_021456.3 [10946842a2]	5'-CCTCCTTTGTGCTACCAAAACC-3'	5'-ATGTATCCACACCATCACGGG-3'
Chga	NM_007693.1 [6680932a2, 6680932a3]	5'-AGCCACCAATACCCAATCACC-3'	5'-CCATCATCTTTCTGGATCGCTTT-3'
Chgb	NM_007694.4 [6680934a1, 6680934a2]	5'-GCTCAGCTCCAGTGGATAACA-3'	5'-ACTCAGGGGTGATCGTTGGAA-3'
Cox2	NM_011198	5'-TGAGCAACTATTCCAAACCAGC-3'	5'-GCACGTAGTCTTCGATCACTATC-3'
Il1b	NM_008361	5'-TGGTGTGTGACGTTCCCATT-3'	5'-CAGCACGAGGCTTTTTTGTTG-3'
Il6	NM_031168	5'-CTTCCATCCAGTTGCCTTCTTG-3'	5'-AATTAAGCCTCCGACTTGTGAAG-3'
Sgk1	NM_011361.1 [6755490a1, 6755490a3]	5'-TGAAAATGTCCCATCCTCAGGA-3'	5'-TCCGCTCTGACATAATATGCTTC-3'
Tnfα	NM_013693	5'-CAACCTCCTCTCTGCCGTCAA-3'	5'-TGACTCCAAAGTAGACCTGCCC-3'
Vnn1	NM_011704.2 [6755979a2, 6755979a3]	5'-CTGGATACCCTGTGATAACCCT-3'	5'-GTCTCCCATGTTCGCCACAA-3'
Reference genes			
U18S		5'-CGGCTACCACATCCAAGGA-3'	5'-CCAATTACAGGGCCTCGAA-3'
Cyclophilin	NM_008907	5'-CAGACGCCACTGTCGCTTT-3'	5'-TGTCTTTGGAACTTTGTCTGCAA-3'

Primer sequences were selected from PrimerBank. *Adra2a* - adrenergic receptor2 alpha, *Ca2* - carbonic anhydrase2, *Cbr1* - carbonyl reductase1, *Cbr3*, *Ces1* - carboxylesterase1, *Chga* – chromograninA, *Chgb*, *Cox2* - cyclooxygenase2, *Il1b* - interleukin1 beta, *Il6*, *Sgk1* - serum/glucocorticoid regulated kinase1, *Tnfα* - tumour necrosis factor alpha, *Vnn1*- vanin1 and U18S – universal 18S. Primers for mouse *Cox2*, *Il1b*, *Il6*, *Tnfα*, U18S and Cyclophilin genes were provided by Wageningen University.

Table 8.2 Selected genes and their level of expression in liver and small intestine of WY14643-treated PPARα wildtype mice and in colon of EPA-fed *Il10*$^{-/-}$ mice

Gene symbol	**GeneBank accession**	**Small intestine 6h WY14643 PPARα wildtype WY *vs.* C FC (*P*-value)**	**Liver 6h WY14643 PPARα wildtype WY *vs.* C FC (*P*-value)**	**Colon 6wks EPA *Il10*$^{-/-}$ EPA *vs.* OA FC (*P*-value)**
Adra2a	NM_007417	1.2 (0.02)	1.0 (0.98)	2.0 (0.001)
Ca2	NM_009801	-1.1 (0.71)	1.7 (<0.00001)	2.2 (0.001)
Cbr1	NM_007620	1.2 (0.15)	1.2 (0.65)	2.0 (<0.00001)
Cbr3	NM_173047	-1.0 (0.82)	-1.1 (0.48)	3.3 (<0.00001)
Ces1	NM_021456	1.1 (0.84)	-1.0 (1.00)	3.0 (0.001)
Chga	NM_007693	-1.1 (0.29)	1.0 (0.86)	1.6 (0.001)
Chgb	NM_007693	-1.1 (0.40)	-1.0 (0.77)	1.9 (0.001)
Sgk1	NM_011361	1.2 (0.48)	-1.1 (0.61)	3.0 (<0.00001)
Vnn1	NM_011704	2.8 (0.01)	10.5 (0.20)	1.9 (0.008)

The fold change (FC) and *P*-values are shown for WY14643-treated PPARα wildtype mice relative to wildtype mice on CMC control (C) diet and EPA-fed *Il10*$^{-/-}$ mice relative to *Il10*$^{-/-}$ mice fed the OA control diet. *Adra2a* - adrenergic receptor2 alpha, *Ca2* - carbonic anhydrase2, *Cbr1* - carbonyl reductase1, *Cbr3* - carbonyl reductase3, *Ces1* - carboxylesterase1, *Chga* - chromograninA, *Chgb* - chromograninB, *Sgk1* - serum/glucocorticoid regulated kinase1, *Vnn1* - vanin1. The gene data for the PPARα wildtype mice were derived from previous microarray experiments from the Nutrition, Metabolism & Genomics Group at Wageningen University.

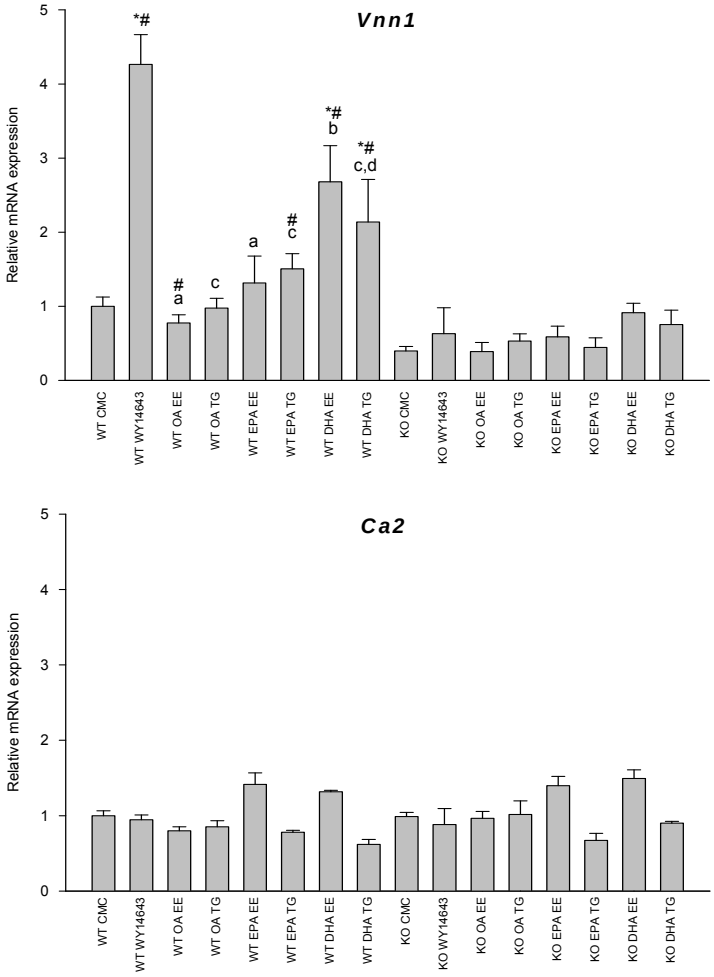


Fig. 8.1 Agonist-induced PPARα-dependent gene regulation in the colon of PPARα$^{-/-}$ (KO) and wildtype (WT) mice. Changes in mRNA abundance of nine genes (listed under 8.4.1) were determined using qRT-PCR. Results are shown as mRNA levels compared to wildtype CMC control for *Vnn1* as a PPARα-dependent gene and for *Ca2* as a PPARα-independent gene. mRNA levels were standardised to Cyclophilin; wildtype control (CMC) was set to 1. Agonists used were the following: WY14643 (synthetic PPARα agonist); the fatty acid ethyl ester (OA-EE, EPA-EE and DHA-EE) and the single unsaturated fatty acid in a synthetic triglyceride (OA-TG, EPA-TG and DHA-TG). Data are presented as mean ± SEM (n=4 to 5). Significant differences (P<0.05) were observed between genotype $^{\#}$ and within wildtype mice *. a, b: different letters indicate a significant difference between EE; c, d: different letters indicate a significant difference between TG within wildtype mice.

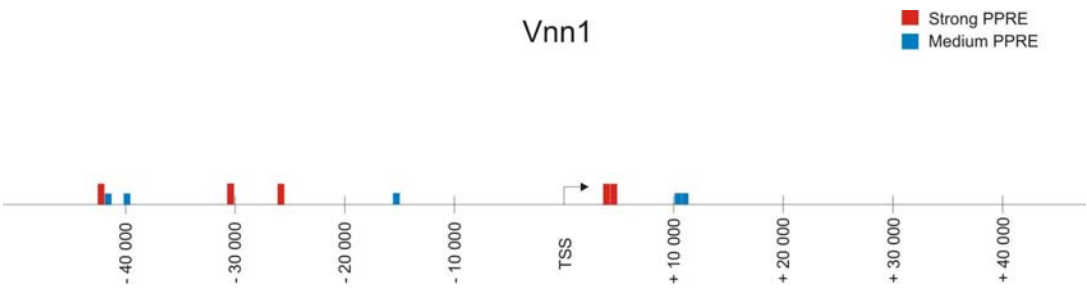


Fig. 8.2 *In silico* analysis of mouse vanin1 (*Vnn1*) as selected putative PPARα target gene. The location of putative strong and medium PPAR response elements (PPRE) specific for binding PPARα 50 kb up- and down-stream of their transcription start site (TSS) are shown. Two strong, adjacent and partially overlapping PPRE at 4175 and 4182 bp down-stream of the TSS were chosen for functionality analysis *in vitro*. The *in silico* approach for PPRE locations and PPAR binding strength prediction has been previously described by Heinäniemi *et al.* (2007).

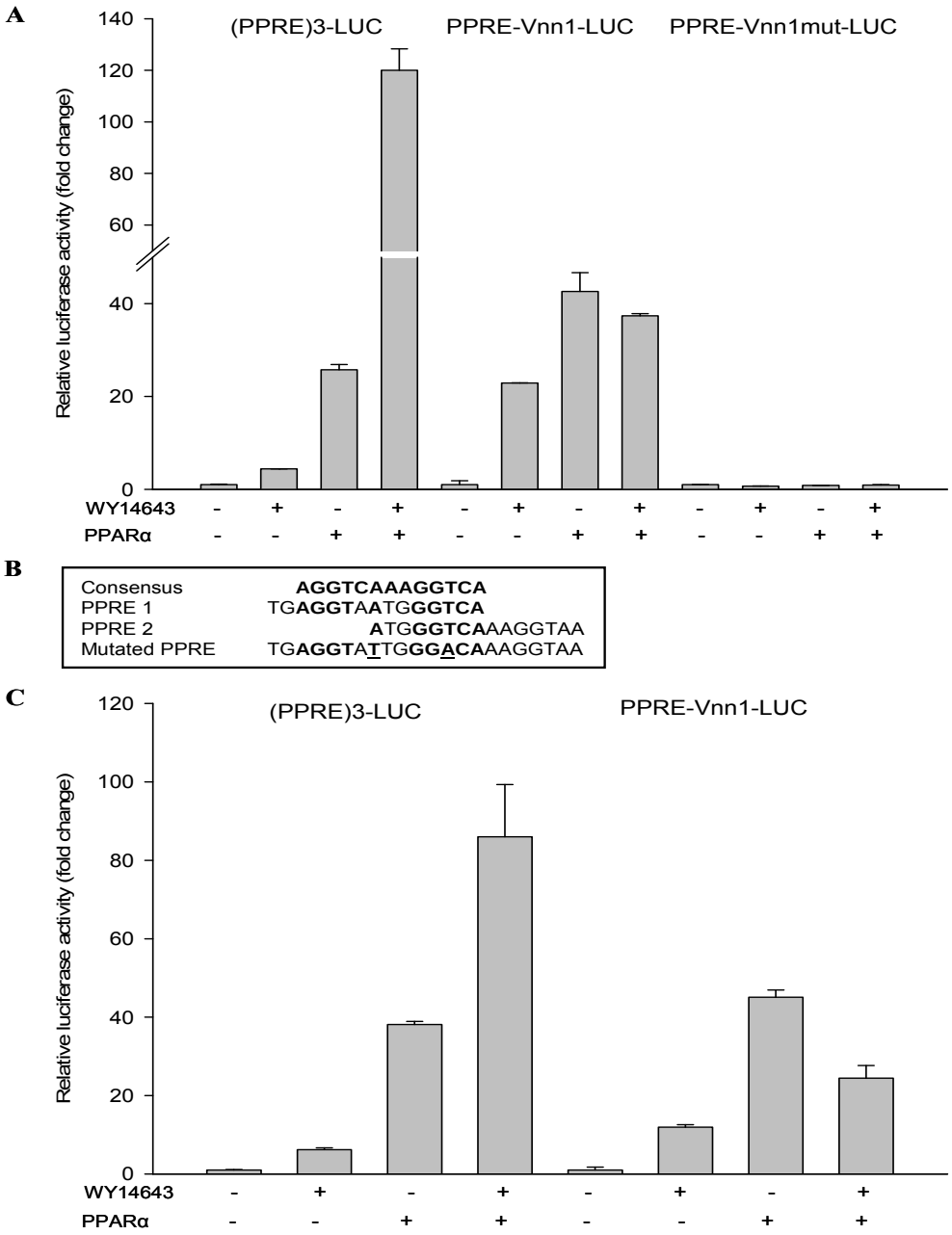


Fig. 8.3 PPARα-mediated increased expression level of mouse *Vnn1* is regulated by PPRE in the *Vnn1* genomic sequence. (A) and (C) HepG2 cells were transiently transfected with the (PPRE)3-LUC as a control or PPRE-Vnn1-LUC or PPRE-Vnn1mut-LUC promoter reporter and PPARα expression plasmid. Cells were treated with WY14643 (50 μM) for 24 h after which luciferase and β-galactosidase activities were determined in the cell lysates. Relative luciferase activities were normalised to β-galactosidase, and the relative luciferase activity of the cells treated with DMSO was set to 1. Error bars represent SEM. (B) Alignment of the consensus PPRE with the 2 overlapping PPRE sequences found in the mouse *Vnn1* genomic region and the mutated PPRE for *Vnn1* used in the transfection experiment.

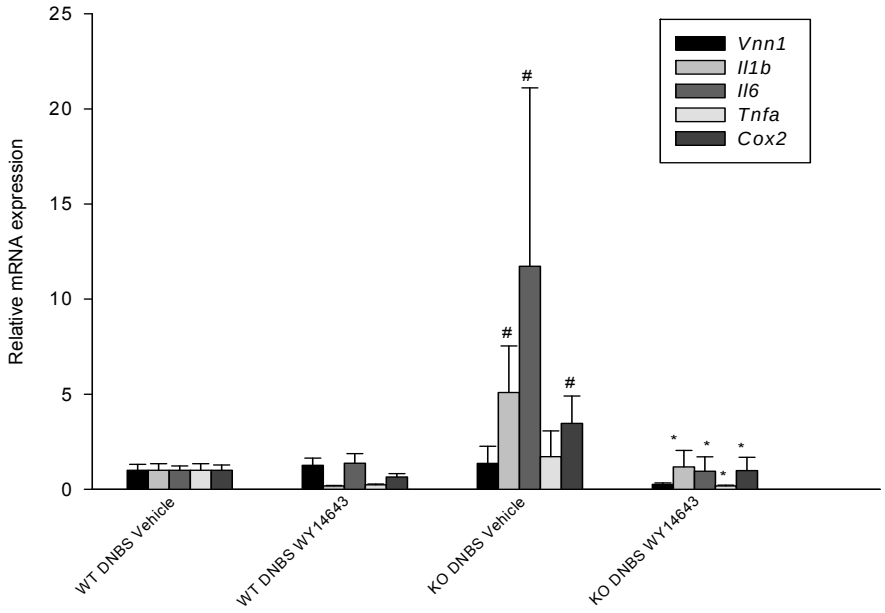


Fig. 8.4 PPARα$^{-/-}$ (KO) and wildtype (WT) mice with DNBS-induced colitis were exposed to WY14643 or vehicle for 4 days. Colon RNA was isolated and expression levels of pro-inflammatory cytokine genes (*Il1b*, *Il6*, *Tnfα* and *Cox2*) and *Vnn1* gene were measured by qRT-PCR. mRNA levels were standardised to 18S; wildtype control (Vehicle) was set to 1. Data are presented as mean ± SEM (n=2 to 5). Significant differences (P<0.05) were observed between genotype $^{\#}$ and within KO mice *.

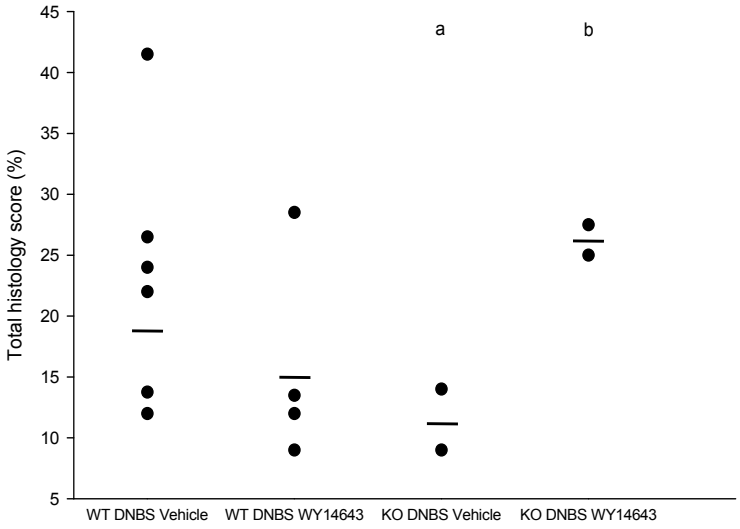


Fig. 8.5 Total colon histology score in the colon of WY14643- or vehicle-treated PPARα$^{-/-}$ (KO) and wildtype (WT) mice with DNBS-induced colitis. The individual scores and the average score (**—**) of each group (n=2-6) are shown. a, b: different letters mean that total histological injury score differs significantly between groups (P<0.05) when comparing between mouse genotypes on the same treatment or within mouse genotypes on different treatments. Group sizes differ because of animal deaths.

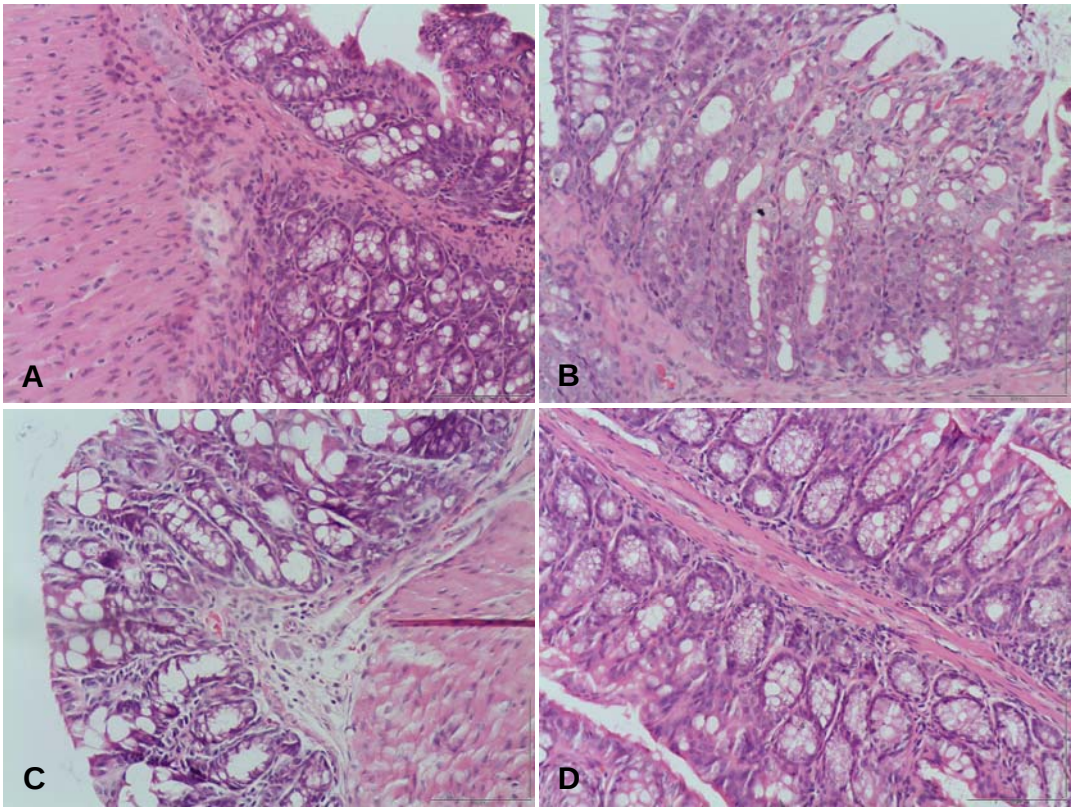


Fig. 8.6 Haematoxylin-eosin stained colon sections of WY14643- and vehicle-treated PPARα$^{-/-}$ and wildtype mice with DNBS-induced colitis. (A) PPARα$^{-/-}$ DNBS vehicle group (×20), (B) PPARα$^{-/-}$ DNBS WY14643 group (×20), (C) Wildtype DNBS vehicle group (×20) and (D) Wildtype DNBS WY14643 group (×20).

8.5 Discussion

The current study focussed on the identification of a putative novel PPARα target gene and its possible functional role in colon inflammation. Several genes for which a direct link with PPARα has not yet been established were tested in colon samples of PPARα$^{-/-}$ and wildtype mice. Only *Vnn1* gene showed increased expression levels in the colon of PPARα agonist-treated wildtype compared to wildtype control mice as well as between wildtype and PPARα$^{-/-}$ mice, especially after treatment with EPA, DHA and WY14643. Global expression analysis of WY14643-treated wildtype relative to wildtype control mice already indicated increased expression levels of *Vnn1* gene in liver and small intestine, which pointed to a potential PPARα-dependent regulation. Based on the increased expression levels of *Ppara* and *Vnn1* genes in the *Il10*$^{-/-}$ colitis mouse model fed dietary EPA (Knoch *et al*., 2009; Chapter 5) a possible role for *Vnn1* in lipid metabolism and colitis development under *Ppara* dependency was suggested. The PPRE identified in the mouse *Vnn1* genomic region showed PPARα sensitivity in the promotor reporter gene assay.

PPARα-dependent vanin1 gene regulation in vivo and in vitro

PPARα is known as a key mediator in lipid metabolism (Latruffe & Vamecq, 1997) and its activity has been shown to respond to changes in dietary fat load and composition in liver (Sanderson *et al*., 2008). Although changes in PPARα-dependent gene expression induced by feeding high fat diets seemed to be minor in liver, pronounced differences in PPARα induction were shown in comparative genome-wide expression analyses between natural and synthetic stimuli using microarrays (Patsouris *et al*., 2006). Sanderson *et al.* (2008) demonstrated that hepatic gene regulation in response to dietary unsaturated fatty acids was highly dependent on PPARα and that the PUFA DHA was the most potent inducer of PPARα in mouse liver. When screening for PPARα-responsive genes using colon tissue of the dietary unsaturated fatty acid intervention study with PPARα$^{-/-}$ and wildtype mice, *Vnn1* mRNA abundance was more strongly increased by DHA and EPA ethyl ester and TG. This was in line with the clear DHA-dependent PPARα target gene regulation in mouse liver and rat hepatoma FAO cells (Sanderson *et al*., 2008) and further suggests *Vnn1* as a PPARα-dependent gene in response to dietary unsaturated fatty acids.

The importance of fatty acid-dependent gene regulation by PPARα and several other transcription factors including RXR, PPARβ/δ, PPARγ, HNF4α, LXR, FXR, SREBP1 and NFκB has mainly been reported in liver (Hertz *et al*., 1998; Yoshikawa *et al*., 2002; Sampath & Ntambi, 2005). A study by Wang *et al*. (2005) demonstrated that PPARα was most abundantly expressed in the jejunum, but also expressed in colon and throughout the entire rat gastrointestinal tract. Also Lee *et al*. (2007) showed PPARα expression in the colon, specifically in immune and epithelial cells lining the lumen. Colon is a less obvious tissue to study the functional role of PPARα and its target genes, because fatty acid digestion, absorption and transport into the body as chylomicrons occur mainly in the small intestine. However, fatty acid binding (FABP) and transporting (FAT/CD36) protein genes are ubiquitously expressed in the rat and human gastrointestinal tract to facilitate the transfer of long chain fatty acids into organ tissues from the peripheral circulation, especially of essential n-3 and n-6 PUFA (Wang *et al*., 2005). PPARγ has been reported to regulate the transcription of FAT/CD36 (Wang *et al*., 2005). The expression of fatty acid transporters under control of PPARα in the colon implies a functional relevance in ensuring fatty acid transfer under normal and diseased physiological conditions.

It has been reported that peroxisome proliferators including WY14643 can induce *Vnn1* gene expression in mouse liver (Moffit *et al*., 2007). In the present study, the PPRE identified within the mouse *Vnn1* gene by *in silico* analysis were experimentally confirmed by a promoter reporter gene assay to be under control of PPARα. Upon binding by specific agonists, PPARα can form a permissive heterodimer with RXR which binds to specific DNA-response elements in the promoter region of target genes and initiates their transcription (Desvergne & Wahli, 1999). The site-directed mutagenesis of the mouse *Vnn1* PPRE resulted in reduced activity which further demonstrated the responsiveness of this genomic region to PPARα. Interestingly, those response elemnets of the mouse *Vnn1* gene displayed no further increase in PPARα-mediated *Vnn1* transactivation by WY14643. Co-transfection with PPARα increased promoter reporter activity for the *Vnn1* genomic regions, but it is unclear why WY14643 does not further stimulate promoter activity.

Potential PPARα-mediated regulatory function of vanin1 in lipid metabolism

The second aim was to identify a possible new regulatory function for *Vnn1* mediated by PPARα in the context of lipid metabolism and colon inflammation. Vanin1 is a pantetheinase expressed by enterocytes and acts as a sensor for oxidative stress through cysteamine, a pro-oxidant in glutathione metabolism (Pouyet *et al*., 2009). Vanin1$^{-/-}$ mice showed decreased NSAID- or *Schistosoma*-induced intestinal inflammation which was associated with higher glutathione levels (Martin *et al*., 2004). Berruyer *et al*. (2006) have demonstrated using Vanin1$^{-/-}$ and wildtype mice with TNBS-induced colitis that vanin1, by antagonising PPARγ activity, stimulates inflammatory mediator production by intestinal epithelial cells controlling the innate immune response. Epithelial vanin1 was also found to regulate inflammation-driven cancer development in a colitis-associated colon cancer model (Pouyet *et al*., 2009). In the present study, the decreased pro-inflammatory cytokine gene expression in the colon of WY14643-treated PPARα$^{-/-}$ mice might indicate an anti-inflammatory effect of the WY14643 agonist, although the colon histology suggested the opposite. The *Vnn1* gene, also considered as pro-inflammatory, tended to have lower mRNA abundance in WY14643- compared to vehicle-treated PPARα$^{-/-}$ mice. It was shown that the PPARα agonist fenofibrate suppressed the expression of pro-inflammatory cytokine genes in *Il10*$^{-/-}$ mice (Lee *et al*., 2007). These authors indicated that this reduction could be due to decreased inflammatory-cytokines producing cells in the colon or those cells suppressing the production of cytokines. The protective effect of WY14643 on rat liver from hepatic ischemia reperfusion injury was associated also with an inhibition of inflammatory response and oxidative stress (Xu *et al*., 2008).

WY14643 is a very specific PPARα agonist which belongs to a group of fibrate drugs used for their ability to lower plasma TG and increase plasma HDL levels and is thus used to treat dyslipidemia (Staels & Fruchart, 2005). Physiological (e.g. fasting) and pharmacological (e.g. synthetic agonist) activation of PPARα using PPARα$^{-/-}$ and wildtype mice show that PPARα plays an important role in hepatic lipid metabolism, but evidence exists that there is only partial overlap between genes up-regulated by physiological and pharmacological activation of PPARα (Rakhshandehroo *et al*., 2007). One possible reason is that another PPAR subtype, e.g. PPARβ/δ may be involved and cross-reacts to some extent with PPARα. Recent data by Sanderson *et al*. (2009) showed that PPARs show different responses and functions to physiological compared

to pharmacological activation. They found that despite overlapping effects of WY14643 and fasting, several WY14643-induced hepatic genes showed increased expression by fasting independent of PPARα but dependent on PPARβ/δ which is sensitive to plasma free fatty acids. PPARβ/δ protein is highly expressed in liver but also in colon, can inhibit inflammation (Burdick *et al*., 2006; Peters *et al*., 2008; Shan *et al*., 2008) and a possible constitutive role for PPARβ/δ in colon epithelial tissue and liver has been suggested (Girroir *et al*., 2008).

Although Vanin1 has not been directly linked to lipid metabolism, a role in the TG lowering mechanism through PPARα in liver has been suggested (Omura *et al*., 2007). It was shown that PPARα activation attenuated inflammatory responses (Wahli *et al*., 1995; Mandard *et al*., 2004). PPARα regulates numerous genes involved in lipid metabolism. Vanin1 was shown here as PPARα-dependent and might be involved in lipid metabolism as well. Indeed the VNN1 gene harbors sequence variants that influence HDL cholesterol concentrations (Gohring *et al*., 2007).

In conclusion, the present data show PPARα-dependent up-regulation of the mouse *Vnn1* gene *in vivo* and *in vitro*, and thus suggest *Vnn1* as a likely PPARα target gene. Further work is required to investigate a possible involvement of PPARβ/δ in the physiological regulation of *Vnn1* in order to distinguish the role between PPARs in colon and under inflammatory conditions.

Chapter 9

Summary and general discussion

9. Summary and general discussion

Findings from previous studies in animal models and humans (as summarised in the general introduction) led to the suggestion that PUFA, particularly n-3 PUFA, may be beneficial for people with IBD such as UC or CD. Much of the evidence regarding immunoregulatory, anti- and pro-inflammatory effects of n-3 and n-6 PUFA, respectively, comes from cell culture studies where single fatty acids were added. Many animal studies have focused on the anti-inflammatory effects of n-3 PUFA from fish oil as assessed by histology, circulating PUFA concentrations, or *ex vivo* production of inflammatory mediators. There is no study that has applied genome-wide expression profiling to define the effects of dietary n-3 and n-6 PUFA and their underlying molecular mechanisms on the disease phenotype. Research is required to investigate the effect of n-3 and n-6 PUFA on whole genome gene expression profiles in the development of colitis, with special emphasis on inflammatory and immune response pathways and to relate gene expression changes with changes in bacterial community profiles.

9.1 Summary of results

The initial aim for studying the effect of PUFA was to define their effects during the initiation phase of colitis. Bacterial inoculation accelerated colitis development in conventional *Il10*$^{-/-}$ mice and resulted in a more consistent and severe colitis peaking between 10 and 12 weeks of age, with no further increase from 12 weeks of age based on histopathological assessment. The combined transcriptomic and proteomic approach provided a more detailed characterisation of the colitis before (7 weeks of age) and after (12 weeks of age) its onset in an *Il10*$^{-/-}$ mouse model of colitis. The transcriptomic and proteomic data showed distinct expression profiles for 7 and 12 week old *Il10*$^{-/-}$ mice compared to C57 mice at the same age with marked changes in the colonic expression profile after onset of colitis (12 weeks of age). Gene and protein expression data linked actin cytoskeleton dynamics of the epithelial barrier and innate immune responses suggesting a delayed remodelling process in the presence of increased expression of inflammatory cytokine genes. Based on histology and gene/protein expression profiles, it was concluded that 11 weeks of age would best reflect the onset stage of colitis in the inoculated C57BL/6J *Il10*$^{-/-}$ mouse model used in this study (**Chapter 3**).

In the subsequent PUFA intervention study, *Il10*$^{-/-}$ and C57 mice were fed EPA- and AA ethyl ester-enriched AIN-76A diets from 5 to 11 weeks of age and their gene expression profile compared to animals fed the OA diet. The OA-enriched AIN-76A diet was the most appropriate control for EPA and AA, due to its composition being closer to the PUFA diets compared to the standard AIN-76A diet based on corn oil. OA has been used before as a fatty acid control. It was confirmed here that OA was an appropriate control fatty acid to compare against dietary PUFA in multi-omic studies based on colonic transcriptome and proteome profiles, which clustered closely between *Il10*$^{-/-}$ mice and C57 mice fed either the AIN-76A or OA diet. Those similarities were associated with inflammation-induced regulatory processes of cellular and humoral immune responses. Comparing transcriptome and proteome profiles between genotypes for each diet resulted in a general lack of overlap between both profiles. Transcriptomic changes mostly involved signalling processes, whereas proteomics data were related to metabolic changes. The proteomic technique used here detected mostly high abundance proteins associated with carbohydrate and lipid metabolism, but not the low abundance proteins such as cytokines associated with inflammatory responses. This highlights the importance for a combined 'omics' approach to obtain a holistic insight into inflammatory processes (**Chapter 4**).

The underlying mechanisms whereby different PUFA mediate their effects are still unclear. The main focus of the PUFA intervention study was the impact of PUFA on inflammatory and immunoregulatory processes involved in the onset of experimental colitis. This was driven by the overall hypothesis that dietary n-3 PUFA EPA may reduce and dietary n-6 PUFA AA may induce colon inflammation. As expected, dietary EPA initiated fatty acid oxidation which along with colitis development might have induced ROS production and lipid peroxidation within the colonocyte. Native and oxidised EPA could have activated PPARα linking to inhibition of down-stream NFκB-mediated pro-inflammatory events. The increased expression levels of detoxification and anti-oxidative enzyme genes supported the oxidative processes in the colon of *Il10*$^{-/-}$ mice as EPA is prone to peroxidation. Whereas the OA diet linked inflammatory and immune responses to fibrosis development and tryptophan degradation when comparing genotypes, dietary EPA did not initiate changes in expression of those genes in the colon of *Il10*$^{-/-}$ mice (**Chapter 5**).

Dietary EPA confirmed the hypothesis of having an anti-inflammatory effect which was exerted mostly through *Ppara* transcription factor activation, whereas dietary AA did not induce a pro-inflammatory response and more colon inflammation as hypothesised. Dietary AA showed beneficial effects in the colon of *Il10*$^{-/-}$ mice when compared to *Il10*$^{-/-}$ mice fed the OA diet by reduced expression levels of genes related to cellular stress and inflammatory and immune responses. Dietary AA increased the mRNA abundance of genes involved in fatty acid transport and oxidation and decreased the expression levels of cholesterol and fatty acid synthesis genes likely via decreasing the expression level of the transcription factor *Srebf1* and its target genes. This study suggested a link between dietary AA and intestinal fibrosis as shown by decreased mRNA levels of fibrotic marker genes, a link which has not previously been made (**Chapter 6**).

Colonic gene expression profiling revealed potential mechanisms underlying the gene expression changes after feeding PUFA-enriched diets, of which some aspects were more thoroughly investigated. One experiment focussed on the identification of a putative novel target gene of PPARα in response to unsaturated fatty acids and a possible functional role of the target gene in colon inflammation. Possible PPARα target genes were selected from the list of up-regulated colonic genes in *Il10*$^{-/-}$ mice fed with EPA compared to the OA diet (Chapter 5). *Vnn1*, but not the other selected genes for which a putative link with PPARα had not been established before, showed increased expression levels in the colon of PPARα wildtype mice in response to dietary OA, EPA and DHA or the synthetic PPARα agonist (WY14643) compared to the control diet. The PPREs identified with an *in silico* screening approach of the mouse *Vnn1* genomic region showed responsiveness to PPARα in the promoter reporter gene assay and site-directed mutagenesis. These findings suggest *Vnn1* as a new PPARα-target gene which may also be involved in lipid metabolism (**Chapter 8**).

Additionally, the time-course and PUFA intervention studies were also used to investigate the hypotheses that the lack of IL10 protein may alter intestinal metabolism causing changes in caecal bacteria communities before and after onset of colitis, and that dietary PUFA may also alter the bacterial community during colitis, thus contributing to their effects on colon mucosal responses. Reduced caecal bacteria diversity was seen in *Il10*$^{-/-}$ compared to C57 mice before (7 weeks of age) and after

(12 weeks of age) onset of colitis in *Il10*$^{-/-}$ mice. The lack of IL10 protein causing changes in metabolic and regulatory processes might alter luminal conditions in the large intestine and cause shifts in the caecal bacteria community before and after colitis onset in *Il10*$^{-/-}$ mice. An increase in total bacterial numbers was observed in the caecum of 12-week-old compared to 7-week-old *Il10*$^{-/-}$ mice and thus occurred in parallel with the colitis development in these mice. Differences in the caecal bacteria community of 7- and 12-week-old *Il10*$^{-/-}$ mice compared to C57 mice of the same age could be linked to increased expression levels of bacteria recognition receptor genes and subsequent increase in expression levels of pro-inflammatory cytokine genes in colitic *Il10*$^{-/-}$ mice at 12 weeks of age. Both AA and EPA diet were capable of modifying the bacterial composition in the caecum of colitic *Il10*$^{-/-}$ mice. It was suggested that changes in the caecal bacteria community in the PUFA-fed *Il10*$^{-/-}$ mice might be associated with the anti-inflammatory effects of dietary EPA and AA compared to the OA diet resulting from whole genome gene expression profiles of *Il10*$^{-/-}$ mice (**Chapters 5 and 6**). The marked increase of *E. coli* cell numbers in *Il10*$^{-/-}$ mice especially in response to dietary AA might be associated with the decreased expression levels of pro-inflammatory cytokine genes *Tnfa* and *Il1b* compared to colitic *Il10*$^{-/-}$ mice fed the OA diet. These associations could not be made for EPA-fed *Il10*$^{-/-}$ mice. For dietary AA, it may imply a protective effect of *E. coli* on colitis in this host (**Chapter 7**).

9.2 General discussion

There are a few points to address when comparing data from the studies described in this thesis. The fact that commensal bacteria are necessary and have different potential for causing colitis in *Il10*$^{-/-}$ mice was one of the arguments for a mix of commensal bacteria to induce more consistent colitis in this IBD mouse model. The time-course study suggested accelerated onset of colitis in *Il10*$^{-/-}$ mice inoculated with commensal bacteria. These *Il10*$^{-/-}$ mice developed colitis with human CD-like features two weeks earlier than noninoculated, conventional *Il10*$^{-/-}$ mice of the same genetic background (C57BL/6J) (Kennedy *et al*., 2000). The inflammation develops mostly in the colon in this *Il10*$^{-/-}$ mouse model. The large number of bacteria, mostly anaerobic, and bacterial proliferations in the large intestine might be the reason that the lesions occur in the colon (Elson & Weaver, 2003; Tannock, 2005) rather than duodenum, jejunum and

ileum (caecum not assessed; Roy *et al*., 2007) and caecum and colon in other studies using *Il10*$^{-/-}$ mice.

At first glance, colon seems a less obvious tissue to study the effects of dietary PUFA, the role of PPARα and its target genes with potential function in lipid metabolism. Most of the dietary lipids (about 98%, including TG, phospholipids, and cholesterol) are digested and absorbed mainly in the small intestine. Luminal lipids are hydrolysed to fatty acids and 2-monoacylglycerols that are taken up by the enterocytes and then resynthesised to TG. The TG are assembled with apolipoproteins into chylomicrons that are secreted from the enterocytes into the lymphatic circulation and then released into the bloodstream (Carrey *et al*., 1983; Phan & Tso, 2001). Only a limited amount (~2%) of dietary lipids in the form of TG will reach the colon via the intestinal lumen.

However, dietary PUFA can exert their effects on colonic cell homeostasis via uptake from circulating blood into colonocytes and incorporation of fatty acids into membrane phospholipids. Once the long chain fatty acids are in the circulatory system, levels of free fatty acids rise and they are transported to various tissues including the colon; there they are taken up for storage, energy production or induction of signalling and metabolic processes. The colonic gene expression changes reflected typical fatty acid responsive genes involved in fatty acid binding, transport and oxidation, including known PPAR target genes and other genes that typically respond to fatty acids via a mechanism that is likely PPAR-independent. Especially in response to EPA, the expression levels of *Ppara* gene and some of its target genes were up-regulated in the colon of *Il10*$^{-/-}$ mice, but not in response to AA. EPA is prone to peroxidation, but the presence of adequate levels of antioxidants in the diet suggested that the oxidative stress that occurred during intestinal inflammation, could be a more likely contributor to oxidised EPA. Native as well as oxidised EPA can serve as ligands for PPARα inhibiting NFκB and down-stream inflammatory and immune response pathways (Sethi *et al*., 2002). The n-3 and n-6 PUFA are both natural ligands for PPARα, whereas n-3 PUFA are considered as more potent activators of PPARα than n-6 PUFA (Volcik *et al*., 2008).

Recent data by Sanderson *et al.* (2008) demonstrated that hepatic gene regulation in response to dietary unsaturated fatty acids was highly dependent on PPARα. PPARα

and its target genes have been less well studied in the small and large intestine than in liver. Bünger *et al.* (2007) were among the first to expand the knowledge of PPARα-dependent gene regulation in the small intestine and suggested an influence of PPARα on enterocytes, immune and inflammatory responses. All PPARs including PPARα are expressed in the intestinal mucosa, and PPARα expression has been shown in the colon, specifically in immune and epithelial cells lining the lumen (Lee *et al*., 2007). Although several studies have linked PPARγ and IBD (Kelly *et al*., 2004; Bassaganya-Riera & Hontecillas, 2006), it has been shown that ligands for PPARγ (Lytle *et al*., 2005) as well as PPARα (Lee *et al*., 2007) have anti-inflammatory activity in the *Il10*$^{-/-}$ mouse model of colitis. The present PUFA study associated increased expression levels of PPARα by dietary EPA with increased expression levels of fatty acid oxidation genes as targets of PPARα to suppress inflammatory responses. Enhanced intestinal fatty acid oxidation has been correlated before with reduced severity of IBD (Lee *et al*., 2007).

In this thesis, mouse *Vnn1* gene has been suggested as a novel PPARα target gene with a potential role in intestinal lipid metabolism based on the PPARα-dependent regulation of the *Vnn1* gene in colon samples of PPARα wildtype mice treated with dietary PPARα ligands DHA, EPA or synthetic PPARα ligand WY14643. There was a good correlation between the quantitative regulation of *Vnn1*, measured as fold change, and the transactivation efficiency of the WY14643 ligand assayed *in vitro*. VNN1 has not directly been linked to lipid metabolism, but peroxisome proliferators such as WY14643 have been shown to induce *Vnn1* gene expression in mouse liver (Moffit *et al*., 2007), and thus a role in the TG decreasing mechanism through PPARα in liver been suggested (Omura *et al*., 2007). PPARα regulates numerous genes involved in lipid metabolism. Therefore, it might be hypothesised that VNN1 is involved in lipid metabolism as well. Indeed the VNN1 gene harbors sequence variants that influence high-density lipoprotein cholesterol concentrations (Goring *et al*., 2007). The potential effect of VNN1 at raising high-density lipoprotein cholesterol concentrations by PPARα-dependent regulation warrants further investigation. PPARα is a molecular target for hypolipidemic drugs such as fibrates (e.g. WY14643) that increase plasma HDL and decrease plasma TG levels.

A lower up-regulation of transcription factors and its target genes involved in lipid metabolism and inflammation occurred in the colon of PUFA- compared to OA-fed

Il10$^{-/-}$ mice. This might be due to the longer term (6 weeks) feeding approach used for PUFA supplementation in this study. The PUFA-enriched AIN-76A diets represented a low fat diet (18 MJ/kg) compared to a normal human diet. Based on the average energy intake of humans (8 MJ/d) and mice (0.07 MJ/d) and their different metabolic rates, the n-3 PUFA density in the mouse diet was about ten times greater (i.e. 1.9 g n-3 PUFA/MJ versus 0.2 g n-3 PUFA/MJ) than in the normal human diet. The sustained intake of PUFA-enriched diets before and during the onset of colitis might have facilitated an adaptation to that PUFA challenge over time, in particular to dietary AA which also showed anti-inflammatory characteristics. The adaptation to this challenge with PUFA, which activates transcription factors (e.g. *Ppara* and *Srebf1*) and their target genes involved in lipid metabolism and inflammation, might contribute to anti-inflammatory mucosal responses in the colon. The study by Ramakers *et al*. (2008) also indicated that an AA-enriched diet had a protective effect in a DSS-induced colitis mouse model, as shown by decreased weight loss and diarrhoea scores compared to fish oil or OA-enriched diets. Their study supplied AA as the ethyl ester, whereas OA, EPA and DHA were used as the TG. In the present PUFA intervention study, pure ethyl esters of EPA and AA as well as OA were used for supplementation. Ramakers *et al*. (2008) argued against the possibility of less efficient absorption of ethyl esters in the intestine compared to TG as reported in other studies (Lawson & Hughes, 1988), because the fatty acid composition of the colon tissue reflected that of the diets used, which indicated adequate AA absorption. It is hypothesised here that the concentration of intracellular AA in the colonocytes might have remained at a level not sufficient to initiate inflammation.

OA was used as a control fatty acid in a previous study evaluating the anti-tumorigenic effect of dietary EPA in *Apc*$^{Min/+}$ mice and the antagonism between AA and EPA (Hansen-Petrik *et al.*, 2000). Some of their unpublished data and later studies from Whelan and coworkers (e.g., McEntee *et al.*, 2008) showed that dietary OA had no effect on tissue content of EPA and AA nor eicosanoid biosynthesis. The greater intake of the AIN-76A diet versus the OA diet on the one hand and the similar growth rates on the other hand for *Il10*$^{-/-}$ and C57 mice indicated that the OA diet might have been utilised with increased efficiency. This might be due to the different fatty acid composition between these two control diets. Thus it is conceivable that the OA diet

was in general an improved diet in terms of fatty acid composition being more similar to the PUFA diets than to the AIN-76A diet. This could also explain the lower magnitude changes in gene expression reported in the colon of the PUFA- versus the OA-fed *Il10*$^{-/-}$ mice as when the PUFA diets were compared to the AIN-76A diet.

A sound interpretation of the data aims to have a confirmation of the salient gene expression changes at the protein level in combination with physiological endpoints. The protein of the same TRIzol processed colonic sample from which RNA was derived has been used to conduct proteome analysis of the time-course and PUFA intervention study. However, the proteomics data for the PUFA intervention study were not included in this thesis due to time constraints and are subject for publication elsewhere. A combination of transcriptomics and proteomics is important given the lack of overlap between gene and protein expression data observed. Their combined use is necessary to obtain a more comprehensive insight into PUFA-mediated effects, since proteins can be expressed at levels and in isoforms that may not be predicted from mRNA analysis.

The present findings regarding the changes in caecal bacteria during onset of colitis could not clearly associate identified bacterial species with colitis development due to the quantity and complexity of bacteria in the large intestine and their interactions. Since the environment can affect the murine microflora, thus the early rearing environmental conditions have to be controlled. The C57 and *Il10*$^{-/-}$ mice were obtained from the same breeding facility where they were kept in the same specific pathogen-free conditions until their arrival at the trial facility, where the mice were randomly allocated to cages and housed conventionally in the same room. Although the mice were kept individually in the cage, the bacterial profiles of the individual mice within an experimental group were similar with only a few outliers. The reduced number and diversity of large intestine bacteria observed between *Il10*$^{-/-}$ and C57 mice before and after onset of colitis agreed with findings from others studying differences in bacterial profiles in IBD mouse models (Nones *et al.*, 2009; Wohlgemuth *et al.*, 2009) or IBD patients compared to healthy subjects (Frank *et al.*, 2007). The bacterial profile changes observed in *Il10*$^{-/-}$ and C57 mice suggest that dietary PUFA and MUFA may alter the bacterial adhesion sites by changing the fatty acid composition of the intestinal epithelial cell membrane, albeit differently, and probably effect the ability of bacteria to attach and thus ameliorate or maintain colitis. A possible protective effect of AA on the

presence of *E. coli* strains and potential changes of the bacterial environment could be a subject for further investigation. The proposed link between dietary AA and intestinal fibrosis based on decreased fibrotic marker gene expression might be associated with the possible protective AA-mediated effect of *E. coli* sp. on colitis. AA-enriched oil has been shown to decrease murine hepatic genes involved in collagen synthesis (Berger *et al*., 2006), an increase of the latter has been observed in CD patients with intestinal fibrosis (Martinez-Augustin *et al*., 2008). Given the fact that normal enteric bacteria differ in their inflammatory and fibrotic potential (Medina *et al*., 2005) and therefore seem to produce different inflammatory responses (Mourelle *et al*., 1998), it will be of interest to correlate changes in large intestine bacteria communities in AA-fed *Il10*$^{-/-}$ mice during colitis development with inflammatory and fibrotic protein responses.

9.3 Future perspectives

By making use of some 'omics' tools and other molecular biology techniques available to nutrigenomics research, the studies described in this thesis reported on the effects of dietary EPA and AA on colon inflammation. EPA and AA showed different activation of transcription factors and their target genes involved in lipid metabolism and inflammation in a systemic adaptation to that PUFA challenge. These new insights improve understanding of molecular mechanisms underlying the effects of dietary PUFA on a CD-like colitis, and may contribute to the development of personalised foods targeting general gut health and wellness.

The focus here was on the effects of EPA and AA supplied as synthetic ethyl esters. Future efforts must be taken to show the effects of these PUFA incorporated as TG, the naturally occurring form or as part of a whole food, on whole transcriptome and also proteome or metabolome. The latter will elucidate the differential effects that macro- and micronutrients as part of the whole food might have on activation of transcription factors and gene expression profiles.

Although microarrays are a powerful technology for screening gene expression changes, the approach of differential gene expression profiling comprises potential flaws. For all studies described here full-thickness, heterogeneous colon tissue samples were used for genome-wide expression analysis. Thus, differences in gene expression levels between

experimental groups may not only account for true changes in gene expression but also for differences in their cellular composition. Especially lower magnitude changes in gene expression, typical with early biomarker discovery (Disset *et al*., 2009) and dietary intervention studies, may not or not easily be detected in heterogeneous samples. The effect of PUFA on the various cell subpopulations (e.g. epithelial, goblet, dendritic and Paneth cells) and the impact of infiltrating inflammatory cells (primarily monocytes and neutrophils) in colon would facilitate identification of the molecular mechanisms of action of the PUFA on colitis development. It is expected that each subpopulation has its cell type-specific function and therefore may have cell type-specific gene expression profiles. This could be approached by isolating the relevant cell types after PUFA intervention of mice. Cell isolation techniques such as laser capture micro-dissection that requires less tissue and time coupled with procedures for high quality RNA isolation could be a valuable tool with application in nutrigenomic studies.

Mouse models of colitis have enhanced our understanding of the pathogenesis of IBD. It will be of interest to study changes in gene expression profiles when PUFA are fed at several time points and doses during colitis development including postprandial intervention. This could establish an efficacious dose for each PUFA within a time course of colon inflammation in order to understand metabolic changes in diet-related disease such as colitis with possible preventive or causative effects of PUFA. Studies using germ-free mice will be required to make clear associations of bacterial species with colitis development, and in particular, the effect of PUFA and also MUFA on the colonisation of certain bacterial strains.

Dietary lipids have varying effects on health, and current nutritional guidelines recommend consumption of unsaturated fatty acids, especially n-3 PUFA from fish oils and less consumption of saturated and *trans* fatty acids. The evidence of significant benefits from n-3 PUFA is not compelling enough to give dietary recommendations for prevention of IBD. There is also an issue concerning a global decline in fish stocks or possible toxins in fish which should be assessed before making clinical and public health recommendations (Jenkins *et al*., 2009). More research is required to clarify the health benefits of PUFA and in the case of n-3 PUFA to also evaluate plant sources such as algae and yeast.

The major goal of Nutrigenomics New Zealand is to develop a nutrigenomics research capability for New Zealand. This will provide the scientific basis and research platform for developing foods that match people's genes and deliver proven health outcomes to consumers. These gene-specific foods will either ameliorate the risk of the genetic disease in subjects who are in the process of developing inflammation but who are not sick yet in order to prevent instead of cure the disease or enhance normal physiological function. For such research, disease models combined with whole genome analyses as described in this thesis are an effective strategy to improve the understanding of molecular mechanisms underlying the effect of specific dietary compounds. A systems biology approach including transcriptomics, proteomics, metabolomics and epigenomics, as well as statistics, bioinformatics and strategic data management, should be utilised to correlate and translate this large amount of data into physiologically relevant information to define a given phenotype.

References

Afman L & Muller M (2006) Nutrigenomics: From molecular nutrition to prevention of disease. *J Am Diet Assoc* **106**, 569-576.

Ahmed S, Macfarlane GT, Fite A, McBain AJ, Gilbert P & Macfarlane S (2007) Mucosa-associated bacterial diversity in relation to human terminal ileum and colonic biopsy samples. *Appl Environ Microbiol* **73**, 7435-7442.

Aitken AE, Richardson TA & Morgan ET (2006) Regulation of drug-metabolizing enzymes and transporters in inflammation. *Annu Rev Pharmacol Toxicol* **46**, 123-149.

Akita H, Chiba H, Tsuji M, Hui S-P, Takahashi Y, Matsuno K & Kobayashi K (1995) Evaluation of G-to-A substitution in the apolipoprotein A-I gene promoter as a determinant of highdensity lipoprotein cholesterol level in subjects with and without cholesteryl ester transfer protein deficiency. *Hum Genet* **96**, 521–6.

Alexander WS & Hilton DJ (2004) The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immuneresponse. *Ann Rev Immunol* **22**, 503-529.

Alwine JC, Kemp DJ & Stark GR (1977) Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc Natl Acad Sci U S A* **74**, 5350-5354.

Amann RI, Krumholz L & Stahl DA (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental-studies in microbiology. *J Bacteriol* **172**, 762-770.

Amann RI, Ludwig W & Schleifer KH (1995) Phylogenetic Identification and in-situ detection of individual microbial-cells without cultivation. *Microbiol Rev* **59**, 143-169.

Amor KB & Vaughan EE (2006) Molecular ecology of the human intestinal microbiota. In Gastrointestinal microbiology, 1st Edition, AC Ouwehand & EE Vaughan (eds.), Taylor & Francis Group, LLC, New York. USA, pp. 1-23.

Andoh A, Tsujikawa T, Ishizuka I, Araki Y, Sasaki M, Koyama S & Fujiyama Y (2003) N-3 fatty acid-rich diet prevents early response of interleukin-6 elevation in trinitrobenzene sulfonic acid-induced enteritis. *Int J Molecular Med* **12**, 721-725.

Andrikopoulos S, Massa CM, Aston-Mourney K, Funkat A (2005) Differential effect of inbred mouse strain (C57BL/6, DBA/2, 129T2) on insulin secretory function in response to a high fat diet. *J Endocrininol* **187**, 45-53.

Anonymous (1977) Report of the American Institute of Nutrition Ad Hoc Committee on standards for nutritional studies. *J Nutr* **107**, 1340-1348.

Arita M, Bianchini F, Aliberti J, Sher A, Chiang N, Hong S, Yang R, Petasis NA & Serhan CN (2005) Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. *J Exp Med* **201**, 713-722.

Auboeuf D, Rieusset J, Fajas L, Vallier P, Frering V, Riou JP, Staels P, Auwerx J, Laville M & Vidal H (1997) Tissue distribution and quantification of the expression of mRNAs of peroxisome proliferator-activated receptors and liver X receptor-alpha in humans - No alteration in adipose tissue of obese and NIDDM patients. *Diabetes* **46**, 1319-1327.

Auwerx J (1992) Regulation Of Gene-Expression By Fatty-Acids And Fibric Acid-Derivatives - An Integrative Role For Peroxisome Proliferator Activated Receptors - The Belgian-Endocrine-Society Lecture 1992. *Hormone Res* **38**, 269-277.

Baer DJ, Judd JT, Clevidence BA & Tracy RP (2004) Dietary fatty acids affect plasma markers of inflammation in healthy men fed controlled diets: a randomized crossover study. *Am J Clin Nutr* **79**, 969-973.

Balish E & Warner T (2002) Enterococcus faecalis induces inflammatory bowel disease in interleukin-10 knockout mice. *Am J Pathol* **160**, 2253-2257.

Bannenberg G, Arita M & Serhan CN (2007) Endogenous receptor agonists: resolving inflammation. *TheScientificWorldJOURNAL* **7**, 1440-1462.

Barber MD, Fearon KCH & Ross JA (2005) Eicosapentaenoic acid modulates the immune response but has no effect on a mimic of antigen-specific responses. *Nutrition* **21**, 588-593.

Baribault H, Penner J, Iozzo RV & Wilsonheiner M (1994) Colorectal hyperplasia and inflammation in keratin 8-deficient Evb/N mice. *Genes Dev* **8**, 2964-2973.

Barraclough D, Obenland D, Laing W & Carroll T (2004) A general method for two-dimensional protein electrophoresis of fruit samples. *Postharv Biol Technol* **32**, 175-181.

Bartsch H, Nair J & Owen RW (1999) Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. *Carcinogenesis* **20**, 2209-2218.

Bassaganya-Riera J & Hontecillas R (2006) CLA and n-3 PUFA differentially modulate clinical activity and colonic PPAR-responsive gene expression in a pig model of experimental IBD. *Clin Nutr* **25**, 454-465.

Bassaganya-Riera J, Reynolds K, Martino-Catt S, Cui YZ, Hennighausen L, Gonzalez F, Rohrer J, Benninghoff AU & Hontecillas R (2004) Activation of PPAR gamma and delta by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. *Gastroenterology* **127**, 777-791.

Beer HD, Bittner M, Niklaus G, Munding C, Max N, Goppelt A & Werner S (2005) The fibroblast growth factor binding protein is a novel interaction partner of FGF-7, FGF-10 and FGF-22 and regulates FGF activity: implications for epithelial repair. *Oncogene* **24**, 5269-5277.

Beg AA, Sha WC, Bronson RT & Baltimore D (1995) Constitutive Nf-Kappa-B activation, enhanced granulopoiesis, and neonatal lethality in I-Kappa-B-Alpha-deficient mice. *Genes Dev* **9**, 2736-2746.

Belluzzi A (2002) N-3 Fatty acids for the treatment of inflammatory bowel disease. *Proc Nutr Soc* **61**, 391 - 395.

Benjamini Y & Hochberg Y (1995) Controlling the false discovery rate - A practical and powerful approach to multiple testing. *J Royal Stat Soc B - Methodological* **57**, 289-300.

Berg DJ, Davidson N, Kuhn R, Muller W, Menon S, Holland G, ThompsonSnipes L, Leach MW & Rennick D (1996) Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest* **98**, 1010-1020.

Berger A, Roberts MA & Hoff B (2006) How dietary arachidonic- and docosahexaenoic-acid rich oils differentially affect the murine hepatic transcriptome. *Lipids Health Dis* **5**.

Berruyer C, Pouyet L, Millet V, Martin FM, LeGoffic A, Canonici A, Garcia S, Bagnis C, Naquet P & Galland F (2006) Vanin-1 licenses inflammatory mediator production by gut epithelial cells and controls colitis by antagonizing peroxisome proliferator-activated receptor gamma activity. *J Exp Med* **203**, 2817-2827.

Bibiloni R, Simon MA, Albright C, Sartor B & Tannock GW (2005) Analysis of the large bowel microbiota of colitic mice using PCR/DGGE. *Lett Appl Microbiol* **41**, 45-51.

Bieri JG (1980) Second Report of the ad hoc Committee on standards for nutritional studies. *J. Nutr.* **110**, 1726.

Bierne H, Gouin E, Roux P, Caroni P, Yin HL & Cossart P (2001) A role for cofilin and LIM kinase in Listeria-induced phagocytosis. *J Cell Biol* **155**, 101-112.

Blanchard RK, Moore JB, Green CL & Cousins RJ (2001) Modulation of intestinal gene expression by dietary zinc status: Effectiveness of cDNA arrays for expression profiling of a single nutrient deficiency. *Proc Nat Acad Sci USA* **98**, 13507-13513.

Boirivant M, Fuss IJ, Chu A & Strober W (1998) Oxazolone colitis: A murine model of T helper cell type 2 colitis treatable with antibodies to interleukin 4. *J Exp Med* **188**, 1929-1939.

Bomprezzi R, Ringner M, Kim S, Bittner ML, Khan J, Chen YD, Elkahloun A, Yu AM, Bielekova B, Meltzer PS, Martin R, McFarland HF & Trent JM (2003) Gene expression profile in multiple sclerosis patients and healthy controls: identifying pathways relevant to disease. *Human Mol Genet* **12**, 2191-2199.

Bordoni A, Di Nunzio M, Danesi F & Biagi PL (2006) Polyunsaturated fatty acids: from diet to binding to ppars and other nuclear receptors. *Genes Nutr* **1**, 95-106.

Bouma G & Strober W (2003) The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* **3**, 521-533.

Bradford MM (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal Biochem* **72**, 248-254.

Bristol IJ, Farmer MA, Cong YZ, Zheng XX, Srom TB, Elson CO, Sundberg JP & Leiter EH (2000) Heritable susceptibility for colitis in mice induced by IL-10 deficiency. *Inflamm Bowel Dis* **6**, 290-302.

Brown JD & Plutzky J (2007) Peroxisome proliferator-activated receptors as transcriptional nodal points and therapeutic targets. *Circulation* **115**, 518-533.

Browning JD & Horton JD (2004) Molecular mediators of hepatic steatosis and liver injury. *J Clin Invest* **114**, 147-152.

Bünger M, van den Bosch HM, van der Meijde J, Kersten S, Hooiveld G & Müller M (2007) Genome-wide analysis of PPAR alpha activation in murine small intestine. *Physiol Genomics* **30**, 192-204.

Burdick AD, Kim DJ, Peraza MA, Gonzalez FJ & Peters JM (2006) The role of peroxisome proliferator-activated receptor-beta/delta in epithelial cell growth and differentiation. *Cell Signal* **18**, 9-20.

Burgess AW (1998) Growth control mechanisms in normal and transformed intestinal cells. *Phil Trans Royal Soc London Series B-Biological Sciences* **353**, 903-909.

Burke JP, Mulsow JJ, O'Keane C, Docherty NG, Watson RWG & O'Connell PR (2007) Fibrogenesis in Crohn's disease. *Am J Gastroenterol* **102**, 439-448.

Burkhardt JK, Carrizosa E & Shaffer MH (2008) The actin cytoskeleton in T cell activation. *Ann Rev Immunol* **26**, 233-259.

Burnens AP, Stanley J, Morgenstern R & Nicolet J (1994) Gastroenteritis associated with Helicobacter-pullorum. *Lancet* **344**, 1569-1570.

Calder PC (2003) n-3 polyunsaturated fatty acids and inflammation: From molecular biology to the clinic. *Lipids* **38**, 343-352.

Calder PC (2006) n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nutr* **83**, 1505S-1519S.

Calder PC (2008) Polyunsaturated fatty acids, inflammatory processes and inflammatory bowel diseases. *Mol Nutr Food Res* **52**, 000-000.

Camandola S, Leonarduzzi G, Musso T, Varesio L, Carini R, Scavazza A, Chiarpotto E, Baeuerle PA & Poli G (1996) Nuclear factor kB is activated by arachidonic acid but not by eicosapentaenoic acid. *Biochem Biophys Res Commun* **229**, 643-647.

Camarero N, Mascaro C, Mayordomo C, Vilardell F, Haro D & Marrero PF (2006) Ketogenic HMGCS2 is a c-Myc target gene expressed in differentiated cells of human colonic epithelium and down-regulated in colon cancer. *Mol Cancer Res* **4**, 645-653.

Campos FG, Waitzberg DL, Habr-Gama A, Logullo AF, Noronha IL, Jancar S, Torrinhas RSM & Furst P (2002) Impact of parenteral n-3 fatty acids on experimental acute colitis. *Brit J Nutr* **87**, S83-S88.

Cantor CR & Schimmel PR (1980) Techniques for the study of biological structure and function. In: Biophysical Chemistry: Part II, CR Cantor & PR Schimmel (eds.), W. H. Freeman.

Caplan MS, Russell T, Xiao Y, Amer M, Kaup S & Jilling T (2001) Effect of polyunsaturated fatty acid (PUFA) supplementation on intestinal inflammation and necrotizing enterocolitis (NEC) in a neonatal rat model. *Pediatr Res* **49**, 647-652.

Carey MC, Small DM & Bliss CM (1983) Lipid digestion and absorption. *Annu Rev Physiol* **45**, 651-677.

Ceelen LM, Decostere A, Van den Buick K, On SLW, Baele M, Ducatelle R & Haesebrouck F (2006) Helicobacter pullorum in chickens, Belgium. *Emerg Infect Dis* **12**, 263-267.

Chadwick VS & Anderson RP (1992) Microorganisms and their products in inflammatory bowel disease. In Inflammatory bowel disease, RP MacDermott (ed.), Elsevier. New York, USA, pp. 241-258.

Chakravarthy MV, Pan ZJ, Zhu YM, Tordjman K, Schneider JG, Coleman T, Turk J & Semenkovich CF (2005) "New" hepatic fat activates PPAR alpha to maintain glucose, lipid, and cholesterol homeostasis. *Cell Metab* **1**, 309-322.

Chapkin RS, Davidson LA, Ly L, Weeks BR, Lupton JR & McMurray DN (2007) Immunomodulatory effects of (n-3) fatty acids: Putative link to inflammation and colon cancer. *J Nutr* **137**, 200S-204S.

Chin EY, Dangler CA, Fox JG & Schauer DB (2000) Helicobacter hepaticus infection triggers inflammatory bowel disease in T cell receptor alpha beta mutant mice. *Comp Med* **50**, 586-594.

Cho JH (2006) Recent progress in inflammatory bowel disease genetics. In Immune mechanisms in inflammatory bowel disease, 1st Edition, RS Blumberg and MF Neurath (eds.), Springer Science and Business Media, NY, USA, pp. 24-31.

Cho JH (2008) The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol* **8**, 458-466.

Clarke SD (2000) Polyunsaturated fatty acid regulation of gene transcription: a mechanism to improve energy balance and insulin resistance. *Brit J Nutr* **83**, S59-S66.

Cobrin GM & Abreu MT (2005) Defects in mucosal immunity leading to Crohn's disease. *Immunol Rev* **206**, 277-295.

Coico RF, Sunshine G & Benajmini E (2003) Immunology: A short course. 5th Edition, John Wiley & Sons. Hoboken, USA.

Colombel JF, Lemann M, Cassagnou M, Bouhnik Y, Duclos B, Dupas JL, Notteghem B & Mary JY (1997) A controlled trial comparing ciprofloxacin with trial mesalazine for the treatment of active Crohn's disease. *Gastroenterology* **112**, A951-A951.

Conte MP, Schippa S, Zamboni I, Penta M, Chiarini F, Seganti L, Osborn J, Falconieri P, Borrelli O & Cucchiara S (2006) Gut-associated bacterial microbiota in paediatric patients with inflammatory bowel disease. *Gut* **55**, 1760-1767.

Cummings JH, Wiggins HS, Jenkins DJA, Houston H, Jivraj T, Drasar BS & Hill MJ (1978) Influence of diets high and low in animal fat on bowel habit, gastrointestinal transit-time, fecal microflora, bile-acid, and fat excretion. *J Clin Investig* **61**, 953-963.

Cuzzocrea S, Di Paola R, Mazzon E, Genovese T, Muia C, Centorrino T & Caputi AP (2004) Role of endogenous and exogenous ligands for the peroxisome proliferators activated receptors alpha (PPAR-alpha) in the development of inflammatory bowel disease in mice. *Lab Investig* **84**, 1643-1654.

Dallas PB, Gottardo NG, Firth MJ, Beesley AH, Hoffmann K, Terry PA, Freitas JR, Boag JM, Cummings AJ & Kees UR (2005) Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR - how well do they correlate? *BMC Genomics* **6**.

Daniel PT, Wieder T, Sturm I & Schulze-Osthoff K (2001) The kiss of death: promises and failures of death receptors and ligands in cancer therapy. *Leukemia* **15**, 1022-1032.

Darfeuille-Michaud A, Neut C, Barnich N, Lederman E, Di Martino P, Desreumaux P, Gambiez L, Joly B, Cortot A & Colombel JF (1998) Presence of adherent Escherichia coli strains in ileal mucosa of patients with Crohn's disease. *Gastroenterology* **115**, 1405-1413.

Das UN (2002) Essential fatty acids as possible enhancers of the beneficial actions of probiotics. *Nutrition* **18**, 786-789.

Davidson NJ, Fort MM, Müller W, Leach MW & Rennick DM (2000) Chronic colitis in IL-10-/- mice: insufficient counter regulation of a Th1 response. *Int Rev Immunol* **19**, 91-121.

de Buhr MF, Mahler M, Geffers R, Hansen W, Westendorf AM, Lauber J, Buer J, Schlegelberger B, Hedrich HJ & Bleich A (2006) Cd14, Gbp1, and Pla2g2a: three major candidate genes for experimental IBD identified by combining QTL and microarray analyses. *Physiol Genomics* **25**, 426-434.

de Graaf AA, Freidig AP, De Roos B, Jamshidi N, Heinemann M, Rullmann JAC, Hall KD, Adiels M & van Ommen B (2009) Nutritional systems biology modeling: From molecular mechanisms to physiology. *PLoS Comput Biol* **5 (11)**.

de Roos B & McArdle HJ (2008) Proteomics as a tool for the modelling of biological processes and biomarker development in nutrition research. *Brit J Nutr* **99**, S66-S71.

De Vogel-van den Bosch HM, Bünger M, De Groot PJ, Bosch-Vermeulen H, Hooiveld GJEJ & Muller M (2008) PPARalpha-mediated effects of dietary lipids on intestinal barrier gene expression. *BMC Genomics* **9**, 231.

De Windt LJ, Cox K, Hofstra L & Doevendans PA (2002) Molecular and genetic aspects of cardiac fatty acid homeostasis in health and disease. *Eur Heart J* **23**, 774-787.

Deckelbaum RJ, Worgall TS & Seo T (2006) n-3 fatty acids and gene expression. *Am. J. Clin. Nutr.* **83**, 1520S-1525S.

Desvergne B, Michalik L & Wahli W (2004) Be fit or be sick: Peroxisome proliferator-activated receptors are down the road. *Mol Endocrinol* **18**, 1321-1332.

Desvergne B & Wahli W (1999) Peroxisome proliferator-activated receptors: Nuclear control of metabolism. *Endocr Rev* **20**, 649-688.

Devchand PR, Keller H, Peters JM, Vazquez M, Gonzalez FJ & Wahli W (1996) The PPAR alpha-leukotriene B-4 pathway to inflammation control. *Nature* **384**, 39-43.

Dianda L, Hanby AM, Wright NA, Sebesteny A, Hayday AC & Owen MJ (1997) T cell receptor-alpha beta-deficient mice fail to develop colitis in the absence of a microbial environment. *Am J Pathol* **150**, 91-97.

Dijkstra G, Zandvoort AJH, Kobold ACM, de Jager-Krikken A, Heeringa P, van Goor H, van Dullemen HM, Tervaert JWC, van de Loosdrecht A, Moshage H & Jansen PLM (2002) Increased expression of inducible nitric oxide synthase in circulating monocytes from patients with active inflammatory bowel disease. *Scand J Gastroenterol* **37**, 546-554.

Disset A, Cheval L, Soutourina O, Duong Van Huyen J-P, Li G, et al. (2009) Tissue compartment analysis for biomarker discovery by gene expression profiling. *Plos One* **4**, e7779

Dobbin K, Shih JH & Simon R (2003) Statistical design of reverse dye microarrays. *Bioinformatics* **19**, 803-810.

Dommels YEM, Alink GM, van Bladeren PJ & van Ommen B (2002) Dietary n-6 and n-3 polyunsaturated fatty acids and colorectal carcinogenesis: results from cultured colon cells, animal models and human studies (vol 11, pg 297, 2002). *Environ Toxicol Pharmacol* **12**, 231-+.

Dommels YEM, Butts CA, Zhu S, Davy M, Martell S, Hedderly D, Barnett MPG, McNabb WC & Roy NC (2007) Characterization of intestinal inflammation and identification of related gene expression changes in mdr1a-/- mice. *Genes Nutr* **2**, 209-223.

Dos Remedios CG, Chhabra D, Kekic M, Dedova IV, Tsubakihara M, Berry DA & Nosworthy NJ (2003) Actin binding proteins: Regulation of cytoskeletal microfilaments. *Physiol Rev* **83**, 433-473.

Duchmann R, Kaiser I, Hermann E, Mayet W, Ewe K & ZumBuschenfelde KHM (1995) Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *Clin Exp Immunol* **102**, 448-455.

Duchmann R, May E, Heike M, Knolle P, Neurath M & zum Buschenfelde KHM (1999) T cell specificity and cross reactivity towards enterobacteria, Bacteroides, Bifidobacterium, and antigens from resident intestinal flora in humans. *Gut* **44**, 812-818.

Dulpus E, Glorian M & Forest C (2000) Fatty acid regulation of gene transcription. *J Biol Chem* **275**, 30749-30752.

Dutta S, Chatterjee A, Dutta P, Rajendran K, Roy S, Pramanik KC & Bhattacharya SK (2001) Sensitivity and performance characteristics of a direct PCR with stool samples in comparison to conventional techniques for diagnosis of Shigella and enteroinvasive Escherichia coli infection in children with acute diarrhoea in Calcutta, India. *J Med Microbiol* **50**, 667-674.

Eastwood MA AG (1995) The effect of olestra on breath gas production and faecal microbial counts. *Eur J Clin Nutr* **49**, 627–639.

Eckburg PB & Relman DA (2007) The role of microbes in Crohn's disease. *Clin Infect Dis* **44**, 256-262.

Ehrlich HP, Allison GM, Page MJ, Kolton WA & Graham M (2000) Increased gelsolin expression and retarded collagen lattice contraction with smooth muscle cells from Crohn's diseased intestine. *J Cell Physiol* **182**, 303-309.

El-Sohemy A, Cornelis MC, Kabagambe EK & Campos H (2007) Coffee, CYP1A2 genotype and risk of myocardial infarction. *Genes Nutr* **2**, 155-156.

Elson CO, Beagley KW, Sharmanov AT, Fujihashi K, Kiyono H, Tennyson GS, Cong YZ, Black CA, Ridwan BW & McGhee JR (1996) Hapten-induced model of murine inflammatory bowel disease - Mucosal immune responses and protection by tolerance. *J Immunol* **157**, 2174-2185.

Elson CO, Cong Y, McCracken VJ, Dimmitt RA, Lorenz RG & Weaver CT (2005) Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunol Rev* **206**, 260-276.

Elson CO & Weaver CT (2003) Experimental mouse models of inflammatory bowel disease: new insights into pathogenic mechanisms. In Inflammatory bowel disease: From bench to bedside. 2nd Edition, SR Targan, F Shanahan & LC Karp (eds.), Kluwer Academic Publishers. Cornwall, Great Britain, pp.67-99.

Embey LR, Jewell LD, Garg ML, Thomson AB & al e (1991) Indomethacin worsens and a leukotriene biosynthesis inhibitor accelerates mucosal healing in rat colitis. *Can J Physiol Pharm* **69**, 480-487.

Endo K, Kumemura M, Nakamura K, Fujisawa T, Suzuki K, Benno Y & Mitsuoka T (1991) Effect of high cholesterol diet and polydextrose supplementation on the microflora, bacteria enzyme activity, putrefactive products, volatile fatty acid (VFA) profile, weight, and pH of the feces in healthy volunteers. *Bifidobact Microflora* **10**, 53-64.

Eng JK, McCormack AL & Yates JR (1994) An approach to correlate tandem mass-spectral data of peptides with amino-acid-sequences in a protein database. *J Am Soc Mass Spectrom* **5**, 976-989.

Ewaschuk JB & Dieleman LA (2006) Probiotics and prebiotics in chronic inflammatory bowel diseases. *World J Gastroenterol* **12**, 5941-5950.

Farnworth ER, Chouinard YP, Jacques H, Venkatramanan S, Maf AA, Defnoun S & Jones PJH (2007) The effect of drinking milk containing conjugated linoleic acid on fecal microbiological profile, enzymatic activity, and fecal characteristics in humans. *Nutr J* **6**, 15.

Favier C, Neut C, Mizon C, Cortot A, Colombel JF & Mizon J (1997) Fecal beta-D-galactosidase production and Bifidobacteria are decreased in Crohn's disease. *Dig Dis Sci* **42**, 817-822.

Ferguson LR (2006) Nutrigenomics - Integrating genomic approaches into nutrition research. *Mol Diagn Ther* **10**, 101-108.

Ferguson LR, Shelling AN, Browning BL, Huebner C & Petermann I (2007) Genes, diet and inflammatory bowel disease. *Mut Res-Fund Mol Mech Mutag* **622**, 70-83.

Ferguson LR, Han DY & Huebner Cea (2008) Single nucleotide polymorphisms in IL4, OCTN1 and OCTN2 genes in association with inflammatory bowel disease phenotypes in a Caucasian population in Canterbury, New Zealand. *Open Gastroenterol J* **2**, 50–56.

Fiocchi C (2003) The normal intestinal mucosa: a state of 'controlled inflammation'. In Inflammatory bowel disease: From bench to bedside, 2nd Edition, SR Targan, F Shanahan & LC Karp (eds.), Kluwer Academic Publishers. Cornwall, Great Britain, pp. 101-120.

Fiore S, Maddox JF, Perez HD & Serhan CN (1994) Identification Of A Human Cdna-Encoding A Functional High-Affinity Lipoxin A(4) Receptor. *J Exp Med* **180**, 253-260.

Fisher SM (1997) Prostaglandins and Cancer. *Front Biosci* **2**, 482-500.

Foitzik T, Kruschewski M, Kroesen A & Buhr HJ (1999) Does microcirculation play a role in the pathogenesis of inflammatory bowel diseases? Answers from intravital microscopic studies in animal models. *Int J Colorectal Dis* **14**, 29-34.

Fox JG, Gorelick PL, Kullberg MC, Ge ZM, Dewhirst FE & Ward JM (1999) Novel urease-negative Helicobacter species associated with colitis and typhlitis in IL-10-deficient mice. *Infect Immun* **67**, 1757-1762.

Franchimont D, Vermeire S, El Housni H, Pierik M, Van Steen K, Gustot T, Quertinmont E, Abramowicz M, Van Gossum A, Deviere J & Rutgeerts P (2004) Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4

Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. *Gut* **53**, 987-992.

Frank DN, Amand ALS, Feldmann RA, Boedeker EC, Harpaz N & Pace NR (2007) Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA* **104**, 13780-13785.

Franks AH, Harmsen HJM, Raangs GC, Jansen GJ, Schut F & Welling GW (1998) Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-Targeted oligonucleotide probes. *Appl Environ Microbiol* **64**, 3336-3345.

Fromin N, Hamelin J, Tarnawski S, Roesti D, Jourdain-Miserez K, Forestier N, Teyssier-Cuvelle S, Gillet F, Aragno M & Rossi P (2002) Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environ Microbiol* **4**, 634-643.

Fukushima K & Fiocchi C (2004) Paradoxical decrease of mitochondrial DNA deletions in epithelial cells of active ulcerative colitis patients. *Am J Physiol-Gastrointest Liver Physiol* **286**, G804-G813.

Fukushima K, Sasaki I, Ogawa H, Naito H, Funayama Y & Matsuno S (1999) Colonization of microflora in mice: mucosal defense against luminal bacteria. *J Gastroenterol* **34**, 54-60.

Furham JA, MacCallum K & Davis AA (1992) Novel major archebacterial group from marine plankton. *Nature* **356**, 148-149.

Ganesan S, Travis SPL, Ahmad T & Jazrawi R (2002) Role of tumor necrosis factor in Crohn's disease. *Curr Opin Invest Drugs* **3**, 1297–1300.

Garcia L, Tercero JC, Legido B, Ramos JA, Alemany J & Sanz M (1998) Rapid detection of Actinobacillus actinomycetemcomitans, Prevotella intermedia and Porphyromona gingivalis by multiplex PCR. *J Periodontal Res* **33**, 59-64.

Gassull MA (2001) New insights in nutritional therapy in inflammatory bowel disease. *Clin Nutr* **20**, 113-121.

Geerling BJ, van Houwelingen AC, Badart-Smook A, Stockbrugger RW & Brummer JM (1999) Fat intake and fatty acid profile in plasma phospholipids and adipose tissue in patients with Crohn's disease, compared with controls. *Am J Gastroenterol* **94**, 410-417.

Gill HS, Shu Q, Lin H, Rutherfurd KJ & Cross ML (2001) Protection against translocating Salmonella typhimurium infection in mice by feeding the immuno-enhancing probiotic Lactobacillus rhamnosus strain HN001. *Med Microbiol Immunol* **190**, 97-104.

Girroir EE, Hollingshead HE, He P, Zhu B, Perdew GH & Peters JM (2008) Quantitative expression patterns of peroxisome proliferator-activated receptor-beta/delta (PPAR beta/delta) protein in mice. *Biochem Biophys Res Commun* **371**, 456-461.

Goodwin JS & Ceuppens J (1983) Regulation of the immune-response by prostaglandins. *J Clin Immunol* **3**, 295-315.

Goring HHH, Curran JE, Johnson MP, Dyer TD, Charlesworth J, Cole SA, Jowett JBM, Abraham LJ, Rainwater DL, Comuzzie AG, Mahaney MC, Almasy L, MacCluer JW, Kissebah AH, Collier GR, Moses EK & Blangero J (2007) Discovery of expression QTLs using large-scale transcriptional profiling in human lymphocytes. *Nat Genet* **39**, 1208-1216.

Gottschalk G (1986) Bacterial fermentation. In Bacterial metabolism. 1st Edition, Springer-Verlag. New York, USA, pp. 210-282.

Goudriaan JR, Dahlmans VEH, Febbraio M, Teusink B, Romijn JA, Havekes LM & Voshol PJ (2002) Intestinal lipid absorption is not affected in CD36 deficient mice. *Mol Cell Biochem* **239**, 199-202.

Grammatikos SIS, P.V.; Victor, T.A. and Miller, W.M. (1994) Diverse effects of essential (n-6 and n-3) fatty acids on cultured cells. *Cytotechnology* **15**, 31-50.

Gui GPH, Thomas PRS, Tizard MLV, Lake J, Sanderson JD & HermonTaylor J (1997) Two-year-outcomes analysis of Crohn's disease treated with rifabutin and macrolide antibiotics. *J Antimicrob Chemother* **39**, 393-400.

Gurtner GJ, Newberry RD, Schloemann SR, McDonald KG & Stenson WF (2003) Inhibition of indoleamine 2,3-dioxygenase augments trinitrobenzene sulfonic acid colitis in mice. *Gastroenterology* **125**, 1762-1773.

Haferlach T, Kohlmann A, Kern W, Hiddemann W, Schnittger S & Schoch C (2003) Gene expression profiling as a tool for the diagnosis of acute leukemias. *Seminars In Hematology* **40**, 281-295.

Hahm KB, Im YH, Parks TW, Park SH, Markowitz S, Jung HY, Green J & Kim SJ (2001) Loss of transforming growth factor beta signalling in the intestine contributes to tissue injury in inflammatory bowel disease. *Gut* **49**, 190-198.

Hansen Petrik MM, MF; Chiu, C and Whelan, J (2000) Antagonism of arachidonic acid is linked to the antitumorigenic effect of dietary eicosapentaenoic acid in Apc Min mice. *J Nutr* **130**, 1153-1158.

Harding HP, Zhang YH & Ron D (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* **397**, 271-274.

Haton C, Francois A, Vandamme M, Wysocki J, Griffiths NM & Benderitter M (2007) Imbalance of the antioxidant network of mouse small intestinal mucosa after radiation exposure. *Radiat Res* **167**, 445-453.

Hegazi RAF, Saad RS, Mady H, Matarese LE, O'Keefe S & Kandil HM (2006) Dietary fatty acids modulate chronic colitis, colitis-associated colon neoplasia and COX-2 expression in IL-10 knockout mice. *Nutrition* **22**, 275-282.

Heimerl S, Moehle C, Zahn A, Boettcher A, Stremmel W, Langmann T & Schmitz G (2006) Alterations in intestinal fatty acid metabolism in inflammatory bowel disease. *Biochim Biophys Acta-Mol Basis Dis* **1762**, 341-350.

Heimesaat MM, Bereswill S, Fischer A, Fuchs D, Struck D, Niebergall J, Jahn HK, Dunay IR, Moter A, Gescher DM, Schumann RR, Gobel UB & Liesenfeld O (2006) Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with Toxoplasma gondii. *J Immunol* **177**, 8785-8795.

Heimesaat MM, Fischer A, Goebel UB, Bereswill S & Rieder G (2007) Shifts to-wards pro-inflammatory bacteria in the intestinal flora of Helicobacter pylori-infected Mongolian gerbils. *Int J Med Microbiol* **297**, 60-60.

Heinaniemi M, Uski JO, Degenhardt T & Carlberg C (2007) Meta-analysis of primary target genes of peroxisome proliferator-activated receptors. *Genome Biol* **8**.

Hermiston ML & Gordon JI (1995) Inflammatory bowel-disease and adenomas in mice expressing a dominant-negative N-cadherin. *Science* **270**, 1203-1207.

Hertz R, Magenheim J, Berman I & Bar-Tana J (1998) Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4 alpha. *Nature* **392**, 512-516.

Hoentjen F, Welling GW, Harmsen HJM, Zhang XY, Snart J, Tannock GW, Lien K, Churchill TA, Lupicki M & Dieleman LA (2005) Reduction of colitis by prebiotics in HLA-1327 transgenic rats is associated with microflora changes and immunomodulation. *Inflamm Bowel Dis* **11**, 977-985.

Hoffmann M, Rath E, Holzlwimmer G, Quintanilla-Martinez L, Loach D, Tannock G & Haller D (2008) *Lactobacillus reuteri* 100-23 transiently activates intestinal epithelial cells of mice that have a complex microbiota during early stages of colonization. *J Nutr* **138**, 1684-1691.

Holland PM, Abramson RD, Watson R & Gelfand DH (1991) Detection of specific polymerase chain-reaction product by utilizing the 5'-3' exonuclease activity of thermus-aquaticus DNA-polymerase. *Proc Natl Acad Sci USA* **88**, 7276-7280.

Hollander GA, Simpson SJ, Mizoguchi E, Nichogiannopoulou A, She JA, Gutierrezramos JC, Bhan AK, Burakoff SJ, Wang BP & Terhorst C (1995) Severe colitis in mice with aberrant thymic selection. *Immunity* **3**, 27-38.

Honda K & Takeda K (2009) Regulatory mechanisms of immune responses to intestinal bacteria. *Mucos Immunol* **2**, 187-196.

Hornquist CE, Lu XH, RogersFani PM, Rudolph U, Shappell S, Birnbaumer L & Harriman GR (1997) G alpha i2-deficient mice with colitis exhibit a local increase in memory CD4(+) T cells and proinflammatory Th1-type cytokines. *J Immunol* **158**, 1068-1077.

Hosack DA, Dennis G, Sherman BT, Lane HC & Lempicki RA (2003) Identifying biological themes within lists of genes with EASE. *Genome Biol* **4**.

Hozapfel WH, Haberer P, Geisen R, Bjorkroth J & Schillinger U (2001) Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am J Clin Nutr* **73**(suppl), 365-373.

Huang CY, Yang LC, Liu KY, Chang IC, Liao PH, Chou JIY, Chou MY, Lin WW & Yang JJ (2009) ZAK negatively regulates RhoGDI beta-induced Rac1-mediated hypertrophic growth and cell migration. *J Biomed Sci* **16**.

Hudault S, Guignot J & Servin AL (2001) *Escherichia coli* strains colonising the gastrointestinal tract protect germfree mice against *Salmonella typhimurium* infection. *Gut* **49**, 47-55.

Hudert CA, Weylandt KH, Lu Y, Wang JD, Hong S, Dignass A, Serhan CN & Kang JX (2006) Transgenic mice rich in endogenous omega-3 fatty acids are protected from colitis. *Proc Natl Acad. Sci. U. S. A.* **103**, 11276-11281.

Huijsdens XW, Linskens RK, Mak MT, Meuwissen SGM, Vandenbroucke-Grauls C & Savelkoul PHM (2002) Quantification of bacteria adherent to gastrointestinal mucosa by real-time PCR. *J Clin Microbiol* **40**, 4423-4427.

Ijpenberg A, Jeannin E, Wahli W & Desvergne B (1997) Polarity and specific sequence requirements of peroxisome proliferator-activated receptor (PPAR) retinoid X receptor heterodimer binding to DNA - A functional analysis of the malic enzyme gene PPAR response element. *J Biol Chem* **272**, 20108-20117.

Inohara N, Ogura Y & Nunez G (2002) Nods: a family of cytosolic proteins that regulate the host response to pathogens. *Curr Opin Microbiol* **5**, 76-80.

Irvine EJ & Marshall JK (2000) Increased intestinal permeability precedes the onset of Crohn's disease in a subject with familial risk. *Gastroenterology* **119**, 1740-1744.

Issemann I & Green S (1990) Activation of a member of the steroid-hormone receptor superfamily by peroxisome proliferators. *Nature* **347**, 645-650.

Itoh K, Wakabayashi N, Katoh Y, Ishii T, O'Connor T & Yamamoto M (2003) Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells* **8**, 379-391.

Izcue A, Coombes JL & Powrie F (2006) Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Rev* **212**, 256-271.

Jackson L, Wahli W, Michalik L, Watson SA, Morris T, Anderton K, Bell DR, Smith JA, Hawkey CJ & Bennett AJ (2003) Potential role for peroxisome proliferator activated receptor (PPAR) in preventing colon cancer. *Gut* **52**, 1317-1322.

James MJ, Gibson RA & Cleland LG (2000) Dietary polyunsaturated fatty acids and inflammatory mediator production. *Am J Clin Nutr* **71**, 343S-348S.

Janeway ChA, Travers P, Walport M & Schlomchik MJ (2001) Immunobiology: the immune system in health and disease. 5th Edition, Garland Publishing. New York, USA.

Jenkins DJA, Sievenpiper JL, Pauly D, Sumaila UR, Kendall CWC & Mowat FM (2009) Are dietary recommendations for the use of fish oils sustainable? *Can Med Assoc J* **180**, 633-637.

Jeenah M, Kessling A, Miller N & Humphries SE (1990) G to A substitution in the promoter region of the apolipoprotein AI gene is associated with elevated serum apolipoprotein AI and high density lipoprotein cholesterol concentrations. *Mol Biol Med* **7**, 233–41.

Jett BD, Huycke MM & Gilmore MS (1994) Virulence of Enterococci. *Clin Microbiol Rev* **7**, 462-&.

Jobin C & Sartor RB (2000) NF-kappa B signaling proteins as therapeutic targets for inflammatory bowel diseases. *Inflamm Bowel Dis* **6**, 206-213.

Johnson LR (2006) Apoptosis in the gastrointestinal tract. In: LR Johnson, KE Barrett, FK Ghisham, JL Merchant, HM Said, JD Wood (eds), Physiology of the Gastrointestinal Tract. 4th ed., San Diego, CA: Elsevier, pp. 345-374.

Jung JH, Yoon T, Choi EC & Lee K (2002) Interaction of cofilin with triose-phosphate isomerase contributes glycolytic fuel for Na,K-ATPase via Rho-mediated signaling pathway. *J Biol Chem* **277**, 48931-48937.

Juran BD & Lazaridis KN (2007) Applying genomics to the study of complex disease. *Seminars In Liver Disease* **27**, 3-12.

Jurjus AR, Khoury NH & Reimund JM (2004) Animal models of inflammatory bowel disease. *J Pharmacol Toxicol Meth* **50**, 81-92.

Kafatos FC, Jones CW & Efstratiadis A (1979) Determination of nucleic-acid sequence homologies and relative concentrations by a dot hybridization procedure. *Nucleic Acids Res* **7**, 1541-1552.

Kagnoff MF & Eckmann L (1997) Epithelial cells as sensors for microbial infection. *J Clin Investig* **100**, S51-S55.

Kajiura T, Takeda T, Sakata S, Sakamoto M, Hashimoto M, Suzuki H, Suzuki M & Benno Y (2009) Change of intestinal microbiota with elemental diet and its impact on therapeutic effects in a murine model of chronic colitis. *Dig Dis Sci* **54**, 1892-1900.

Kankaanpaa PE, Salminen SJ, Isolauri E & Lee YK (2001) The influence of polyunsaturated fatty acids on probiotic growth and adhesion. *FEMS Microbiol Lett* **194**, 149-153.

Kaput J (2007) Nutrigenomics - 2006 update. *Clin Chem Lab Med* **45**, 279-287.

Kaput J (2008) Nutrigenomics research for personalized nutrition and medicine. *Curr Opin Biotechnol* **19**, 110-120.

Kaput J & Rodriguez RL (2004) Nutritional genomics: the next frontier in the postgenomic era. *Physiol Genomics* **16**, 166-177.

Karlsson A, Jägervall Ä, Pettersson M, Andersson A-K, Gillberg P-G & Melgar S (2008) Dextran sulphate sodium induces acute colitis and alters hepatic function in hamsters. *Int Immunopharmacol* **8**, 20–27.

Katz KD, Hollander D, Vadheim CM, McElree C, Delahunty T, Dadufalza VD, Krugliak P & Rotter JI (1989) Intestinal permeability in patients with Crohn's disease and their healthy relatives. *Gastroenterology* **97**, 927-931.

Kelly D, Campbell JI, King TP, Grant G, Jansson EA, Coutts AGP, Pettersson S & Conway S (2004) Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. *Nat Immunol* **5**, 104-112.

Kennedy RJ, Hoper M, Deodhar K, Erwin PJ, Kirk SJ & Gardiner KR (2000) Interleukin 10-deficient colitis: new similarities to human inflammatory bowel disease. *Br J Surg* **87**, 1346-1351.

Kersten S, Desvergne B & Wahli W (2000) Roles of PPARs in health and disease. *Nature* 405, 421-424.

Keshavarzian A, Banan A, Farhadi A, Komanduri S, Mutlu E, Zhang Y & Fields JZ (2003) Increases in free radicals and cytoskeletal protein oxidation and nitration in the colon of patients with inflammatory bowel disease. *Gut* **52**, 720-728.

Kett K, Rognum TO & Brandtzaeg P (1987) Mucosal subclass distribution of immunoglobulin G-producing cells is different in ulcerative colitis and Crohn's disease of the colon. *Gastroenterology* **93**, 919-924.

Khovidhunkit W, Kim MS, Memon RA, Shigenaga JK, Moser AH, Feingold KR & Grunfeld C (2004) Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. *J Lipid Res* **45**, 1169-1196.

Kim SC, Tonkonogy SL, Albright CA, Tsang J, Balish EJ, Braun J, Huycke MM & Sartor RB (2005) Variable phenotypes of enterocolitis in interleukin 10-deficient mice monoassociated with two different commensal bacteria. *Gastroenterology* **128**, 891-906.

Kim SC, Tonkonogy SL, Karrasch T, Jobin C & Sartor RB (2007) Dual-association of gnotobiotic IL-10-/- mice vith 2 nonpathogenic commensal bacteria induces aggressive pancolitis. *Inflamm Bowel Dis* **13**, 1457-1466.

Kishi D, Takahashi I, Kai Y, Tamagawa H, Iijima H, Obunai S, Nezu R, Ito T, Matsuda H & Kiyono H (2000) Alteration of V beta usage and cytokine production of CD4(+) TCR beta beta homodimer T cells by elimination of *Bacteroides vulgatus* prevents colitis in TCR alpha-chain-deficient mice. *J Immunol* **165**, 5891-5899.

Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM & Lehmann JM (1997) Fatty acids and eicosanoids regulate

gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Nat Acad Sci USA* **94**, 4318-4323.

Knoch B, Barnett MPG, Zhu S, Park ZA, Nones K, Dommels YEM, Knowles SO, McNabb WC & Roy NC (2009) Genome-wide analysis of dietary eicosapentaenoic acid- and oleic acid-induced modulation in colon inflammation of interleukin-10 gene-deficient mice. *J Nutrigenet Nutrigenomics* **2**, 9-28.

Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F & Kollias G (1999) Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: Implications for joint and gut-associated immunopathologies. *Immunity* **10**, 387-398.

Kraus TA, Toy L, Chan L, Childs J & Mayer L (2004) Failure to induce oral tolerance to a soluble protein in patients with inflammatory bowel disease. *Gastroenterology* **126**, 1771-1778.

Kroman N & Green A (1980) Epidemiological studies in the Upernauik district, Greenland. *Acta Med Scand* **208**, 401-406.

Kufer TA, Banks DJ & Philpott DJ (2006) Innate immune sensing of microbes by Nod proteins. *Inflamm Bowel Dis: Genetics, Barrier Function, Immunologic Mechanisms, And Microbial Pathways* **1072**, 19-27.

Kühn R, Löhler J, Rennick D, Rajewsky K & Müller W (1993) Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**, 263-274.

Kullberg MC, Ward JM, Gorelick PL, Caspar P, Hieny S, Cheever A, Jankovic D & Sher A (1998) *Helicobacter hepaticus* triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and gamma interferon-dependent mechanism. *Infect Immun* **66**, 5157-5166.

Kuroki F, Iida M, Matsumoto T, Aoyagi K, Kanamoto K & Fujishima M (1997) Serum n3 polyunsaturated fatty acids are depleted in Crohn's disease. *Dig Dis Sci* **42**, 1137-1141.

Kussmann M, Raymond F & Affolter M (2006) OMICS-driven biomarker discovery in nutrition and health. *J Biotechnol* **124**, 758-787.

Laharie D, Asencio C, Asselineau J, Bulois P, Bourreille A, Moreau J, Bonjean P, Lamarque D, Pariente A, Soule JC, Charachon A, Coffin B, Perez P, Megraud F & Zerbib F (2009) Association between entero-hepatic Helicobacter species and Crohn's disease: a prospective cross-sectional study. *Aliment Pharmacol Ther* **30**, 283-293.

Lands WEM (1987) Proceedings of the AOCS short course on polyunsaturated fatty acids and eicosanoids. *American Oil Chemists' Society: Champaign, IL*.

Lang R, Patel D, Morris JJ, Rutschman RL & Murray PJ (2002) Shaping gene expression in activated and resting primary macrophages by IL-10. *J Immunol* **169**, 2253-2263.

Langmann T, Moehle C, Mauerer R, Scharl M, Liebisch G, Zahn A, Stremmel W & Schmitz G (2004) Loss of detoxification in inflammatory bowel disease: Dysregulation of pregnane X receptor target genes. *Gastroenterology* **127**, 26-40.

Langmann T & Schmitz G (2006) Loss of detoxification in inflammatory bowel disease. *Nat Clin Pract Gastroenterol Hepatol* **3**, 358-359.

Latruffe N & Vamecq J (1997) Peroxisome proliferators and peroxisome proliferator activated receptors (PPARs) as regulators of lipid metabolism. *Biochimie* **79**, 81-94.

Laubach VE, Shesely EG, Smithies O & Sherman PA (1995) Mice lacking inducible nitric-oxide synthase are not resistant to lipopolysaccharide-induced death. *Proc Natl Acad Sci USA* **92**, 10688-10692.

Lawson LD & Hughes BG (1988) Human absorption of fish oil fatty-acids as triacylglycerols, free acids, or ethyl-esters. *Biochem Biophys Res Commun* **152**, 328-335.

Lee G & Buchman AL (2009) DNA-driven nutritional therapy of inflammatory bowel disease. *Nutrition* **25**, 885-891.

Lee CK, Klopp RG, Weindruch R & Prolla TA (1999) Gene expression profile of aging and its retardation by caloric restriction. *Science* **285**, 1390-1393.

Lee J, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, Lee HK, Shen C, Cojocaru G, Shenouda S, Kagnoff M, Eckmann L, Ben-Neriah Y & Raz E (2006) Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. *Nat Cell Biol* **8**, 1327-U1327.

Lee JW, Bajwa PJ, Carson MJ, Jeske DR, Cong Y, Elson CO, Lytle C & Straus DS (2007) Fenofibrate represses interleukin-17 and interferon-gamma expression and improves colitis in interleukin-10-deficient mice. *Gastroenterology* **133**, 108-123.

Lee K, Jung JH, Kim M & Guidotti G (2001) Interaction of the alpha subunit of Na,K-ATPase with cofilin. *Biochem J* **353**, 377-385.

Lee TS & Chau LY (2002) Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat Med* **8**, 240-246.

Li BB, C. and Whelan, J. (1994) Antithetic relationship of dietary arachidonic acid and eicosapentaenoic acid on eicosanoid production in vivo. *J Lipid Res* **35**, 1869-1877.

Linskens RK, Huijsdens XW, Savelkoul PH, Vandenbroucke-Grauls CM & Meuwissen SG (2001) The bacterial flora in inflammatory bowel disease: current insights in pathogenesis and the influence of antibiotics and probiotics. *Scand J Gastroenterol* **234**, s29-s40.

Liu WT, Marsh TL, Cheng H & Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol* **63**, 4516-4522.

Loftus EV (2004) Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology* **126**, 1504-1517.

Löhler J, Kühn R, Rennick D, Rajewsky K & Müller W (1995) Interleukin-10-deficient mice: a model of chronic mucosal inflammation. In Inflammatory bowel disease. G Tygat, J Bartelsman & S van Deventer (eds.), Kluwer Academic Publishers. Dordrecht, The Netherlands, pp. 401-407.

Lu J, Jilling T, Li D & Caplan MS (2007) Polyunsaturated fatty acid supplementation alters proinflammatory gene expression and reduces the incidence of necrotizing enterocolitis in a neonatal rat model. *Pediat Res* **61**, 427-432.

Lytle C, Tod TJ, Vo KT, Lee JW, Atkinson RD & Straus DS (2005) The peroxisome proliferator-activated receptor gamma ligand rosiglitazone delays the onset of inflammatory bowel disease in mice with interleukin 10 deficiency. *Inflamm Bowel Dis* **11**, 231-243.

MacBain AJ & Macfarlane GT (1998) Ecological and physiological studies on large intestinal bacteria in relation to production of hydrolytic and reductive enzymes involved in formation of genotoxic metabolites. *J Med Microbiol* **47**, 407-416.

Macdonald TT (2003) The mucosal immune system. *Paras Immunol* **25**, 235-246.

Macfarlane GT, Gibson GR & Cummings JH (1992) Comparison of fermentation reactions in different regions of the human colon. *J Appl Bacteriol* **72**, 57-64.

Macfarlane S, Steed H & Macfarlane GT (2009) Intestinal bacteria and inflammatory bowel disease. *Crit Rev Clin Lab Sci* **46**, 25-54.

Madara JL (2000) Modulation of tight junctional permeability - Foreword. *Adv Drug Deliv Rev* **41**, 251-253.

Madsen K (2002) Combining T cells and IL-10: A new therapy for Crohn's disease? *Gastroenterology* **123**, 2140-2144.

Madsen KL, Doyle JS, Tavernini MM, Jewell LD, Rennie RP & Fedorak RN (2000) Antibiotic therapy attenuates colitis in interleukin 10 gene-deficient mice. *Gastroenterology* **118**, 1094-+.

Madsen KL, Malfair D, Gray D, Doyle JS, Jewell LD & Fedorak RN (1999) Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. *Inflamm Bowel Dis* **5**, 262-270.

Maeda S, Hsu LC, Liu HJ, Bankston LA, Iimura M, Kagnoff MF, Eckmann L & Karin M (2005) Nod2 mutation in Crohn's disease potentiates NF-kappa B activity and IL-10 processing. *Science* **307**, 734-738.

Mahler M, Bristol IJ, Leiter EH, Workman AE, Birkenmeier EH, Elson CO & Sundberg JP (1998) Differential susceptibility of inbred mouse strains to dextran sulfate sodium-induced colitis. *Am J Physiol-Gastrointest Liver Physiol* **274**, G544-G551.

Mahler M & Leiter EH (2002) Genetic and environmental context determines the course of colitis developing in IL-10-deficient mice. *Inflamm Bowel Dis* **8**, 347-355.

Malfatti MA & Felton JS (2006) Susceptibility to exposure to heterocyclic amines from cooked food: role of UDP-glucuronosyltransferases. In Nutritional genomics. Discovering the path to personlized nutrition. J Kaput, RL Rodriquez (eds.), Hoboken, NJ. John Wiley and Sons, pp. 331-352.

Malhotra JD & Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: A vicious cycle or a double-edged sword? *Antioxid Redox Signal* **9**, 2277-2293.

Mandard S, Muller M & Kersten S (2004) Peroxisome proliferator-activated receptor alpha target genes. *Cell Mol Life Sci* **61**, 393-416.

Mangin I, Bonnet R, Seksik P, Rigottier-Gois L, Sutren M, Bouhnik Y, Neut C, Collins MD, Colombel JF, Marteau P & Dore J (2004) Molecular inventory of faecal microflora in patients with Crohn's disease. *FEMS Microbiol Ecol* **50**, 25-36.

Mannick EE, Bonomolo JC, Horswell R, Lentz JJ, Serrano MS, Zapata-Velandia A, Gastanaduy M, Himel JL, Rose SL, Udall JN, Hornick CA & Liu ZY (2004) Gene expression in mononuclear cells from patients with inflammatory bowel disease. *Clin Immunol* **112**, 247-257.

Manson JM, Rauch M & Gilmore MS (2008). The commensal microbiology of the gastrointestinal tract. *Adv Exp Med Biol* **635**, 15-28.

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer ML, Jarvie TP, Jirage KB, Kim JB, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu P, Begley RF & Rothberg JM (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**, 376-380.

Mariadason JM, Barkla DH & Gibson PR (1997) Effect of short-chain fatty acids on paracellular permeability in Caco-2 intestinal epithelium model. *Am J Physiol-Gastrointest Liver Physiol* **272**, G705-G712.

Mariman ECM (2006) Nutrigenomics and nutrigenetics: the 'omics' revolution in nutritional science. *Biotechnol Appl Biochem* **44**, 119 - 128.

Mariotto S, Suzuki Y, Persichini T, Colasanti M, Suzuki H & Cantoni O (2007) Cross-talk between NO and arachidonic acid in inflammation. *Curr Med Chem* **14**, 1940-1944.

Martin F, Penet MF, Malergue F, Lepidi H, Dessein A, Galland F, de Reggi M, Naquet P & Gharib B (2004) Vanin-1(-/-) mice show decreased NSAID- and Schistosoma-induced intestinal inflammation associated with higher glutathione stores. *J Clin Investig* **113**, 591-597.

Martinez-Augustin O, Merlos M, Zarzuelo A, Suarez MD & de Medina FS (2008) Disturbances in metabolic, transport and structural genes in experimental colonic inflammation in the rat: a longitudinal genomic analysis. *BMC Genomics* **9**.

Marx N, Duez H, Fruchart JC & Staels B (2004) Peroxisome proliferatoractivated receptors and atherogenesis: regulators of gene expression in vascular cells. *Circulation Research* **94**, 1168–1178.

Mathur D, Malik G & Garg LC (2006) Biochemical and functional characterization of triosephosphate isomerase from Mycobacterium tuberculosis H37Rv. *FEMS Microbiol Lett* **263**, 229-235.

Matsumoto S, Okabe Y, Setoyama H, Takayama K, Ohtsuka J, Funahashi H, Imaoka A, Okada Y & Umesaki Y (1998) Inflammatory bowel disease-like enteritis and caecitis in a senescence accelerated mouse P1/Yit strain. *Gut* **43**, 71-78.

Matsunaga A, Sasaki J, Mori T, Moriyama K, Nishi K, Hidaka K & Arakawa K (1995) Apolipoprotein A-I gene promoter polymorphism in patients with coronary artery disease and healthy controls. *Nutr Metab Cardiovasc Dis* **5**, 269–75.

Matsuzaki G & Umemura M (2007) Interleukin-17 as an effector molecule of innate and acquired immunity against infections. *Microbiol Immunol* **51**, 1139-1147.

McBurney W, Mangold M, Munro K, Schultz M, Rath HC & Tannock GW (2006) PCR/DGGE and 16S rRNA gene library analysis of the colonic microbiota of HLA-B27/beta 2-microglobulin transgenic rats. *Lett Appl Microbiol* **42**, 165-171.

McBurney WT (2003) PCR/DGGE analysis of the intestinal microflora of experimental animal models: Potential applications in the study of inflammatory diseases. PhD thesis, University of Otago, Dunedin, New Zealand.

McCarthy J, O'Mahony L, O'Callaghan L, Sheil B, Vaughan EE, Fitzsimons N, Fitzgibbon J, O'Sullivan GC, Kiely B, Collins JK & Shanahan F (2003) Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. *Gut* **52**, 975-980.

McEntee MF, Ziegler C, Reel D, Tomer K, Shoieb A, Ray M, Li X, Neilsen N, Lih FB, O'Rourke D & Whelan J (2008) Dietary n-3 polyunsaturated fatty acids enhance hormone ablation therapy in androgen-dependent prostate cancer. *Am J Pathol* **173**, 229-241.

Medina C, Santana A, Llopis M, Paz-Cabrera MC, Antolin M, Mourelle M, Guarner F, Vilaseca J, Gonzalez C, Salas A, Quintero E & Malagelada JR (2005) Induction of colonic transmural inflammation by bacteroides fragilis - Implication of matrix metalloproteinases. *Inflamm Bowel Dis* **11**, 99-105.

Mellor AL & Munn DH (2003) Tryptophan catabolism and regulation of adaptive immunity. *J Immunol* **170**, 5809-5813.

Miller AM, Elliot PR, Connell W, Desmond PV & D'Apice AJ (2000) A novel model of ulcerative colitis based on genetic modification of the glycosylation of colonic mucins. *Gastroenterology* **118**, 3769.

Mills SC, Windsor AC & Knight SC (2005) The potential interactions between polyunsaturated fatty acids and colonic inflammatory processes. *Clin Exp Immunol* **142**, 216-228.

Mine Y, Miyashita K & Shahidi F (2009) Nutrigenomics and proteomics in health and disease: An overview. In Nutrigenomics and proteomics in health and disease: food factors and gene interactions. Y Mine, K Miyashita, F Shahidi (eds.), Ames, Iowa, USA. Wiley-Blackwell, pp. 3-9.

Mitsuyama K, Suzuki A, Tomiyasu N, Takaki K, Toyonaga A & Sata M (2001) Transcription factor-targeted therapies in inflammatory bowel disease. *Digestion* **63**, 68-72.

Moffit JS, Koza-Taylor PH, Holland RD, Thibodeau MS, Beger RD, Lawton MP & Manautou JE (2007) Differential gene expression in mouse liver associated with the hepatoprotective effect of clofibrate. *Toxicol Appl Pharmacol* **222**, 169-179.

Mombaerts P, Mizoguchi E, Grusby MJ, Glimcher LH, Bhan AK & Tonegawa S (1993) Spontaneous development of inflammatory bowel disease in T-cell receptor mutant mice. *Cell* **75**, 275-282.

Montalto MC, Hart ML, Jordan JE, Wada K & Stahl GL (2003) Role for complement in mediating intestinal nitric oxide synthase-2 and superoxide dismutase expression. *Am J Physiol-Gastrointest Liver Physiol* **285**, G197-G206.

Morrison TB, Weis JJ & Wittwer CT (1998) Quantification of low-copy transcripts by continuous SYBR (R) green I monitoring during amplification. *Biotechniques* **24**, 954-+.

Mourelle M, Salas A, Guarner F, Crespo E, Garcia-Lafuente A & Malagelada JR (1998) Stimulation of transforming growth factor beta 1 by enteric bacteria in the pathogenesis of rat intestinal fibrosis. *Gastroenterology* **114**, 519-526.

Mueller C (2002) Tumor necrosis factor in mouse models of chronic intestinal inflammation. *Immunology* **105**, 1-8.

Mueller C & Macpherson AJ (2006) Layers of mutualism with commensal bacteria protect us from intestinal inflammation. *Gut* **55**, 276-284.

Muller M & Kersten S (2003) Nutrigenomics: goals and strategies. *Nat Rev Genet* **4**, 315-322.

Murray PJ (2006) Understanding and exploiting the endogenous interleukin-10/STAT3-mediated anti-inflammatory response. *Curr Opin Pharmacol* **6**, 379-386.

Mutch DM, Grigorov M, Berger A, Fay LB, Roberts MA, Watkins SM, Williamson G & German JB (2005) An integrative metabolism approach identifies stearoyl-CoA desaturase as a target for an arachidonate-enriched diet. *FASEB Journal* **19**, 599-+.

Muyzer G, Dewaal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal RNA. *Appl Environ Microbiol* **59**, 695-700.

Muyzer G & Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Leeuwenhoek Int J Gen Mol Microbiol* **73**, 127-141.

Nagler-Anderson C (2006) Functional aspects of the mucosal immune system. In Immune mechanisms in inflammatory bowel disease, 1st Edition, RS Blumberg and MF Neurath (eds.), Springer Science and Business Media, NY, USA, pp. 15-23.

Netting AG (2002) pH, abscisic acid and the integration of metabolism in plants under stressed and non-stressed conditions. II. Modifications in modes of metabolism induced by variation in the tension on the water column and by stress. *J Exp Bot* **53**, 151-173.

Neuhoff V, Arold N, Taube D & Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric-focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9**, 255-262.

New Zealand Health Information Service (2007).

Niess JH & Reinecker HC (2006) Dendritic cells: the commanders-in-chief of mucosal immune defenses. *Curr Opin Gastroenterol* **22**, 354-360.

Nieto N, Torres MI, Rios A & Gil A (2002) Dietary polyunsaturated fatty acids improve histological and biochemical alterations in rats with experimental ulcerative colitis. *J Nutr* **132**, 11-19.

Nones K, Dommels YEM, Martell S, Butts C, McNabb WC, Park ZA, Zhu ST, Hedderley D, Barnett MPG & Roy NC (2009) The effects of dietary curcumin and rutin on colonic inflammation and gene expression in multidrug resistance gene-deficient (mdr1a(-/-)) mice, a model of inflammatory bowel diseases. *Brit J Nutr* **101**, 169-181.

Nones K, Knoch B, Dommels YEM, Paturi G, Butts C, McNabb WC & Roy NC (2009) Multidrug resistance gene deficient (mdr1a(-/-)) mice have an altered caecal microbiota that precedes the onset of intestinal inflammation. *J Appl Microbiol* **107**, 557-566.

Nubel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, Ludwig W & Backhaus H (1996) Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. *J Bacteriol* **178**, 5636-5643.

Nutrient Requirements of Laboratory Animals, 4th Revised Edition, 1995 Institute for Laboratory Animal Research (ILAR), The National Academies Press.

O'Neil D & Steidler L (2003) Cytokines, chemokines and growth factors in the pathogenesis and treatment of inflammatory bowel disease. *Cytokines Chemokines Autoimmune Dis* **520**, 252-285.

Ogawa H, Fukushima K, Naito H, Funayama Y, Unno M, Takahashi K, Kitayama T, Matsuno S, Ohtani H, Takasawa S, Okamoto H & Sasaki I (2003) Increased expression of HIP/PAP and regenerating gene III in human inflammatory bowel disease and a murine bacterial reconstitution model. *Inflamm Bowel Dis* **9**, 162-170.

Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G & Cho JH (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* **411**, 603-606.

Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y & Nakaya R (1990) A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* **98**, 694-702.

Omura K, Kiyosawa N, Uehara T, Hirode M, Shimizu T, Miyagishima T, Ono A, Nagao T & Urushidani T (2007) Gene expression profiling of rat liver treated with serum triglyceride-decreasing compounds. *J Toxicol Sci* **32**, 387-399.

Ordovas JM, Corella D, Cupples LA, Demissie S, Kelleher A, Coltell O, Wilson PWF, Schaefer EJ & Tucker K (2002) Polyunsaturated fatty acids modulate the effects of the *APOA1* G-A polymorphism on HDL-cholesterol concentrations in a sex-specific manner: the Framingham Study. *Am J Clin Nutr* **75**, 38–46.

Osborne TF (2000) Sterol regulatory element-binding proteins (SREBPs): Key regulators of nutritional homeostasis and insulin action. *J Biol Chem* **275**, 32379-32382.

Pacheco S, Hillier K & Smith C (1987) Increased arachidonic acid levels in phospholipids of human colonic mucosa in inflammatory bowel disease. *Clin Sci* **73**, 361-364.

Panwala CM, Jones JC & Viney JL (1998) A novel model of inflammatory bowel disease: Mice deficient for the multiple drug resistance gene, mdr1a, spontaneously develop colitis. *J Immunol* **161**, 5733-5744.

Park EI, Paisley EA, Mangian HJ, Swartz DA, Wu M, O'Morchoe PJ, Behr SR, Visek WJ & Kaput J (1997) Lipid level and type alter stearoyl CoA desaturase mRNA abundance differently in mice with distinct susceptibilities to diet-influenced diseases. *J Nutr* **127**, 566 - 573.

Pasetti MF, Salerno-Goncalves R & Sztein MB (2005) Mechanisms of adaptive immunity that prevent colonization at mucosal surfaces. In Colonization of mucosal surfaces, 1st Edition, JP Nataro, PS Cohen, HLT Mobley & JN Weiser (eds.), ASM Press. Washington, D.C., USA, pp. 35-47.

Patsouris D, Reddy JK, Muller M & Kersten S (2006) Peroxisome proliferator-activated receptor alpha mediates the effects of high-fat diet on hepatic gene expression. *Endocrinology* **147**, 1508-1516.

Pearson JP & Brownlee IA (2005) Structure and function of mucosal surfaces. In Colonization of mucosal surfaces, 1st Edition, JP Nataro, PS Cohen, HLT Mobley & JN Weiser (eds.), ASM Press. Washington, D.C., USA, pp. 3-16.

Pease AC, Solas D, Sullivan EJ, Cronin MT, Holmes CP & Fodor SPA (1994) Light-generated oligonucleotide arrays for rapid DNA-sequence analysis. *Proc Natl Acad Sci USA* **91**, 5022-5026.

Peltekova VD, Wintle RF, Rubin LA, Amos CI, Huang QQ, Gu XJ, Newman B, Van Oene M, Cescon D, Greenberg G, Griffiths AM, St George-Hyslop PH & Siminovitch KA (2004) Functional variants of OCTN cation transporter genes are associated with Crohn disease. *Nat Genet* **36**, 471-475.

Pessi T, Sutas Y, Marttinen A & Isolauri E (1998) Probiotics reinforce mucosal degradation of antigens in rats: Implications for therapeutic use of probiotics. *J Nutr* **128**, 2313-2318.

Petermann I, Triggs CM, Huebner C, Han DY, Gearry RB, Barclay ML, Demmers PS, McCulloch A & Ferguson LR (2009) Mushroom intolerance: a novel diet-gene interaction in Crohn's disease. *Brit J Nutr* **102**, 506-508.

Peters JM, Hollingshead HE & Gonzalez FJ (2008) Role of peroxisome-proliferator-activated receptor beta/delta (PPAR beta/delta) in gastrointestinal tract function and disease. *Clin Sci* **115**, 107-127.

Pfaffl MW (2004) Quantification strategies in real-time PCR. In A-Z of quantitative PCR. SA Bustin (ed.), International University Line (IUL), La Jolla, California, USA, pp. 87-112.

Phan CT & Tso P (2001) Intestinal lipid absorption and transport. *Front Biosci* **6**, D299-D319.

Philpott M, Mackay L, Ferguson LR, Forbes D & Skinner M (2007) Cell culture models in developing nutrigenomics foods for inflammatory bowel disease. *Mutat Res: Fundam Mol Mech Mutagen* **622**, 94-102.

Pianetti S, Guo SQ, Kavanagh KT & Sonenshein GE (2002) Green tea polyphenol epigallocatechin-3 gallate inhibits Her-2/neu signaling, proliferation, and transformed phenotype of breast cancer cells. *Cancer Res* **62**, 652-655.

Podolsky DK (2002) Inflammatory bowel disease. *N Engl J Med* **347**, 417-429.

Poussier P, Ning T, Chen J, Banerjee D & Julius M (2000) Intestinal inflammation observed in IL-2R/IL-2 mutant mice is associated with impaired intestinal T lymphopoiesis. *Gastroenterology* **118**, 880-891.

Pouyet L, Roisin-Bouffay C, Clément A, Millet V, Garcia S, Chasson L, Issaly N, Rostan A, Hofman P, Naquet P & Galland F (2009) Epithelial vanin-1 controls inflammation-

driven carcinogenesis in the colitis-associated colon cancer model. *Inflamm Bowel Dis* **000**, 000-000.

Powrie F, Leach MW, Mauze S, Caddle LB & Coffman RL (1993) Phenotypically Distinct Subsets Of Cd4(+) T-Cells Induce Or Protect From Chronic Intestinal Inflammation In C - B-17 Scid Mice. *Int Immunol* **5**, 1461-1471.

Prüfer K, Muetzel B, Do HH, Weiss G, Khaitovich P, Rahm E, Pääbo S, Lachmann M & Enard W (2007) FUNC: a package for detecting significant associations between gene sets and ontological annotations. *BMC Bioinformatics* **8**.

Qi W, Ebbert KVJ, Craig AWB, Greer PA & McCafferty DM (2005) Absence of Fer protein tyrosine kinase exacerbates endotoxin induced intestinal epithelial barrier dysfunction in vivo. *Gut* **54**, 1091-1097.

Rader DJ, Hoeg JM, Brewer HB Jr. (1994) Quantitation of plasma apolipoproteins in the primary and secondary prevention of coronary artery disease. *Ann Intern Med* **120**, 1012–25.

Rakhshandehroo M, Sanderson LM, Matilainen M, Stienstra R, Carlberg C, de Groot PJ, Muller M & Kersten S (2007) Comprehensive analysis of PPARa-dependent regulation of hepatic lipid metabolism by expression profiling. *PPAR Res* **26839**.

Ramakers JD, Mensink RP, Verstege MI, Te Velde AA & Plat J (2008) An arachidonic acid-enriched diet does not result in more colonic inflammation as compared with fish oil- or oleic acid-enriched diets in mice with experimental colitis. *Brit J Nutr* **100**, 347-54.

Rao L, Puschner B & Prolla TA (2001) Gene expression profiling of low selenium status in the mouse intestine: Transcriptional activation of genes linked to DNA damage, cell cycle control and oxidative stress. *J Nutr* **131**, 3175-3181.

Rath HC, Wilson KH & Sartor RB (1999) Differential induction of colitis and gastritis in HLA-B27 transgenic rats selectively colonized with *Bacteroides vulgatus* or *Escherichia coli*. *Infect Immun* **67**, 2969-2974.

Reeves PG, Nielsen FH & Fahey GC (1993) Ain-93 purified diets for laboratory rodents - Final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the Ain-76A rodent diet. *J Nutr* **123**, 1939-1951.

Ren B, Thelen AP, Peters JM, Gonzalez FJ & Jump DB (1997) Polyunsaturated fatty acid suppression of hepatic fatty acid synthase and S14 gene expression does not require peroxisome proliferator-activated receptor alpha. *J Biol Chem* **272**, 26827-26832.

Rieder F, Brenmoehl J, Leeb S, Scholmerich J & Rogler G (2007) Healing and fibrosis in intestinal disease. *Gut* **56**, 130-139.

Rieder F & Fiocchi C (2009) Intestinal fibrosis in IBD-a dynamic, multifactorial process. *Nat Rev Gastroenterol Hepatol* **6**, 228-235.

Riley LK, Franklin CL, Hook RR & BeschWilliford C (1996) Identification of murine Helicobacters by PCR and restriction enzyme analyses. *J Clin Microbiol* **34**, 942-946.

Rinttila T, Kassinen A, Malinen E, Krogius L & Palva A (2004) Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J Appl Microbiol* **97**, 1166-1177.

Riordan AM, Hunter JO, Cowan RE, Crampton JR, Davidson AR, Dickinson RJ, Dronfield MW, Fellows IW, Hishon S, Kerrigan GNW, Kennedy HJ, McGouran RCM, Neale G & Saunders JHB (1993) Treatment Of Active Crohns-Disease By Exclusion Diet - East Anglian Multicenter Controlled Trial. *Lancet* **342**, 1131-1134.

Rioux V, Catheline D, Beauchamp E, Le Bloc'h J, Pedrono F & Legrand R (2008) Substitution of dietary oleic acid for myristic acid increases the tissue storage of alpha-linolenic acid and the concentration of docosahexaenoic acid in the brain, red blood cells and plasma in the rat. *Animal* **2**, 636-644.

Rivera E, Flores I, Rivera E & Appleyard CB (2006) Molecular profiling of a rat model of colitis: Validation of known inflammatory genes and identification of novel disease-associated targets. *Inflamm Bowel Dis* **12**, 950-966.

Roberts RL, Gearry RB, Hollis-Moffatt JE, Miller AL, Reid J, Stat M, Abkevich V, Timms KM, Gutin A, Lanchbury JS, Merriman TR, Barclay ML & Kennedy MA (2007) IL23R R381Q and ATG16L1 T300A are strongly associated with Crohn's disease in a study of

New Zealand Caucasians with inflammatory bowel disease. *Am J Gastroenterol* **102**, 2754-2761.

Roedinger WEW & Duncan A (1993) Reducing sulfur compounds of the colon impair colonocyte nutrition: implications for ulcerative colitis. *Gastroenterol* **104**, 802-809.

Rose DP & Connolly JM (1999) Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacol Ther* **83**, 217-244.

Roy N, Barnett M, Knoch B, Dommels Y & McNabb W (2007) Nutrigenomics applied to an animal model of inflammatory bowel diseases: Transcriptomic analysis of the effects of eicosapentaenoic acid and arachidonic acid-enriched diets. *Mutat Res: Fundam Mol Mech Mutagen* **622**, 103-116.

Rozen S & Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics Methods and Protocols: Methods in Molecular Biology, S Krawetz & S Misener (eds.), Totowa, NJ:Humana Press, pp. 365-386.

Rudolph U, Finegold MJ, Rich SS, Harriman GR, Srinivasan Y, Brabet P, Boulay G, Bradley A & Birnbaumer L (1995) Ulcerative colitis and adenocarcinoma of the colon in G-alpha(I2)-deficient mice. *Nat Genet* **10**, 143-150.

Sacks FM, Tonkin AM, Craven T, Pfeffer MA, Shepherd J, Keech A, Furberg CD & Braunwald E (2002) Coronary heart disease in patients with low LDL-cholesterol - Benefit of pravastatin in diabetics and enhanced role for HDL-cholesterol and triglycerides as risk factors. *Circulation* **105**, 1424-1428.

Sadlack B, Lohler J, Schorle H, Klebb G, Haber H, Sickel E, Noelle RJ & Horak I (1995) Generalized autoimmune disease in interleukin-2-deficient mice is triggered by an uncontrolled activation and proliferation of Cd4(+) T-cells. *Eur J Immunol* **25**, 3053-3059.

Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC & Horak I (1993) Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell* **75**, 253-261.

Saha N, Tay JSH, Low PS & Humphries SE (1994) Guanidine to adenine (*G/A*) substitution in the promoter region of the apolipoprotein AI gene is associated with elevated serum apolipoprotein AI levels in Chinese non-smokers. *Genet Epidemiol* **11**, 255–64.

Salter A & Tarling E (2007) Regulation of gene transcription by fatty acids. *Animal* **1**, 1314–1320.

Salzman NH, de Jong H, Paterson Y, Harmsen HJM, Welling GW & Bos NA (2002) Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. *Microbiol-SGM* **148**, 3651-3660.

Sampath H & Ntambi JM (2005) Polyunsaturated fatty acid regulation of genes of lipid metabolism. *Ann Rev Nutr* **25**, 317-340.

Samstag Y, Eibert SM, Klemke M & Wabnitz GH (2003) Actin cytoskeletal dynamics in T lymphocyte activation and migration. *J Leukoc Biol* **73**, 30-48.

Sanchez-Munoz F, Dominguez-Lopez A & Yamamoto-Furusho JK (2008) Role of cytokines in inflammatory bowel disease. *World J Gastroenterol* **14**, 4280-4288.

Sanderson LM, De Groot PJ, Hooiveld GJEJ, Koppen A, Kalkhoven E, Muller M & Kersten S (2008) Effect of synthetic dietary triglycerides: A novel research paradigm for nutrigenomics. *PLoS ONE* **3**, 1681.

Sanderson LM, Degenhardt T, Koppen A, Kalkhoven E, Desvergne B, Muller M & Kersten S (2009) PPARb/d but not PPARa serves as plasma free fatty acid sensor in liver. *Mol Cell Biol 2009 Oct 5*.

Sands BE (2002) From symptoms to diagnosis: clinical distinctions among various forms of intestinal inflammation. *Gastroenterology* **126**, 1518-1532.

Sands BE (2007) Inflammatory bowel disease: past, present, and future. *J Gastroenterol* **42**, 16-25.

Sartor RB (2006) Living with the flora in IBD: Loss of tolerance to normal bacteria or immunity to low pathogenic bacteria? *Inflamm Bowel Dis* **12**, S3-S4.

Schmitz G & Ecker J (2008) The opposing effects of n-3 and n-6 fatty acids. *Progr Lipid Res* **47**, 147-155.

Schorle H, Holtschke T, Hunig T, Schimpl A & Horak I (1991) Development and function of T-cells in mice rendered interleukin-2 deficient by gene targeting. *Nature* **352**, 621-624.

Schroder K, Hertzog PJ, Ravasi T & Hume DA (2004) Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* **75**, 163-189.

Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M & Ragg T (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol* **7**.

Schultz M, Munro K, Tannock GW, Melchner I, Gottl C, Schwietz H, Scholmerich J & Rath HC (2004) Effects of feeding a probiotic preparation (SIM) containing inulin on the severity of colitis and on the composition of the intestinal microflora in HLA-B27 transgenic rats. *Clin Diagn Lab Immunol* **11**, 581-587.

Schultz M & Sartor RB (2000) Probiotics and inflammatory bowel diseases. *Am J Gastroenterol* **95**, S19-S21.

Sellon RK, Tonkonogy S, Schultz M, Dieleman LA, Grenther W, Balish E, Rennick DM & Sartor RB (1998) Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun* **66**, 5224-5231.

Seo T, Blaner WS & Deckelbaum RJ (2005) Omega-3 fatty acids: molecular approaches to optimal biological outcomes. *Curr Opin Lipidol* **16**, 11-18.

Serhan CN, Clish CB, Brannon J, Colgan SP, Chiang N & Gronert K (2000) Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. *J Exp Med* **192**, 1197-1204.

Serhan CN & Chiang N (2008) Endogenous pro-resolving and anti-inflammatory lipid mediators: a new pharmacologic genus. *Brit J Pharmacol* **153**, S200-S215.

Sethi S, Ziouzenkova O, Ni HY, Wagner DD, Plutzky J & Mayadas TN (2002) Oxidized omega-3 fatty acids in fish oil inhibit leukocyte-endothehal interactions through activation of PPAR alpha. *Blood* **100**, 1340-1346.

Sha WC, Liou HC, Tuomanen EI & Baltimore D (1995) Targeted disruption of the P50 subunit of Nf-Kappa-B leads to multifocal defects in immune responses. *Cell* **80**, 321-330.

Shaikh SR, Mitchell D, Carroll E, Li M, Schneck J & Edidin M (2008) Differential effects of a saturated and a monounsaturated fatty acid on MHC class I antigen presentation. *Scand J Immunol* **68**, 30-42.

Shan WW, Nicol CJ, Ito S, Bility MT, Kennett MJ, Ward JM, Gonzalez FJ & Peters JM (2008) Peroxisome proliferator-activated receptor-beta/delta protects against chemically induced liver toxicity in mice. *Hepatology* **47**, 225-235.

Shanahan F (2002) Gut flora in gastrointestinal disease. *Eur J Surg* **168**, 47-52.

Sheehan AL, Warren BF, Gear MWL & Shepherd NA (1992) Fat-wrapping in Crohn's disease - pathological basis and relevance to surgical practice. *Brit J Surg* **79**, 955-958.

Shen L & Turner JR (2006) Role of epithelial cells in initiation and propagation of intestinal inflammation. Eliminating the static: tight junction dynamics exposed. *Am J Physiol-Gastrointest Liver Physiol* **290**, G577-G582.

Shimizu T, Fujii T, Suzuki R, Igarashi J, Ohtsuka Y, Nagata S & Yamashiro Y (2003) Effects of highly purified eicosapentaenoic acid on erythrocyte fatty acid composition and leukocyte and colonic mucosa leukotriene B-4 production in children with ulcerative colitis. *J Pediatr Gastroenterol Nutr* **37**, 581-585.

Shkoda A, Werner T, Daniel H, Gunckel M, Rogler G & Haller D (2007) Differential protein expression profile in the intestinal epithelium from patients with inflammatory bowel disease. *J Proteome Res* **6**, 1114-1125.

Shoda R, Matsueda K, Yamato S & Umeda N (1995) Therapeutic efficacy of n-3 polyunsaturated fatty acid in experimental Crohn's disease. *J Gastroenterol* **30**, 98-101.

Shoda R, Matsueda K, Yamato S & Umeda N (1996) Epidemiologic analysis of Crohn disease in Japan: increased dietary intake of n-6 polyunsaturated fatty acids and animal protein relates to the increased incidence of Crohn disease in Japan. *Am J Clin Nutr* **63**, 741-745.

Silverberg MS (2006) OCTNs: Will the real IBD5 gene please stand up? *World J Gastroenterol* **12**, 3678-3681.

Simopoulos AP (2002) Omega-3 fatty acids in inflammation and autoimmune diseases. *J Am Coll Nutr* **21**, 495 - 505.

Simpson JM, McCracken VJ, White BA, Gaskins HR & Mackie RI (1999) Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. *J Microbiol Methods* **36**, 167-179.

Singh I, Paintlia AS, Khan M, Stanislaus R, Paintlia MK, Haq E, Singh AK & Contreras MA (2004) Impaired peroxisomal function in the central nervous system with inflammatory disease of experimental autoimmune encephalomyelitis animals and protection by lovastatin treatment. *Brain Res* **1022**, 1-11.

Smyth GK (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3** (1) article 3. http://www.bepress.com/sagmb/vol3/iss1/art3.

Smyth GK (2005) Limma: linear models for microarray data, in: Bioinformatics and Computational Biology Solutions using R and Bioconductor; Gentleman R, Carey V, Dudoit S, Irizarry R and Huber W (Eds.). *Springer, New York*, 397-420.

Smyth GK & Speed T (2003) Normalization of cDNA microarray data. *Methods* **31**, 265-273.

Snapper SB, Rosen FS, Mizoguchi E, Cohen P, Khan W, Liu CH, Hagemann TL, Kwan SP, Ferrini R, Davidson L, Bhan AK & Alt FW (1998) Wiskott-Aldrich syndrome protein-deficient mice reveal a role for WASP in T but not B cell activation. *Immunity* **9**, 81-91.

Soupene E & Kuypers FA (2008) Mammalian long-chain Acyl-CoA synthetases. *Exp Biol Med* **233**, 507-521.

Spielbauer B & Stahl F (2005) Impact of microarray technology in nutrition and food research. *Mol Nutr Food Res* **49**, 908-917.

Staels B & Fruchart JC (2005) Therapeutic roles of peroxisome proliferator-activated receptor agonists. *Diabetes* **54**, 2460-2470.

Stahl A (2004) A current review of fatty acid transport proteins (SLC27). *Pflugers Archl* **447**, 722-727.

Sterrenburg E, Turk R, Boer JM, van Ommen GB & den Dunnen JT (2002) A common reference for cDNA microarray hybridizations. *Nucleic Acids Res* **30**.

Stienstra R, Mandard S, Tan NS, Wahli W, Trautwein C, Richardson TA, Lichtenauer-Kaligis E, Kersten S & Muller M (2007) The Interleukin-1 receptor antagonist is a direct target gene of PPAR alpha in liver. *J Hepatol* **46**, 869-877.

Strober W, Fuss I & Mannon P (2007) The fundamental basis of inflammatory bowel disease. *J Clin Investig* **117**, 514-521.

Strup-Perrot C, Mathe D, Linard C, Violot D, Milliat F, Francois A, Bourhis J & Vozenin-Brotons MC (2004) Global gene expression profiles reveal an increase in mRNA levels of collagens, MMPs, and TIMPs in late radiation enteritis. *Am J Physiol-Gastroint Liver Physiol* **287**, G875-G885.

Stulnig TM (2003) Immunomodulation by polyunsaturated fatty acids: Mechanisms and effects. *Int Arch Allergy Immunol* **132**, 310-321.

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES & Mesirov JP (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* **102**, 15545-15550.

Sukhija PS & Palmquist DL (1988) Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. *J Agric Food Chem* **36**, 1202-1206.

Sülzle A, Hirche F & Eder K (2004) Thermally oxidized dietary fat upregulates the expression of target genes of PPARa in rat liver. *J Nutr* **134**, 1375-1383.

Sutherland LR, Robinson M, Onstad G, Peppercorn M, Greenberger N, Goodman M & Martin F (1991) A double-blind, placebo-controlled multicenter study of the efficacy and safety of 5-aminosalicylic acid tablets in the treatment of ulcerative colitis. *Trends Inflamm Bowel Dis Ther* **56**, 335-340.

Swidsinski A, Weber J, Loening-Baucke V, Hale LP & Lochs H (2005) Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *J Clin Microbiol* **43**, 3380-3389.

Takahashi M, Tsuboyama-Kasaoka N, Nakatani T, Ishii M, Tsutsumi S, Aburatani H & Ezaki O (2002) Fish oil feeding alters liver gene expressions to defend against PPAR alpha activation and ROS production. *Am J Physiol-Gastroint Liver Physiol* **282**, G338-G348.

Takano T, Fiore S, Maddox JF, Brady HR, Petasis NA & Serhan CN (1997) Aspirin-triggered 15-epi-lipoxin A(4) (LXA(4)) and LXA(4) stable analogues are potent inhibitors of Acute inflammation: Evidence for anti-inflammatory receptors. *J Exp Med* **185**, 1693-1704.

Takeda K, Clausen BE, Kaisho T, Tsujimura T, Terada N, Forster I & Akira S (1999) Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* **10**, 39-49.

Tannock G (2005) Microbiota of mucosal surfaces in the gut of monogastric animals. In Colonization of mucosal surfaces, 1st Edition, JP Nataro, PS Cohen, HLT Mobley & JN Weiser (eds.), ASM Press. Washington, D.C., USA, pp. 163-178.

Tannock GW, Munro K, Harmsen HJM, Welling GW, Smart J & Gopal PK (2000) Analysis of the fecal microflora of human subjects consuming a probiotic product containing Lactobacillus rhamnosus DR20. *Appl Environ Microbiol* **66**, 2578-2588.

Tao F (2009) Challenges and current solutions in proteomic sample preparation. In: Nutrigenomics and proteomics in health and disease: food factors and gene interactions. 1st Edition, Y Mine, K Miyashita & F Shahidi (eds.), Wiley-Blackwell. Iowa, USA, pp. 351-365.

Taurog JD, Richardson JA, Croft JT, Simmons WA, Zhou M, Fernandezsueiro JL, Balish E & Hammer RE (1994) The germ-free state prevents development of gut and joint inflammatory disease in Hla-B27 transgenic rats. *J Exp Med* **180**, 2359-2364.

Taylor KD, Rotter JI & Yang H (2003) Genetics of inflammatory bowel disease. In Inflammatory bowel disease: From bench to bedside, 2nd Edition, SR Targan, F Shanahan & LC Karp (eds.), Kluwer Academic Publishers. Cornwall, Great Britain, pp. 21-65.

Te Velde AA, De Kort F, Sterrenburg E, Pronk I, Ten Kate FJW, Hommes DW & Van Deventer SJH (2007) Comparative analysis of colonic gene expression of three experimental colitis models mimicking inflammatory bowel disease. *Inflamm Bowel Dis* **13**, 325-330.

Torres MI, Lopez-Casado MA, Lorite P & Rios A (2007) Tryptophan metabolism and indoleamine 2,3-dioxygenase expression in coeliac disease. *Clin Exp Immunol* **148**, 419-424.

Torres MI & Ríos A (2008) Current view of the immunopathogenesis in inflammatory bowel disease and its implications for therapy. *World J Gastroenterol* **14**, 1972-1980.

Triggs CM, Munday K, Hu R, Fraser AG, Gearry RB, Barclay ML & Ferguson LR (2010) Dietary factors in chronic inflammation: Food tolerances and intolerances of a New Zealand Caucasian Crohn's disease population. *Mutat Res: Fundam Mol Mech Mutagen, in press*, doi:10.1016/j.mrfmmm.2010.01.020.

Trujillo E, Davis, C., Milner, J. (2006) Nutrigenomics, proteomics, metabolomics, and the practice of dietetics. *J Am Diet Assoc* **106**, 403 - 413.

Tsao CC, Coulter SJ, Chien A, Luo G, Clayton NP, Maronpot R, Goldstein JA & Zeldin DC (2001) Identification and localization of five CYP2Cs in murine extrahepatic tissues and their metabolism of arachidonic acid to regio- and stereoselective products. *J Pharmacol Exp Ther* **299**, 39-47.

Vaahtovuo J, Toivanen P & Eerola E (2001) Study of murine faecal microflora by cellular fatty acid analysis; effect of age and mouse strain. *Antonie Van Leeuwenhoek Int J Gen Mol Microbiol* **80**, 35-42.

Vaahtovuo J, Toivanen P & Eerola E (2003) Bacterial composition of murine fecal microflora is indigenous and genetically guided. *FEMS Microbiol Ecol* **44**, 131-136.

Valk PJM, Verhaak RGW, Beijen MA, Erpelinck CAJ, van Doorn-Khosrovani SBV, Boer JM, Beverloo HB, Moorhouse MJ, van der Spek PJ, Lowenberg B & Delwel R (2004) Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med* **350**, 1617-1628.

van der Heijden M, Versteilen AMG, Sipkema P, Amerongen GP, Musters RJP & Groeneveld ABJ (2008) Rho-kinase-dependent F-actin rearrangement is involved in the inhibition of PI3-kinase/Akt during ischemia-reperfusion-induced endothelial cell apoptosis. *Apoptosis* **13**, 404-412.

van Ommen B (2004) Nutrigenomics: Exploiting systems biology in the nutrition and health arenas. *Nutrition* **20**, 4-8.

Varon C, Duriez A, Lehours P, Ménard A, Layé S, Zerbib F, Mégraud F & Laharie D (2009) Study of Helicobacter pullorum proinflammatory properties on human epithelial cells in vitro. *Gut* **58**, 629-635.

Vilaseca J, Salas A, Guarner F, Rodriguez R, Martinez M & Malagelada JR (1990) Dietary fish oil reduces progression of chronic inflammatory lesions in a rat model of granulomatous colitis. *Gut* **31**, 539-544.

Viola A, Schroeder S, Sakakibara Y & Lanzavecchia A (1999) T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* **283**, 680-682.

Volcik KA, Nettleton JA, Ballantyne CM & Boenvinkle E (2008) Peroxisome proliferator-activated receptor a genetic variation interacts with n-6 and long-chain n-3 fatty acid intake to affect total cholesterol and LDL-cholesterol concentrations in the Atherosclerosis Risk in Communities Study. *Am J Clin Nutr* **87**, 1926-1931.

Vozenin-Brotons MC, Milliat F, Linard C, Strup C, Francois A, Sabourin JC, Lasser P, Lusinchi A, Deutsch E, Girinsky T, Aigueperse J, Bourhis J & Mathe D (2004) Gene expression profile in human late radiation enteritis obtained by high-density cDNA array hybridization. *Radiat Res* **161**, 299-311.

Wachtershauser A & Stein J (2000) Rationale for the luminal provision of butyrate in intestinal diseases. *Eur J Nutr* **39**, 164-171.

Wada K, Montalto MC & Stahl GL (2001) Inhibition of complement C5 reduces local and remote organ injury after intestinal ischemia/reperfusion in the rat. *Gastroenterology* **120**, 126-133.

Wahle KWJ, Caruso D, Ochoa JJ & Quiles JL (2004) Olive oil and modulation of cell signaling in disease prevention. *Lipids* **39**, 1223-1231.

Wahli W, Braissant O & Desvergne B (1995) Peroxisome proliferator activated receptors - transcriptional regulators of adipogenesis, lipid metabolism and more. *Chem Biol* **2**, 261-266.

Waidmann M, Bechtold O, Frick JS, Lehr HA, Schubert S, Dobrindt U, Loffler J, Bohn E & Autenrieth IB (2003) *Bacteroides vulgatus* protects against *Escherichia coli*-induced colitis in gnotobiotic interleukin-2-deficient mice. *Gastroenterology* **125**, 162-177.

Wander RC & Du SL (2000) Oxidation of plasma proteins is not increased after supplementation with eicosapentaenoic and docosahexaenoic acids. *Am J Clin Nutr* **72**, 731-737.

Wang Q, Herrera-Ruiz D, Mathis AS, Cook TJ, Bhardwaj RK & Knipp GT (2005) Expression of PPAR and RXR isoforms and fatty acid transporting proteins in the rat and human gastrointestinal tracts. *J Pharm Sci* **94**, 363-372.

Wang J, Li D, Dangott LJ & Wu G (2006) Proteomics and its role in nutrition research. *J Nutr* **136**, 1759-1762.

Wang RF, Cao WW & Cerniglia CE (1996) PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. *Appl Environ Microbiol* **62**, 1242-1247.

Wang XW & Seed B (2003) A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res* **31**.

Warren GW, Van Ess PJ, Watson AM, Mattson MP & Blouin RA (2001) Cytochrome P450 and antioxidant activity in interleukin-6 knockout mice after induction of the acute-phase response. *J Interferon Cytokine Res* **21**, 821-826.

Watanabe M, Ueno Y, Yajima T, Okamoto S, Hayashi T, Yamazaki M, Iwao Y, Ishii H, Habu S, Uehira M, Nishimoto H, Ishikawa H, Hata J & Hibi T (1998) Interleukin 7 transgenic mice develop chronic colitis with decreased interleukin 7 protein accumulation in the colonic mucosa. *J Exp Med* **187**, 389-402.

Watkins BA, Hannon K, Ferruzzi M & Li Y (2007) Dietary PUFA and flavonoids as deterrents for environmental pollutants. *J Nutr Biochem* **18**, 196-205.

Wellcome Trust Case Control Consortium (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* **447**, 661-78.

Wennerberg K & Der CJ (2004) Rho-family GTPases: it's not only Rac and Rho (and I like it). *J Cell Sci* **117**, 1301-1312.

Werner T & Haller D (2007) Intestinal epithelial cell signalling and chronic inflammation: From the proteome to specific molecular mechanisms. *Mutat Res* **622**, 42-57.

Werner T, Shkoda A & Haller D (2007) Intestinal epithelial cell proteome in IL-10 deficient mice and IL-10 receptor reconstituted epithelial cells: Impact on chronic inflammation. *J Proteome Res* **6**, 3691-3704.

Whiting CV, Bland PW & Tarlton JE (2005) Dietary n-3 polyunsaturated fatty acids reduce disease and colonic proinflammatory cytokines in a mouse model of colitis. *Inflamm Bowel Dis* **11**, 340-349.

Wiercinska-Drapalo A, Jaroszewicz J, Siwak E, Pogorzelska J & Prokopowicz D (2008) Intestinal fatty acid binding protein (I-FABP) as a possible biomarker of ileitis in patients with ulcerative colitis. *Regul Pept* **147**, 25-28.

Wild GE, Drozdowski L, Tartaglia C, Clandinin MT & Thomson AB (2007) Nutritional modulation of the inflammatory response in inflammatory bowel disease - From the molecular to the integrative to the clinical. *World J Gastroenterol* **13**, 1-7.

Wilkins MR, Sanchez JC, Gooley AA, Appel RD, HumpherySmith I, Hochstrasser DF & Williams KL (1996) Progress with proteome projects: Why all proteins expressed by a genome should be identified and how to do it. *Biotechnol Genet Eng Rev* **13**, 19-50.

Wilson KH & Blitchington RB (1996) Human colonic biota studied by ribosomal DNA sequence analysis. *Appl Environ Microbiol* **62**, 2273-2278.

Wirtz S, Finotto S, Kanzler S, Lohse AW, Blessing M, Lehr HA, Galle PR & Neurath MF (1999) Cutting edge: Chronic intestinal inflammation in STAT-4 transgenic mice: Characterization of disease and adoptive transfer by TNF- plus IFN-gamma-producing CD4(+) T cells that respond to bacterial antigens. *J Immunol* **162**, 1884-1888.

Wohlgemuth S, Haller D, Blaut M & Loh G (2009) Reduced microbial diversity and high numbers of one single *Escherichia coli* strain in the intestine of colitic mice. *Environ Microbiol* **11**, 1562-1571.

Wolber PK, Collins PJ, Lucas AB, De Witte A & Shannon KW (2006) The agilent in situ-synthesized microarray platform. In *DNA Microarrays Part A: Array Platforms And Wet-Bench Protocols*, pp. 28-57.

Wolf AM, Wolf D, Rumpold H, Moschen AR, Kaser A, Obrist P, Fuchs D, Brandacher G, Winkler C, Geboes K, Rutgeerts P & Tilg H (2004) Overexpression of indoleamine 2,3-dioxygenase in human inflammatory bowel disease. *Clin Immunol* **113**, 47-55.

Wu F, Dassopoulos T, Cope L, Maitra A, Brant SR, Harris ML, Bayless TM, Parmigiani G & Chakravarti S (2007) Genome-vide gene expression differences in Crohn's disease and ulcerative colitis from endoscopic pinch biopsies: Insights into distinctive pathogenesis. *Inflamm Bowel Dis* **13**, 807-821.

Wu J, James JDW, Dooner HK & Browse J (1994) A mutant of Arabidpsis deficient in the elongation of palmitic acid. *Plant Physiol* **106**, 143-150.

Xavier R, Brennan T, Li QQ, McCormack C & Seed B (1998) Membrane compartmentation is required for efficient T cell activation. *Immunity* **8**, 723-732.

Xu SQ, Li YH, Hu SH, Chen K & Dong LY (2008) Effects of Wy14643 on hepatic ischemia reperfusion injury in rats. *World J Gastroenterol* **14**, 6936-6942.

Yamamoto S, Isuzugawa K, Takahashi Y, Murase Y, Iwata M, Arisawa T, Nakano H, Nishimura N, Yamato S, Ohta M, Ina K, Murata T, Hori M, Ozaki H & Imakawa K

(2005) Intestinal gene expression in TNBS treated mice using genechip and subtractive cDNA analysis: Implications for Crohn's disease. *Biol Pharm Bull* **28**, 2046-2053.

Yang X, Letterio JJ, Lechleider RJ, Chen L, Hayman R, Gu H, Roberts AB & Deng CX (1999) Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *EMBO J* **18**, 1280-1291.

Yang YH & Speed T (2002) Design issues for cDNA microarray experiments. *Nat Rev Genet* **3**, 579-588.

Yates JR, Eng JK, McCormack AL & Schieltz D (1995) Method to correlate tandem mass-spectra of modified peptides to amino acid sequences in the protein database. *Analyt Chem* **67**, 1426-1436.

Yen D, Cheung J, Scheerens H, Poulet F, McClanahan T, McKenzie B, Kleinschek MA, Owyang A, Mattson J, Blumenschein W, Murphy E, Sathe M, Cua DJ, Kastelein RA & Rennick D (2006) IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Investig* **116**, 1310-1316.

Yoshikawa T, Shimano H, Yahagi N, Ide T, Amemiya-Kudo M, Matsuzaka T, Nakakuki M, Tomita S, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, Takahashi A, Sone H, Osuga J, Gotoda T, Ishibashi S & Yamada N (2002) Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. *J Biol Chem* **277**, 1705-1711.

Yu ZT & Morrison M (2004) Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Appl Environ Microbiol* **70**, 4800-4806.

Yuceyar H, Ozutemiz O, Huseyinov A, Saruc M, Alkanat M, Bor S, Coker I & Batur Y (1999) Is administration of n-3 fatty acids by mucosal enema protective against trinitrobenzene-induced colitis in rats? *Prostaglandins Leukot Essent Fatty Acids* **61**, 339-345.

Zeldin DC, Foley J, Goldsworthy SM, Cook ME, Boyle JE, Ma JX, Moomaw CR, Tomer KB, Steenbergen C & Wu S (1997) CYP2J subfamily cytochrome P450s in the gastrointestinal tract: Expression, localization, and potential functional significance. *Mol Pharmacol* **51**, 931-943.

Zoetendal EG, Akkermans ADL, Akkermans-van Vliet WM, deVisser JAGM & deVos WM (2001) The host genotype affects the bacterial community in the human gastrointestinal tract. *Microb Ecol Health Dis* **13**, 129-134.