# Nutrigenomics applied to an animal model of Inflammatory Bowel Diseases: Transcriptomic analysis of the effects of eicosapentaenoic acid- and arachidonic acid-enriched diets

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## Abstract

In vivo models of Inflammatory Bowel Diseases (IBD) elucidate important mechanisms of chronic inflammation. Complex intestinal responses to food components create a unique "fingerprint" discriminating health from disease. Five-week-old IL10−/− and C57BL/6J (C57; control) mice were inoculated orally with complex intestinal microflora (CIF) and/or pure cultures of Enterococcus faecalis and E. faecalis (EF) aiming for more consistent inflammation of the intestinal mucosa. Inoculation treatments were compared to non-inoculated IL10−/− and C57 mice, either kept in specific pathogen free (SPF) or conventional conditions (2×5 factorial design). At 12 weeks of age, mice were sacrificed for intestinal histological (HIS) and transcriptomic analysis using limma and Ingenuity Pathway Analysis Software. Colonic HIS was significantly affected ($P < 0.05$) in inoculated IL10−/− mice and accounted for approximately 60% of total intestinal HIS. Inoculation showed a strong effect on colonic gene expression, with more than 2000 genes differentially expressed in EF·CIF-inoculated IL10−/− mice. Immune response gene expression was altered ($P < 0.05$) in these mice. The second study investigated the effect of arachidonic (AA) and eicosapentaenoic acid (EPA) on colonic HIS and gene expression to test whether EPA, contrary to AA, diminished intestinal inflammation in EF·CIF IL10−/− mice (2×4 factorial design). AIN-76A (5% corn oil) and AIN-76A (fat-free) +1% corn oil supplemented with either 3.7% oleic acid (OA), AA or EPA were used. IL10−/− mice fed EPA- and AA-enriched diets had at least 40% lower colonic HIS ($P < 0.05$) than those fed control diets (AIN-76A and OA diets). The expression of immune response and 'inflammatory disease' genes (down-regulated: TNF, IL6, S100A8, FGF7, PTGS2; up-regulated: PPAR, MGLL, MYLK, PPSS23, ABCB4 with EPA and/or AA) was affected in IL10−/−mice fed EPA- and AA-enriched diets, compared to those fed AIN-76A diet.

## Keywords

nutrigenomics, analysis, effects, eicosapentaenoic, inflammatory, model, acid, animal, arachidonic, enriched, diets, applied, bowel, diseases, transcriptomic

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## Abstract

In vivo models of Inflammatory Bowel Diseases (IBD) elucidate important mechanisms of chronic inflammation. Complex intestinal responses to food components create a unique "fingerprint" discriminating health from disease. Five-week-old IL10$^{-/-}$ and C57BL/6J (C57; control) mice were inoculated orally with complex intestinal microflora (CIF) and/or pure cultures of *Enterococcus faecalis* and *E. faecalis* (EF) aiming for more consistent inflammation of the intestinal mucosa. Inoculation treatments were compared to non-inoculated IL10$^{-/-}$ and C57 mice, either kept in specific pathogen free (SPF) or conventional conditions ($2 \times 5$ factorial design). At 12 weeks of age, mice were sacrificed for intestinal histological (HIS) and transcriptomic analysis using limma and Ingenuity Pathway Analysis Software. Colonic HIS was significantly affected ($P < 0.05$) in inoculated IL10$^{-/-}$ mice and accounted for approximately 60% of total intestinal HIS. Inoculation showed a strong effect on colonic gene expression, with more than 2000 genes differentially expressed in EF·CIF-inoculated IL10$^{-/-}$ mice. Immune response gene expression was altered ($P < 0.05$) in these mice. The second study investigated the effect of arachidonic (AA) and eicosapentaenoic acid (EPA) on colonic HIS and gene expression to test whether EPA, contrary to AA, diminished intestinal inflammation in EF·CIF IL10$^{-/-}$ mice ($2 \times 4$ factorial design). AIN-76A (5% corn oil) and AIN-76A (fat-free) +1% corn oil supplemented with either 3.7% oleic acid (OA), AA or EPA were used. IL10$^{-/-}$ mice fed EPA- and AA-enriched diets had at least 40% lower colonic HIS ($P < 0.05$) than those fed control diets (AIN-76A and OA diets). The expression of immune response and 'inflammatory disease' genes (down-regulated: TNFα, IL6, S100A8, FGF7, PTGS2; up-regulated: PPARα, MGLL, MYLK, PPSS23, ABCB4 with EPA and/or AA) was affected in IL10$^{-/-}$ mice fed EPA- and AA-enriched diets, compared to those fed AIN-76A diet.
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*Keywords:* Inflammatory Bowel Diseases; IL10$^{-/-}$ mice; Colon; Gene expression; Dietary polyunsaturated fatty acids

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# 1. Introduction

The intestinal tract is the largest interface where human beings interact with their environment. This complex system consists of different cell types, harbors up to $10^{13}$ bacteria – more than 10 times the number of cells in the body [1] – and comprises 70% of the body's immune system [2]. Four protective barriers maintain the integrity of this interface: the epithelial barrier [3], the chemical barrier [4], the microbiological barrier [1] and the immunological barrier [5,6]. The disruption of one or more of these barriers can lead to chronic inflammation which is a hallmark of intestinal disorders such as Ulcerative Colitis [7] and Crohn's Disease [8,9], collectively referred to as Inflammatory Bowel Diseases (IBD). Current *in vitro* models, while useful for screening components, are unable to mimic the level of complexity of the intestinal system. Thus, *in vivo* models of IBD are important for unraveling the complex interactions between food, intestinal bacteria and intestinal cells, thereby elucidating the mechanisms by which IBD are initiated and maintained.

While the exact etiology and pathogenesis of IBD is still unclear, there is strong epidemiological evidence for a genetic contribution to disease susceptibility. Since $IL10^{-/-}$ mice spontaneously develop intestinal enterocolitis, they have been used extensively as a model of IBD [10–12]. IL10 is an anti-inflammatory cytokine which affects the growth and differentiation of various cell types of the immune system in vitro [13] and suppresses their function by inhibiting production of numerous cytokines by these cells [14]. Thus, IL10 is an important regulator of intestinal mucosal responses [15]. The severity of enterocolitis in $IL10^{-/-}$ mice is dependent on the breeding background, with BALB/c mice showing the most severe symptoms and C57BL/6J the least severe [16]. In the case of the latter, $IL10^{-/-}$ mice develop CD-like colitis by 12 weeks of age but this is not the case when they are raised under specific pathogen free (SPF) conditions [16]. Other studies report that under SPF conditions, $IL10^{-/-}$ mice do develop colitis, but it is less severe and is not observed before 20 weeks of age [17]. The mechanistic defect causing inflammation in $IL10^{-/-}$ mice is unclear but intestinal bacteria are necessary for the initiation and progression of inflammation [18].

Results from our laboratory using $IL10^{-/-}$ mice (C57BL/6J background) showed individual variability between animals with respect to the occurrence and severity of intestinal inflammation [19]. In the first part of this paper, we describe an experiment in which the effect of inoculation with intestinal bacteria was tested in $IL10^{-/-}$ mice to determine whether this would result in a more consistent and reproducible mucosal inflammation in the intestine. Histological injury score and gene expression in intestinal tissues of $IL10^{-/-}$ mice inoculated with populations of bacteria commonly found in the intestinal lumen, either complex intestinal microflora (CIF) and/or pure cultures of intestinal bacteria such as *Enterococcus faecalis* and *E. faecium* (EF) [20], were measured. These bacteria were introduced into the intestinal tract at a similar level to wild-type animals in normal conditions, with the aim of ensuring that bacterial colonization and subsequent exposure to potential inflammatory factors occurred at a similar level across all treatments.

In the second part of this paper, preliminary findings on the effect of two polyunsaturated fatty acids (PUFA), arachidonic (AA) and eicosapentaenoic acid (EPA), on intestinal inflammation are presented. AA is a precursor for the synthesis of eicosanoids, which are modulators of defensive and inflammatory processes in the intestinal mucosa [21]. EPA can inhibit AA metabolism competitively via the cyclo-oxygenase enzymatic pathways and thereby suppress the production of AA inflammatory mediators [21]. We hypothesize that contrary to AA, EPA diminishes both the severity of the lesions and the progression of induced intestinal inflammation. The aim was to identify these interactions using bacterially inoculated $IL10^{-/-}$ mice, with principal outcomes being histological injury score and differential gene expression using transcriptomic analysis.

# 2. Material and methods

The experimental procedures for these trials were reviewed and approved by the Crown Research Institute Animal Ethics Committee in Hamilton, New Zealand, according to the Animal Protection Act (1960) and Animal Protection Regulations (1987) and amendments.

## 2.1. Animals

Male mice, 4–5 weeks of age were used for all experiments. $IL10^{-/-}$ mice (B6.129P2-IL10<tm1Cgn>/J) and C57 controls (C57BL/6J) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA), and individually housed under conventional conditions (12-h light:12-h dark cycle (lights on from 08:00 to 20:00 h), temperature of $22 \pm 1\,^{\circ}C$ and approximately 50% relative humidity) in shoebox-style cages containing untreated wood shavings (Hi Tech Security Disposals Ltd., Auckland, New Zealand) and a plastic tube for environmental enrichment. All mice were given water and, unless otherwise stated, fed a non-sterile, AIN-76A powdered diet made in-house (Table 1). Food intake

Table 1

Composition of the AIN-76A diet and the AIN-76A diets enriched with oleic acid (OA), arachidonic acid (AA) and eicosapentaenoic acid (EPA)

| Dietary ingredient | Percentage in the diet | | | |
|---|---|---|---|---|
| | AIN-76A | OA | EPA | AA |
| Casein[a] | 20 | 20 | 20 | 20 |
| DL-methionine[b] | 0.3 | 0.3 | 0.3 | 0.3 |
| Sucrose[c] | 50 | 50 | 50 | 50 |
| Dextrin[d] | 15 | 15 | 15 | 15 |
| Arbocel, non-nutritive bulk[e] | 5 | 5 | 5 | 5 |
| Corn oil[f] | 5 | 1 | 1 | 1 |
| C18:2, 99%+[g] | 0 | 0.20 | 0.20 | 0.20 |
| C18:3, 99%+[h] | 0 | 0.06 | 0.06 | 0.06 |
| Oleic acid, 99%+ | 0 | 3.74 | 0 | 0 |
| Eicosapentaenoic acid, 98%+ | 0 | 0 | 3.74 | 0 |
| Arachidonic acid, 99%+ | 0 | 0 | 0 | 3.74 |
| Choline bitartrate[b] | 0.2 | 0.2 | 0.2 | 0.2 |
| AIN-76A Mineral Mix[i] | 3.5 | 3.5 | 3.5 | 3.5 |
| AIN-76A Vitamin Mix[i] | 1 | 1 | 1 | 1 |
| Ethoxyquin[b] | 0.01 | 0 | 0 | 0 |
| TBHQ[j] | 0 | 0.001 | 0.001 | 0.001 |

All diets were prepared in-house using ingredients from the suppliers listed, and according to the compositions shown.

[a] Alacid, lactic casein 30 mesh, NZMP Ltd., Wellington, New Zealand.

[b] Sigma, Sigma–Aldrich Inc., St. Louis, MO, USA.

[c] Caster sugar, Chelsea, New Zealand Sugar Company Limited, Auckland, New Zealand.

[d] Wheaten cornstarch, Golden Harves, Primary Foods Ltd., Auckland, New Zealand.

[e] Arbocell B600, J. Rettenmaier & Sohne GmbH+ Co, Rosenberg, Germany.

[f] Tradewinds, Davis Trading, Palmerston North, New Zealand.

[g] C18:2, linoleic acid.

[h] C18:3, alpha-linolenic acid.

[i] Prepared in-house based on the AIN-76A diet formulation [46].

[j] TBHQ, Tertiary butylhydroquinone as antioxidant.

was measured daily and water was refreshed twice a week. The food intake of all mice was adjusted daily to equal the mean amount of food consumed by the IL10$^{-/-}$ mice on the previous day. Growth performance was measured a minimum of thrice weekly. All mice were carefully monitored for disease symptoms (weight loss, soft faeces, inactivity). Tissue sampling and downstream analyses were performed as described below.

### 2.2. Inoculation study in IL-10$^{-/-}$ mice

Twenty-five male C57 mice and 25 male IL10$^{-/-}$ mice (C57BL/6J background) were used (Fig. 1). Upon arrival (4 weeks of age), five C57 and five IL10$^{-/-}$ mice were housed in SPF conditions (isolator cages). All mice were fed AIN-76A powdered diet (Table 1) sterilized by gamma radiation suitable for SPF (Schering-Plough, Wellington, New Zealand). The other 20 C57 and 20 IL10$^{-/-}$ mice were housed in conventional conditions. At 5 weeks of age, 15 of each strain were

inoculated orally with the three different preparations of bacteria commonly found in the intestinal lumen (5 IL10$^{-/-}$ mice and 5 C57 mice each for each different bacterial preparation).

Five C57 mice and five IL10$^{-/-}$ mice housed in conventional conditions were inoculated with a culture of *E. faecalis* and *E. faecium* (EF; 12 calf and chicken strains obtained from AgResearch Microbiology Group in Palmerston North; $1.2 \times 10^8$ colony forming units). The *Enterococcus* strains were subcultured onto fresh Slanetz & Bartley medium (Oxoid, Hampshire, UK) and incubated at 42 °C for 48 h, then transferred to Todd-Hewitt Broth (Oxoid, Hampshire, UK) and incubated at 37 °C for 24 h. All strains were then centrifuged ($2000 \times g$, 10 min, 4 °C), re-suspended in sterile $1 \times$ PBS, and pooled for inoculation.

Another five C57 and five IL10$^{-/-}$ mice were inoculated with complex intestinal flora (CIF; 200 μL). The CIF solution was prepared by harvesting digesta from age-matched C57BL/6 mice raised under conventional conditions following humane euthanasia by CO$_2$ asphyxiation and cervical dislocation. Briefly, the intestinal tract (from pyloric sphincter to caecum) was removed and digesta were collected from the intestine and caecum and suspended in sterile $1 \times$ PBS.

The final group of five C57 and five IL10$^{-/-}$ mice was inoculated with 200 μL of an inoculation solution consisting of a 1:1 (v/v) mixture of the EF ($6.0 \times 10^7$ CFU) and CIF solutions described above (EF·CIF).

The EF, CIF and EF·CIF treatments were compared with each other and to non-inoculated C57 and IL10$^{-/-}$ mice and C57 and IL10$^{-/-}$ mice kept in SPF according to a $2 \times 5$ factorial design. At 12 weeks of age, all mice were sacrificed and sampling performed to harvest tissues and body fluids for histology evaluation and transcriptomic analysis of intestinal tissues as described below.

### 2.3. PUFA experiment with IL10$^{-/-}$ mice

Twenty-four male C57 mice and 24 male IL10$^{-/-}$ mice were inoculated according to the EF·CIF protocol described above to obtain consistent and reproducible intestinal inflammation. A powdered diet based on AIN-76A (Table 1) was used to facilitate the mixing of EPA and AA compounds. Mice were randomly assigned to the following diets (Table 1) according to a $2 \times 4$ factorial design: (1) AIN-76A (5% corn oil), (2) AIN-76A (fat-free) + 1% corn oil + 3.7% oleic acid (OA), (3) AIN-76A (fat-free) + 1% corn oil + 3.7% AA and (4) AIN-76A (fat-free) + 1% corn oil + 3.7% EPA (Fig. 1). The 1% corn oil was supplemented with purified linoleic acid and alpha-linolenic acid to meet the nutritional requirements of mice for these essential fatty acids [22]; the OA, EPA and AA diets had 5% fat as in the AIN-76A diet. The ethyl ester of OA was used to make an OA-enriched control diet so that AA and EPA in PUFA-enriched diets could be substituted for OA. OA was chosen as the control fatty acid because it has no effect on tissue contents of AA and EPA and on eicosanoid biosynthesis [23]. Esters of AA, OA, linoleic acid and alpha-linolenic acid (99% purity, NuCheck Inc., Elysian, MN, USA) and EPA (98% purity, Brainfats Biotechnology Limited, Auckland, New
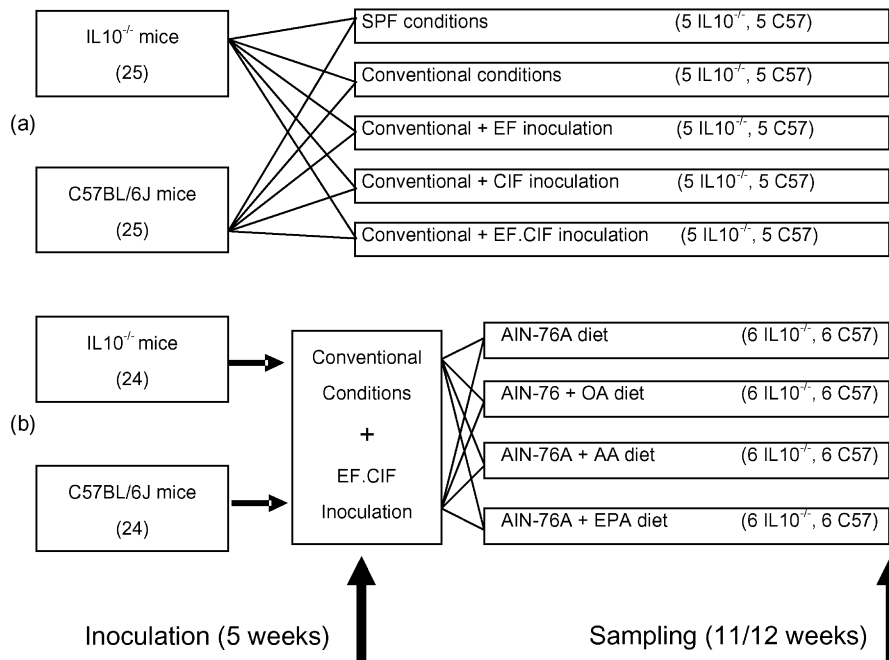
Fig. 1. Overall study design, showing the inoculation (a) and PUFA (b) experiments. For both experiments, mice were fed experimental diets from 4.5 weeks of age. In the case of the inoculation study, the diet was AIN-76A for all groups, while for the PUFA trial the diets are as shown in the figure and described in Table 1. Inoculation was performed at 5 weeks of age; for the inoculation experiment, this was as described in Section 2.2, while for the PUFA experiment all animals were inoculated with a mixture of *Enterococcus faecalis* and *Enterococcus faecium* strains and conventional intestinal flora derived from normal C57BL/6 mice raised under conventional conditions (EF·CIF). Tissue sampling was performed at 11 and 12 weeks of age, respectively, for the PUFA and inoculation experiments.

Zealand) were used. Between 5 and 11 weeks of age food consumption and growth performance were recorded as described previously. At 11 weeks of age, all mice were sacrificed and sampling performed to harvest tissue and body fluids for histology evaluation and transcriptomic analysis of intestinal tissues as described below. This tissue sampling time was selected based on a previous study, where IL10$^{-/-}$ mice inoculated with EF·CIF at 5 weeks of age showed an acceptable and consistent level of colonic inflammation by 11 weeks of age [24].

### 2.4. Tissue sampling

Before sacrifice, mice were fasted overnight (14 h), after which food was re-offered for 2 h. Food was again removed for the 2 h prior to sampling. This was to minimize the variation in time between the last food intake and tissue sampling [25]. A maximum of 12 mice were euthanized on each day; 14 h overnight fasting and tissue sampling were staggered in three groups of four mice, with 1 h between each group. Animals were euthanized via $CO_2$ asphyxiation and cervical dislocation. Blood was sampled via cardiac puncture (0.5–1 mL), and cells were separated from plasma by centrifugation ($2000 \times g$, 3 min, room temperature). Plasma was removed, immediately frozen in liquid nitrogen and stored at $-85\,^{\circ}$C. Pelleted blood cells were re-suspended in RNAlater® (Ambion, Austin, TX, USA) and frozen for subsequent RNA extraction. Spleen, liver,

thymus, kidneys and skeletal muscle were quickly removed and frozen in liquid nitrogen. The intestine was quickly removed, cut open lengthwise and flushed with 0.9% NaCl to remove any traces of digesta, then carefully laid out on an ice cold stainless steel tray. Sections of duodenum, jejunum, ileum and colon were fixed in 10% phosphate-buffered formalin and stored at room temperature until histological evaluation of inflammation. Samples of the same tissue were also rapidly frozen in liquid nitrogen before storage at $-85\,^{\circ}$C for gene and protein profiling.

### 2.5. Histopathology

Formalin-fixed intestinal tissue samples were embedded in paraffin and processed for sectioning [26]. Embedded tissue samples were sliced to obtain 5 μm thick sections which were stained with haematoxylin and eosin for light microscopic examination. Stained sections were assessed for inflammation based on a combination of inflammatory cell infiltration (monocytes, neutrophils, fibrin exudation and lymphangiectasis), tissue destruction (oedema and mucosal atrophy) and tissue repair (hyperplasia, angiogenesis, granulomas and fibrosis) [26]. A rating score between 0 (no change from normal tissue) and 3 (lesions involved most areas and all the layers of the intestinal section including mucosa, muscle and omental fat) was applied to each intestinal section (ileum, colon, etc.) for each aspect of inflammation (e.g.

monocytes, oedema, hyperplasia, etc.). The sum of scores for inflammatory cell infiltration (multiplied by 2), tissue destruction and tissue repair was used to represent the total histological injury score (HIS) for each intestinal section. The sum of the inflammatory cell infiltration was multiplied by 2 to give more weight to this value, as this represented the main characteristic of the observed inflammation. Gene expression analysis was performed only if significant changes in histopathology were observed in any intestinal section.

## 2.6. Gene expression hybridization

For microarray hybridization, total RNA from intestinal tissues was isolated by homogenizing the samples in TRIzol (Invitrogen, Auckland, New Zealand), a modification of the acid guanidinium thiocyanate–phenol–chloroform extraction method [27], according to the manufacturer's instructions. RNA was quantified with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was assessed with an RNA 6000 Nano LabChip kit using the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). Extracted RNAs were purified using RNeasy spin columns (QIAGEN, San Diego, CA, USA). Only total RNA with an OD 260/280 ratio > 2.0, a Bioanalyzer 28s/18s peak ratio > 1.2 and a RIN number superior to 8 was used for microarray hybridization. An equimolar pool of two to three total RNA extracts from intestinal tissues of two to three different mice per treatment was made. Reference RNA was used for microarray hybridization. For the inoculation trial, RNA extracts from control and treated mice were pooled in an equimolar proportion and used as the reference sample. For the PUFA trial, equimolar RNA extracts from small intestine, colon, kidney and liver of normal healthy growing Swiss mice plus RNA extracts from Swiss mouse fetuses were pooled to make a universal reference RNA for use in all subsequent trials.

RNA samples (experimental and reference pools) were labeled using the Agilent Low RNA Input Linear Amplification Kit (5184-3523) according to the manufacturer's instructions. Briefly, 500 ng of total RNA was amplified and reverse transcribed *in vitro* to cDNA using T7-polymerase, which was subsequently labeled with either cyanine3 (experimental) or cyanine5 (reference)-labeled CTP (10 mM; Perkin-Elmer/NEN Life Science (NEL 580, 581), Boston, MA, USA) dye. A Nanodrop ND-1000 spectrophotometer was used to monitor dye incorporation for the experimental samples (500 ng) which was between 9 and 22 pmol/$\mu$g. Targets consisting of amplified and fluorescent-labeled cRNA were hybridized using the Agilent oligonucleotide microarray in situ hybridization plus kit (5184-3568), following the manufacturer's protocols. Briefly 750 ng of pooled treatment cRNA was mixed with 750 ng of pooled reference cRNA in the presence of a control target, fragmented for 30 min at 60 °C in the dark and hybridized onto Agilent Technologies 44k (G4122-60510) mouse 60mer oligonucleotide arrays in a rotation oven (60 °C, 17 h, 4 rpm in the dark). Following hybridization, chambers were disassembled and the slides sequentially washed in solutions I, II and III (Agilent Technologies, Santa Clara, CA, USA) and air-dried. Slides were then scanned using a GenePix Professional 4200A scanner (Molecular Devices, Sunnyvale, CA, USA); a photomultiplier tube voltage of 550 (red laser) and 545 (green laser) was applied for the inoculation trial, and a photomultiplier tube voltage of 585 (red laser) and 600 (green laser) for the PUFA trial. Spot identification and quantification was performed with GenePix 6.0 software (Molecular Devices).

## 2.7. Statistical analysis

Statistical analyses of body weight and histological injury score were performed in GenStat (eighth ed., VSN International, Hemel Hempstead, UK, 2005). One-way ANOVA was used to detect significant differences between treatments. Log transformed values were used for the statistical analysis of HIS scores. A probability value less than 0.05 was regarded as significant and probability less than 0.10 but higher than 0.05 was declared a trend.

Intensity ratio values for all microarray spots were normalized using global lowess normalization without background correction (inoculation trial) and with background correction (PUFA trial). Relative amounts of the two dyes were used to determine the level of expression of a particular gene in IL10$^{-/-}$ mice compared to C57 mice. For each comparison a candidate list of differentially expressed genes was generated using linear models for microarray analysis (limma) within the Bioconductor framework [28]. For IL10$^{-/-}$ versus C57 (control diet) comparisons in both the inoculation and PUFA experiments, moderated *t*-statistics and false discovery rate control with probability less than 0.05- and 1.5-fold-change as statistical filtering criteria were used. For IL10$^{-/-}$ versus C57 (PUFA diets) comparisons, moderated *t*-statistics and probability less than 0.05- and 1.3-fold-change as statistical filtering criteria were used due to fewer genes being differently expressed, often with a lower fold-change compared to the inflammation comparison [28]. Differentially expressed genes from the inoculation and PUFA experiments were clustered into pathways using Ingenuity Pathway Analysis (Ingenuity Systems Inc., Redwood City, CA, USA).

This paper presents preliminary microarray results for both the inoculation and PUFA trial. Validation of gene expression changes is on-going; in this paper, only genes with a fold-change in expression greater than 1.5-fold for the inoculation trial and 1.3-fold for the PUFA experiment are presented. The gene expression data presented in the tables represent differentially expressed genes within a particular pathway ranked in the first 1000 differentially expressed genes according to the probability value obtained using limma analysis. PUFA comparisons are shown for IL10$^{-/-}$ mice fed PUFA-enriched diets and IL10$^{-/-}$ mice fed the AIN-76A diet. AIN-76A is the diet common to both inoculation and PUFA experiments; any reference to a control diet in Sections 3 and 4 applies to the AIN-76A diet.

## 3. Results

### 3.1. Inoculation trial

Mean live weight gain, measured between 5 and 10 weeks of age, was lower in $IL10^{-/-}$ mice compared to C57 mice in the C and EF·CIF groups (Table 2). EF·CIF inoculated $IL10^{-/-}$ mice and non-inoculated $IL10^{-/-}$ mice kept in conventional condition (C group) gained less weight than non-inoculated $IL10^{-/-}$ mice kept in SPF and EF- or CIF-inoculated $IL10^{-/-}$ mice (Table 2). For the C57 group, only the EF inoculation significantly reduced ($P < 0.05$) live weight gain compared to non-inoculated C57 mice either in SPF or conventional conditions (Table 2). There was no difference in live weight gain between the non-inoculated C57 mice kept in SPF or conventional conditions (Table 2). All C57 mice inoculated with a bacterial preparation (EF, CIF or EF·CIF) had the same live weight gain (Table 2).

There was no significant difference between the two mouse strains with respect to total intestinal inflammation in the C group ($IL10^{-/-}$ 4.7 ± 3.0 (S.D.) versus C57 4.3 ± 1.4 (S.D.), $P = 0.65$). As a result of inoculation, total intestinal HIS was higher in $IL10^{-/-}$ mice, and this effect was significant in both the EF ($IL10^{-/-}$ 12.5 ± 5.9 (S.D.) versus C57 2.3 ± 1.3 (S.D.), $P < 0.05$) and EF·CIF ($IL10^{-/-}$ 13.7 ± 5.4 (S.D.) versus C57 4.9 ± 2.3 (S.D.), $P < 0.05$) groups. Colon was the most inflamed intestinal section (Fig. 2a), accounting for approximately 60% of total intestinal HIS in inoculated $IL10^{-/-}$ mice. Colon HIS was greater in $IL10^{-/-}$ mice in each of the inoculation groups compared to C57 mice, with the largest difference being in the EF·CIF group ($IL10^{-/-}$ 8.1 ± 3.8 (S.D.) versus C57 0.4 ± 0.5 (S.D.), $P < 0.01$; Fig. 2b). Colon was therefore used for subsequent microarray analysis.

Initial microarray limma analysis showed a strong effect of inoculation on gene expression; 155 genes were differentially expressed (fold-change > 1.5, $P < 0.05$) in non-inoculated $IL10^{-/-}$ mice compared to non-inoculated C57 mice. In contrast, approximately 2000 genes showed differential expression in EF- and EF·CIF-inoculated $IL10^{-/-}$ mice compared to C57 mice with the same inoculations. For the CIF inoculation, over 1700 genes showed differential expression in $IL10^{-/-}$ mice compared to CIF-inoculated C57 mice. $IL10^{-/-}$ mice inoculated with EF·CIF had the greatest number of differentially expressed genes (2105) when compared with non-inoculated $IL10^{-/-}$ mice (group C), followed by EF (1641) and CIF (929). When the first 1000 ranked differentially expressed genes were compared between the three inoculation protocols using Ingenuity Pathway
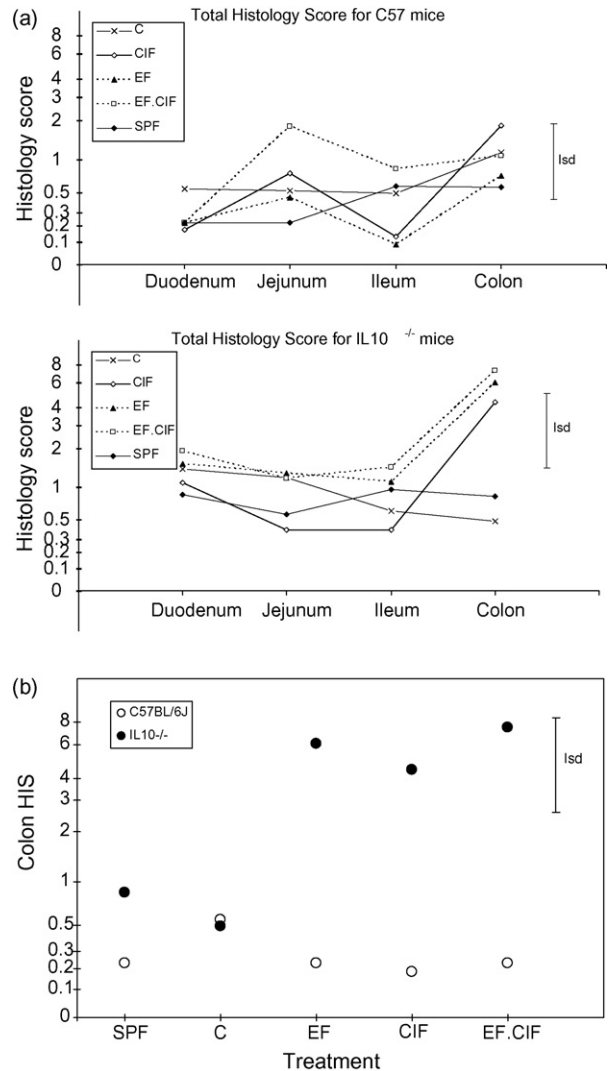


Fig. 2. (a) Total intestinal histological injury score (HIS) and (b) colon HIS of $IL10^{-/-}$ and C57 mice at 12 weeks of age after different inoculations at 5 weeks of age. Data represent mean for 5 mice fed the AIN-76A diet, and housed in specific pathogen free (SPF) or conventional (C) conditions and orally inoculated with 12 *Enterococcus* strains (EF), with conventional intestinal flora from normal C57BL/6 mice (CIF), or a 1:1 combination (EF·CIF) at 5 weeks of age. Error bar represents the least significant differences (LSD) for the overall comparison of data.

Analysis, changes in gene expression were largely consistent across the inoculation protocols (Table 3).

Clustering of the 155 differentially expressed genes between $IL10^{-/-}$ and C57 mice in the C group using Ingenuity Pathway Analysis identified lipid metabolism, molecular transport and small molecule biochemistry as the most significant pathways that were affected (Table 3). In contrast, cellular movement, haematological system development and function and immune

Table 2
Mean live weight gain (g) of IL10$^{-/-}$ and C57 mice between 5 and 10 weeks of age

| | Mouse strain | | Anova *P*-value (57 vs. IL10$^{-/-}$) |
|---|---|---|---|
| | C57 | IL10$^{-/-}$ | |
| Inoculation experiment[a] | | | |
| SPF | 5.40 ± 0.73[a] | 4.76 ± 1.25[a] | *P* = 0.49 |
| C | 5.94 ± 1.46[a] | 1.98 ± 2.30[b] | *P* < 0.05 |
| EF | 3.86 ± 0.79[b] | 2.98 ± 3.29[a,b] | *P* = 0.59 |
| CIF | 4.86 ± 3.04[a,b] | 2.03 ± 1.86[a,b] | *P* = 0.23 |
| EF·CIF | 5.26 ± 1.79[a,b] | 2.12 ± 1.45[b] | *P* < 0.01 |
| PUFA experiment[b] | | | |
| AIN-76A | 6.09 ± 2.37[a] | 4.85 ± 0.73[a] | *P* = 0.36 |
| OA | 5.68 ± 1.87[a] | 5.20 ± 0.92[a] | *P* = 0.31 |
| EPA | 5.27 ± 0.34[a] | 2.19 ± 1.36[b] | *P* < 0.001 |
| AA | 3.82 ± 0.93[b] | 3.70 ± 0.96[c] | *P* = 0.41 |

[a] Data represent mean ± S.D. for 5 mice fed the AIN-76A diet, and housed in specific pathogen free (SPF) or conventional (C) conditions, or housed in conventional conditions and orally inoculated with 12 *Enterococcus* strains (EF), with conventional intestinal flora from normal C57 mice (CIF), or a 1:1 combination (EF·CIF). Superscript letters (a and b) denote C57 and IL10$^{-/-}$ mice with values significantly different from mice of the same strain under SPF conditions.

[b] Data represent mean ± S.D. for six mice fed the AIN-76A diet, or AIN-76A diet supplemented with oleic acid (OA), or the PUFAs eicosapentaenoic acid (EPA) or arachidonic acid (AA). All mice were inoculated at 5 weeks of age with a combination of 12 *Enterococcus* strains and conventional intestinal flora from normal C57 mice. Superscript letters (a–c) that are different indicate a statistical difference (*P* < 0.05) between IL10$^{-/-}$ mice fed AIN-76A, OA, EPA or AA diets and between C57 mice fed AIN-76A, OA, EPA or AA diets.

response were the most significant pathways altered in IL10$^{-/-}$ mice as a result of the EF·CIF inoculation compared to similarly inoculated C57 controls (Table 3). Genes and fold-changes from the immune response, 'inflammatory diseases' and antigen presentation pathways are shown in Tables 4 and 5.

For the antigen presentation pathway, 12 genes were up-regulated in IL10$^{-/-}$ mice as a result of EF·CIF inoculation compared to C57 mice receiving the same inoculation; no genes showed evidence of down-regulation in this pathway (Table 5). In the case of immune response, 140 genes were differentially expressed in EF·CIF inoculated IL10$^{-/-}$ mice compared to EF·CIF inoculated C57 mice. Only the genes within this pathway that were differentially expressed with the EPA- and/or AA-enriched diets are presented in Section 3.3 and in Table 4. For example, the ATP-binding cassette sub-family B member 4 gene (ABCB4) and S100 calcium binding protein A8 gene (S100A8), which ranked in the top 30 according to its limma probability value, are shown in Table 4. In comparison, only 13 immune response genes were differentially expressed in non-inoculated IL10$^{-/-}$ mice compared to the non-inoculated C57 mice (data not shown). Seventy-three genes associated with the Ingenuity Pathway Analysis grouping of 'inflammatory diseases' showed differential expression in the EF·CIF IL10$^{-/-}$ mice compared to EF·CIF C57 mice (including ABCB4, SLP1 and TNFRSFIB; Table 4). This is contrasted with only six

genes differentially expressed genes in this pathway for the non-inoculated IL10$^{-/-}$ mice compared to non-inoculated C57 mice (C comparison; data not shown).

### 3.2. PUFA trial

Mean live weight gain from 5 to 10 weeks of age for C57 and IL10$^{-/-}$ mice in the various dietary treatments is shown in Table 2. Live weight gain was significantly lower in IL10$^{-/-}$ mice fed the EPA-enriched diet (*P* < 0.05) compared to IL10$^{-/-}$ mice fed the AA-enriched diet (Table 2). Reduction in live weight gain was also obtained when the IL10$^{-/-}$ mice fed the EPA-enriched diet or AA-enriched diet was compared to the control diets (AIN-76A and OA). There was no difference between IL10$^{-/-}$ mice fed either control diet (Table 2). Within the C57 mice, there was no significant difference in live weight gain between the AIN-76A, OA and EPA diets but C57 mice fed the AA diet gained less weight than C57 mice fed the AIN-76A, OA and EPA diets (Table 2). There was no significant difference in mean live weight gain between C57 and IL10$^{-/-}$ mice in the AIN-76A, OA or AA diet groups but IL10$^{-/-}$ mice fed the EPA-enriched diet had a reduced live weight gain compared to C57 mice fed the same diet (Table 2).

Total intestinal HIS among IL10$^{-/-}$ mice was higher than for C57 mice (IL10$^{-/-}$ 9.3 ± 1.5 (S.D.) versus C57 1.3 ± 0.4 (S.D.), *P* < 0.01) with colon being the most

Table 3

Summary of differentially expressed genes in colon tissue of IL-10$^{-/-}$ mice compared to C57 mice, and in IL10$^{-/-}$ mice inoculated with EF·CIF in response to dietary PUFAs, and the key functions represented by these genes

| Comparison | Differentially expressed genes | | | Major functions (number differentially expressed) |
|---|---|---|---|---|
| | Increase | Decrease | Total | |
| Inoculation experiment | | | | |
| C (IL10$^{-/-}$ vs. C57) | 104 | 51 | 155 | Lipid metabolism (11)<br>Molecular transport (7)<br>Small molecule biochemistry (18) |
| EF (IL10$^{-/-}$ vs. C57) | 1020 | 1222 | 2242 | Cellular movement (110)<br>Haematological system development and function (127)<br>Immune response (140) |
| CIF (IL10$^{-/-}$ vs. C57) | 864 | 839 | 1703 | Cellular movement (109)<br>Haematological system development and function (129)<br>Immune response (147) |
| EF·CIF (IL10$^{-/-}$ vs. C57) | 967 | 1032 | 1999 | Cellular movement (107)<br>Haematological system development and function (115)<br>Immune response (140) |
| IL10$^{-/-}$ (EF vs. C) | 633 | 1008 | 1641 | Cellular movement (98)<br>Haematological system development and function (104)<br>Immune response (120) |
| IL10$^{-/-}$ (CIF vs. C) | 450 | 479 | 929 | Immune response (127)<br>Cellular movement (110)<br>Haematological system development and function (116) |
| IL10$^{-/-}$ (EF·CIF vs. C) | 604 | 1501 | 2105 | Cellular movement (97)<br>Haematological system development and function (106)<br>Immune response (118) |
| PUFA experiment | | | | |
| AIN-76 (IL10$^{-/-}$ vs C57) | 1046 | 889 | 1935 | Inflammatory disease (94)<br>Haematological system development and function (121)<br>Immune response (143) |
| IL10$^{-/-}$ (OA vs. AIN-76A) | 264 | 257 | 521 | Cellular growth and proliferation (15)<br>Cell-to-cell signaling and interaction (31)<br>Immune response (10) |
| IL10$^{-/-}$ (EPA vs. AIN-76A) | 597 | 389 | 986 | Cell-to-cell signaling and interaction (42)<br>Inflammatory diseases (15)<br>Amino acid metabolism (4) |
| IL10$^{-/-}$ (AA vs. AIN-76A) | 852 | 575 | 1427 | Cell-to-cell signaling and interaction (79)<br>Inflammatory diseases (49)<br>Amino acid metabolism (8) |

Data represent numbers of differentially expressed genes for each comparison, calculated using limma analysis within the Bioconductor framework. For the inoculation experiment, a fold-change of >1.5 and false discovery rate control of $P < 0.05$ were used as filtering criteria; for the PUFA experiment, fold-change >1.3 and $P$-value <0.05. The most significant functions were identified using Ingenuity Pathway Analysis Software. Total RNA from colon tissue was used for microarray hybridization to Agilent Technologies 44k (G4122-60510) mouse 60mer oligonucleotide arrays. For the inoculation (EF·CIF-inoculated IL10$^{-/-}$ vs. similarly treated C57 mice) and PUFA (PUFA-fed IL10$^{-/-}$ vs. AIN-76A-fed IL10$^{-/-}$ mice) experiments data represent mean values from two pools of RNA for each mouse strain, with RNA from two and three mice, respectively, in each pool.

inflamed intestinal tissue (Fig. 3a). Intestinal inflammation scores were mainly derived from inflammatory cell infiltration (75%, monocytes and neutrophils) and to a lesser extent, tissue destruction (20%, enterocyte loss and edema) and tissue repair (5%, hyperplasia). IL10$^{-/-}$ mice on the EPA and AA diets had at least 40% lower colonic HIS scores ($P < 0.05$) than IL10$^{-/-}$ mice on control diets, AIN-76A and OA (Fig. 3b). There was also a greater variation in HIS in the colon of IL10$^{-/-}$ mice compared to C57 mice (Fig. 3b).

Table 4
Differentially expressed immune response and 'inflammatory diseases' genes in colon tissue of IL-10$^{-/-}$ mice compared to C57 mice (both with EF·CIF bacterial inoculation), and in PUFA-fed EF·CIF-inoculated IL10$^{-/-}$ mice compared to similarly inoculated IL10$^{-/-}$ mice fed AIN-76A

| Gene name | Symbol | Refseq ID | Fold-change | | |
|---|---|---|---|---|---|
| | | | EF·CIF IL10$^{-/-}$ vs. C57 | IL10$^{-/-}$ EPA vs. AIN-76A | IL10$^{-/-}$ AA vs. AIN-76A |
| ATP-binding cassette, subfamily B, member 4 | ABCB4 | NM_008830 | −4.1 | +2.8 | +1.7 |
| Calpastatin | CAST | NM_009817 | +1.9 | +1.4 | +1.3 |
| CD38 molecule | CD38 | NM_007646 | +3.3 | +1.7$^{NS}$ | +2.2 |
| Fibroblast growth factor 7 | FGF7 | NM_008008 | +1.1$^{NS}$ | −1.3$^{NS}$ | −1.6 |
| Inducible T-cell costimulator ligand | ICOSLG | NM_015790 | +1.1$^{NS}$ | +1.4$^{NS}$ | +1.6 |
| Interleukin 6 | IL6 | NM_031168 | −1.2$^{NS}$ | −1.1$^{NS}$ | −2.7 |
| Monoglyceride lipase | MGLL | NM_011844 | −2.3 | +2.1 | +1.8 |
| Myosin, light polypeptide kinase | MYLK | NM_139300 | −2.7 | +1.4 | +1.4 |
| Peroxisome proliferative activated receptor, alpha | PPARα | NM_011144 | −2.3 | +1.9 | +1.7 |
| Peroxisome proliferative activated receptor, gamma, coactivator 1, alpha | PPARGC1A | AK032149 | −1.2$^{NS}$ | +1.3$^{NS}$ | +1.4 |
| Protease, serine, 23 | PRSS23 | NM_029614 | −3.8 | +2.0$^{NS}$ | +2.8 |
| Prostaglandin-endoperoxide synthase 2 (Cox-2) | PTGS2 | NM_011198 | +1.5$^{NS}$ | −1.1$^{NS}$ | −3.1 |
| S100 calcium binding protein A8 | S100A8 | NM_013650 | +8.9 | +1.4$^{NS}$ | −2.8 |
| Secretory leukocyte peptidase inhibitor | SLPI | NM_011414 | +6.0 | +1.7 | −1.1$^{NS}$ |
| Tumor necrosis factor, alpha | TNFα | NM_013693 | +2.3 | −1.1$^{NS}$ | −1.5 |
| Tumor necrosis factor receptor superfamily, member 1B | TNFRSF1B | NM_011610 | +1.9 | −1.3 | −1.3$^{NS}$ |

Data represent the fold-change in gene expression for the particular comparison, calculated using limma analysis according to the Bioconductor framework and clustered in pathways according the Ingenuity Pathway Analysis Software. Total RNA from colon tissue was used for microarray hybridization to Agilent Technologies 44k (G4122-60510) mouse 60mer oligonucleotide arrays. A NS superscript means the reported fold-change was not significant between the two groups being compared. For the inoculation (EF·CIF-inoculated IL10$^{-/-}$ vs. similarly treated C57 mice) and PUFA (PUFA-fed IL10$^{-/-}$ vs. AIN-76A-fed IL10$^{-/-}$ mice) experiments data represent mean values from two pools of RNA for each mouse strain, with RNA from two and three mice, respectively, in each pool.

Gene expression analysis using limma and pathway clustering using Ingenuity Pathway Analysis Software revealed 'cell-to-cell signaling and interaction', 'inflammatory diseases' and 'amino acid metabolism' as the key functions affected in IL10$^{-/-}$ mice fed EPA- and AA-enriched diets (Table 3). This paper focused on the expression data for genes in the ranked first 1000 list that are significant according to the criteria stated in Section 2.7 and that clustered in the 'immune response' and 'inflammatory diseases' pathways (Table 4). The gene list for EF·CIF IL10$^{-/-}$ versus C57 comparison presented in Table 4 is data from the inoculation trial as similar results were obtained from the PUFA trial for that comparison.

### 3.3. Specific differentially expressed genes in the inoculation or PUFA trial

The expression of the monoglyceride lipase gene (MGLL) which is involved in lipid metabolism and inflammatory responses was down-regulated in IL10$^{-/-}$ mice compared to C57 mice, both fed the control diet but MGLL expression was up-regulated in the IL10$^{-/-}$ mice fed PUFA diets (Table 4). The S100A8 protein plays a role in inflammatory responses and its gene expression was up-regulated by 8.9-fold in IL10$^{-/-}$ mice fed the control diet compared to C57 mice fed the same diet. In contrast, the expression of the S100A8 gene showed a significant down-regulation in the IL10$^{-/-}$ mice fed the AA-enriched diet, but its expression was unchanged in the EPA-fed IL10$^{-/-}$ mice (Table 4). The expression of the prostaglandin-endoperoxide synthase 2 gene (PTGS2, also named COX-2), which is a major mediator of inflammation, was unchanged in IL10$^{-/-}$ mice fed the control diet, but when this diet was enriched with AA, PTGS2 gene expression was down-regulated.

Table 4 also shows which genes involved in innate immune responses and T-cell responses had altered expression as a consequence of the dietary PUFA treatments. This includes genes such as tumor necrosis factor alpha (TNFα; only significant for the AA-enriched diet) and its receptor (tumor necrosis factor receptor super-

Table 5

'Antigen presentation pathway' genes differentially expressed in colon tissue of EF·CIF-inoculated IL-10$^{-/-}$ mice compared with EF·CIF-inoculated C57 mice

| Gene name | Symbol | Refseq ID | EF·CIF IL10$^{-/-}$ vs. EF·CIF C57 |
|---|---|---|---|
| CD74 molecule, MHC Class II invariant chain | CD74 | NM_010545 | +5.7 |
| Histocompatability 2, Q region locus 1 | H2-Q1 | NM_010390 | +1.5 |
| MHC Class II, DM alpha | HLA-DMA | NM_010386 | +2.9 |
| MHC Class II, DM beta | HLA-DMB | NM_010387 | +7.1 |
| MHC Class II, DQ alpha 1 | HLA-DQA1 | NM_010378 | +4.4 |
| MHC Class II, DQ beta 2 | HLA-DQB2 | NM_207105 | +6.3 |
| MHC Class II, DR beta 1 | HLA-DRB1 | NM_010382 | +4.6 |
| HLA-G histocompatability antigen, Class I, G | HLA-G | NM_010391 | +1.8 |
| Proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7) | PSMB8 | NM_010724 | +3.9 |
| Proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2) | PSMB9 | NM_013585 | +3.8 |
| Transporter 1, ATP-binding cassette, sub-family B | TAP1 | NM_013683 | +2.5 |
| Transporter 1, ATP-binding cassette, sub-family B | TAP2 | NM_011530 | +1.7 |

Data represent the fold-change of gene expression for the particular comparison, calculated using limma analysis according to the Bioconductor framework and clustered in pathways using Ingenuity Pathway Analysis Software. Total RNA from colon tissue was used for microarray hybridization to Agilent Technologies 44k (G4122-60510) mouse 60mer oligonucleotide arrays. Data represent mean values from two pools of RNA for each mouse strain, with RNA from two and three mice, respectively, in each pool.

family, member 1B (TNFRF1B); only significant for the EPA-enriched diet), which were over-expressed in IL10$^{-/-}$ mice fed the control diet compared to C57 mice fed the same diet, but under-expressed in IL10$^{-/-}$ mice fed either PUFA diet (Table 4). There was no change in interleukin 6 (IL6) expression in IL10$^{-/-}$ mice fed the control diet, but this gene showed a lower expression in mice fed the AA-enriched diet (not statistically significant for the EPA-enriched diet). The secretory leukocyte peptidase inhibitor (SLPI) gene, expression of which was up-regulated by 6-fold in IL10$^{-/-}$ mice compared to C57 mice, was still up-regulated but by only 1.7-fold in IL10$^{-/-}$ mice fed the EPA-enriched diet, but decreased in IL10$^{-/-}$ mice fed the AA-enriched diet. The expression of the inducible T-cell costimulator ligand (ICOSLG) gene was unchanged in the IL10$^{-/-}$ mice fed the control diet, but up-regulated in IL10$^{-/-}$ mice fed the PUFA diets (Table 4).

Other genes involved in immune response and inflammation that were up-regulated in IL10$^{-/-}$ mice fed either PUFA-enriched diet were the ABCB4 gene (also named multidrug resistance gene 2 (mouse); down-regulated in IL10$^{-/-}$ mice compared to C57 mice fed the control diet) and the PPARα gene (Table 3). The other isoform of the PPAR gene, PPARγ, was down-regulated in IL10$^{-/-}$ mice fed the control diet but unchanged with the PUFA-enriched diet (data not shown). In contrast, myosin light polypeptide kinase (MYLC) and protease serine 23 genes were under-expressed in the IL10$^{-/-}$ mice fed

the control diet but over-expressed when the IL10$^{-/-}$ mice were fed the PUFA diets (Table 4). Expression of the genes encoding calpastatin and CD38 molecule were up-regulated irrespective of the strain effect or dietary PUFAs (Table 4).

## 4. Discussion

Our study shows for the first time that oral inoculation of IL10$^{-/-}$ mice (C57 background) with an EF·CIF bacterial preparation at 5 weeks of age results in increased and consistent inflammation compared to non-inoculated IL10$^{-/-}$ mice, with the most highly inflamed area of intestine being the colon. These histological changes were accompanied by significant changes in expression of genes involved in 'immune response', 'inflammatory diseases' and 'antigen presentation' by 11 weeks of age. These results support the suggestion that our refined IL10$^{-/-}$ mouse model shows characteristics of chronic inflammatory diseases, such as a dysregulated immune response acting in combination with inflammatory mechanisms that can cause tissue damage. The up-regulation of antigen presentation genes is consistent with increased expression of HLA Class II molecules that typically occurs in IBD in humans [29]. HLA Class II genes have been associated with risk of IBD in a number of populations [30,31] (reviewed in ref. [32]). In addition, MHC Class II antigen expression
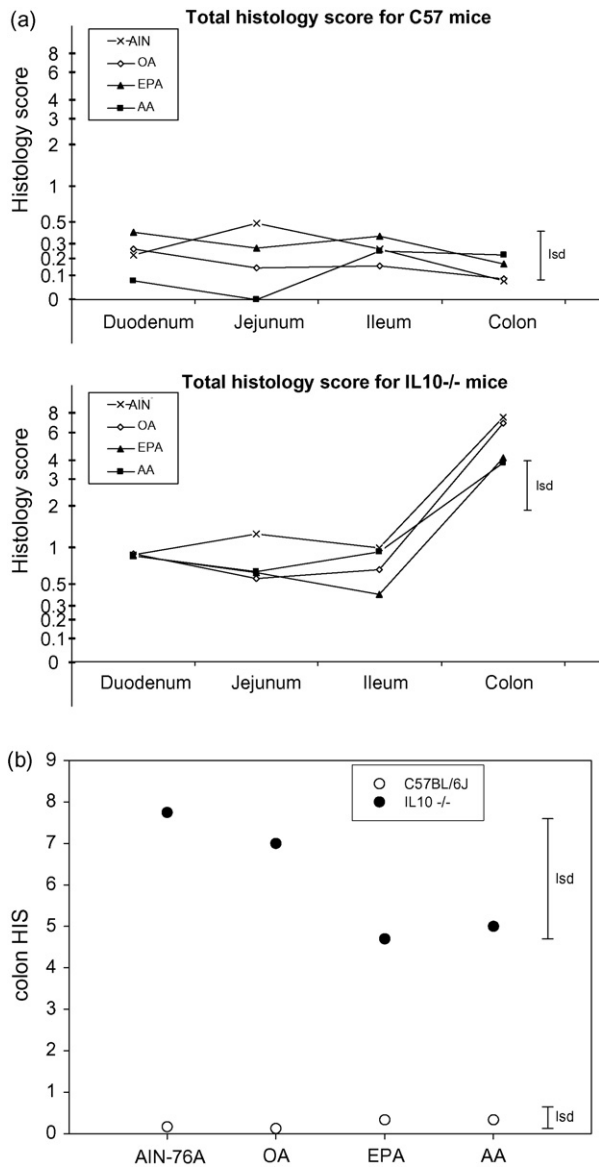
Fig. 3. (a) Total intestinal histological injury score (HIS) and (b) colonic HIS of IL10$^{-/-}$ and C57BL/6J mice at 11 weeks of age after PUFA treatments from 5 to 11 weeks of age. Data represent means for 6 mice fed AIN-76A diet, or AIN-76A diet supplemented with oleic acid (OA) or the PUFAs eicosapentaenoic acid (EPA) or arachidonic acid (AA). AIN-76A and OA are both control diets. Bars represent the least significant differences (LSD) for each mouse strain. All mice were inoculated at 5 weeks of age with a combination of 12 *Enterococcus* strains and conventional intestinal flora from normal C57BL/6 mice.

has previously been observed in the colon epithelial cells of 3–6 month-old IL10$^{-/-}$ mice [16]. Overall, our findings indicate that this refined IL10$^{-/-}$ mouse model represents an improved model of IBD, due to increased intestinal inflammation as a result of the inoculation

procedure. This model is therefore more suitable for testing various food components to identify those with potential beneficial effects for IBD patients.

Using this model, we have shown that dietary EPA and AA lowered the colonic inflammation observed in IL10$^{-/-}$ mice. Unexpectedly, both AA- and EPA-fed IL10$^{-/-}$ mice lost more body weight than IL10$^{-/-}$ mice fed the control diets (AIN-76A and OA diets), but AA-fed IL10$^{-/-}$ mice did not lose as much body weight as those fed EPA. Why growth was reduced with the EPA diet is unclear. Improved colonic health (occurrence and incidence of colonic tumors) in Apc(min) mice fed an EPA-enriched diet similar to this study has also been reported in the literature [23]. However, in that study, a similar diet enriched with AA increased the occurrence and incidence of colonic tumors in these mice compared to OA-enriched diet [23] whereas we did not observe similar effects of AA on colonic inflammation.

In IL10$^{-/-}$ mice fed the control diet, expression of PTGS2, a gene coding for an enzyme that catalyses the formation of prostaglandins from AA, was unchanged. This is in contrast with literature reports showing that PTGS2 is over-expressed in sites of early inflammation [33]. Results of another study by our group indicated that EF·CIF IL10$^{-/-}$ mice fed a control diet similar to the control (AIN-76A) diet used in this study showed early signs of inflammation at 8 weeks of age [24] so it is possible that an early increase in PTGS2 gene expression occurred but did not persist to 11 weeks of age. When AA-enriched diet was fed to IL-10$^{-/-}$ mice, expression of the PTGS2 gene was down-regulated at 11 weeks of age. It has been shown *in vitro* that unsaturated fatty acids such as EPA are the most potent inhibitors of AA-induced PTGS2 protein activity [34] and lipopolysaccharide-induced PTGS2 protein expression [35]. In Lee et al.'s study, lipopolysaccharide-induced PTGS2 protein expression was also inhibited by $n-6$ PUFA such as AA but to a lesser degree than EPA and DHA while saturated fatty acids induced its expression [35]. As PTGS2 catalyses the formation of pro-inflammatory prostaglandin E2 from AA, higher dietary AA should induce colonic inflammation but this was not the observation in IL10$^{-/-}$ mice fed the AA-enriched diet. Why AA reduced the colonic expression of PTGS2 and inflammation in IL10$^{-/-}$ mice in this study is unclear. It has been shown that prostaglandin E2 also suppresses production of TNFα and IL6 by monocytes and macrophages, and inhibits 5-lipoxygenase, thereby interfering with leukotriene X4 production; in this way, AA could give rise to anti-inflammatory lipoxins [36].

In IL10$^{-/-}$ mice fed the control diet, there was a four-fold down-regulation of ABCB4 in colonic tissues.

Another gene which is part of the ABC family, ABCB1, was also down-regulated in the same mice (data not shown). Like several other ABC transporters, ABCB4 is a lipid translocator and it has been shown to translocate phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane of hepatocytes; ABCB4 deficiency results in severe liver disease [37]. In colonic tissue, to our knowledge, there is no report of ABCB4 gene expression in the literature. As in this study, chronic colonic inflammation as observed in IBD patients and in animal models of IBD can result in a down-regulation of other ABC genes, such as ABCB1A and ABCB1B [38–40] suggesting a dysregulation of the ABC transporters during inflammation. In contrast, PUFA-enriched diets resulted in an up-regulation of the ABCB4 gene and likely, in a more optimal lipid translocation.

Furthermore, the expression of the S100A8 gene that codes for a calcium binding protein was up-regulated by nine-fold in $IL10^{-/-}$ mice fed the control diet. Murine S100A8 is a potent chemo-attractant (calcium binding protein) and its up-regulation has been associated with a number of acute and chronic inflammatory conditions and may protect against oxidative tissue damage [41]. In contrast, the down-regulation of the S100A8 gene observed in $IL10^{-/-}$ mice fed the AA diet could be explained by the additional free extracellular AA available to the inflamed tissue reducing the need for intracellular AA to be transported through the S100A8/A9-AA complex at the site of inflammatory lesions [42].

Down-regulation of the expression of FGF7 (also called keratinocyte growth factor) was observed in the colon of $IL10^{-/-}$ mice fed AA-enriched diet but its expression was unchanged in the colon of $IL10^{-/-}$ and C57 mice fed the control diet. FGF7 is involved in epithelial regeneration, and could be less active in $IL10^{-/-}$ mice fed AA because of the reduced colonic inflammation and tissue damage observed in this diet group, indicating a reduced requirement for regeneration. No mRNA for the FGF7 gene was detected in colon of dextran sodium sulfate challenged pigs (another model of IBD) fed a control diet or diets supplemented with $n-3$ PUFA (no data was available for $n-6$ PUFA) despite increased colonic tissue regeneration [43]. This result indicates that epithelial regeneration may in this case be FGF7-independent although its occurrence might depend on other growth factors and cytokines [44]. In the Bassaganya-Riera and Hontecillas study [43], an $n-3$ PUFA-enriched diet failed to protect from IBD, and instead promoted remission from IBD possibly by activating PPARδ. The same study showed that a mixture of conjugated linoleic acid and $n-3$

PUFAs accelerated recovery from IBD by activating PPARδ and up-regulating the expression of FGF7 in the colon, while CLA alone delayed the onset of IBD by activating another isoform of PPAR, PPARγ. These results together might indicate a synergistic regulation of inflammatory pathways by unsaturated lipids.

The colonic expression of PPARα was down-regulated by at least two-fold in $IL10^{-/-}$ mice fed the control diet but up-regulated when fed the PUFA-enriched diets. PPAR molecules have three isoforms (α, γ, δ) and act as a sensor for intracellular nutrient concentration through a nuclear receptor, thus regulating gene expression [36]. Up-regulation of PPARα by AA corroborates previous findings where PPAR isoforms in the intestine can be activated by PUFAs to ameliorate inflammatory disease severity [43]. PPARα gene activation is not specific to PUFA as a large number of fatty acids have been shown to activate this gene [36]. PPARα genes can be activated by lipids or their metabolites (e.g. oxidized phospholipids, raised AA concentrations or eicosanoids) following binding to the PPAR receptor [36]. For example, PPARα could have been activated indirectly through the increased AA concentration as a result of feeding the AA-enriched diet, or because of eicosanoid formation from AA via the CYP4A family [45], although the expression of the genes coding for the CYP4As was unchanged in $IL10^{-/-}$ mice fed this diet (data not shown). In contrast to PPARα, PPARγ has selectivity for unsaturated fatty acids including PUFAs [36]. However, in $IL10^{-/-}$ mice fed PUFA-enriched diet, PPARγ expression was unchanged.

The beneficial effect of PUFAs on colonic inflammatory responses seen in this study agrees with literature reports which show that PUFAs have immunomodulatory effects on cells of the immune system [36]. The results from the current study therefore suggest that, contrary to the proposed hypothesis, dietary AA supplementation had a beneficial effect on intestinal inflammation similar to that of EPA, and showed similar gene expression results to those of CLA reported in inflamed pigs [43]. AA activated colonic PPARα but also diminished the production of genes involved in immunomodulation and inflammation (TNFα, IL6, etc.). Comparing gene expression changes in tissues of animals fed $n-3$ versus $n-6$ PUFAs diets often reveals only minor differences [36]. However, the preliminary results from this study suggest that the refined mouse model of IBD developed here, $IL10^{-/-}$ mice inoculated with EF·CIF, allows important gene expression changes occurring as a result of $n-3$ and $n-6$ PUFA-enriched diets to be identified.

## 5. Conclusions

The studies described in this paper show the development of an improved mouse model of inflammation, and one example of using this model to investigate food components with potential for the amelioration of IBD. These results represent early steps in the development of foods which may interact with an individual's genetic make-up in a beneficial manner, to improve health and well-being.

## Acknowledgements

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