Diversity of caecal bacteria is altered in interleukin-10 gene-deficient mice before and after colitis onset and when fed polyunsaturated fatty acids

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Interleukin-10 gene-deficient (*Il10*^{−/−}) mice show a hyper-reaction to normal intestinal bacteria and develop spontaneous colitis similar to that of human Crohn's disease when raised under conventional (but not germ-free) conditions. The lack of IL10 protein in these mice leads to changes in intestinal metabolic and signalling processes. The first aim of this study was to identify changes in the bacterial community of the caeca at 7 weeks of age (preclinical colitis) and at 12 weeks of age (when clinical signs of colitis are present), and establish if there were any changes that could be associated with the mouse genotype. We have previously shown that dietary n-3 and n-6 polyunsaturated fatty acids (PUFA) have anti-inflammatory effects and affect colonic gene expression profiles in *Il10*^{−/−} mice; therefore, we also aimed to test the effect of the n-3 PUFA eicosapentaenoic acid (EPA) and the n-6 PUFA arachidonic acid (AA) on the bacterial community of caeca in both *Il10*^{−/−} and C57 mice fed these diets. The lower number of caecal bacteria observed before colitis (7 weeks of age) in *Il10*^{−/−} compared to C57 mice suggests differences in the intestinal bacteria that might be associated with the genotype, and this could contribute to the development of colitis in this mouse model. The number and diversity of caecal bacteria increased after the onset of colitis (12 weeks of age). The increase in caecal *Escherichia coli* numbers in both inflamed *Il10*^{−/−} and healthy C57 mice might be attributed to the dietary PUFA (especially dietary AA), and thus not be a cause of colitis development. A possible protective effect of *E. coli* mediated by PUFA supplementation and associated changes in the bacterial environment could be a subject for further investigation to define the mode of action of PUFA in colitis.

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Abbreviations: AA, arachidonic acid; CD, Crohn's disease; DGGE, denaturing gradient gel electrophoresis; *D*_{SC}, Dice similarity coefficient; EPA, eicosapentaenoic acid; IBD, inflammatory bowel disease; IL10, interleukin-10; *Il10*^{−/−}, IL10 gene-deficient; OA, oleic acid; PUFA, polyunsaturated fatty acid(s); qPCR, real-time quantitative PCR; SPF, specific pathogen free; UPGMA, unweighted pair group method of arithmetic averages.

INTRODUCTION

The interactions between intestinal bacteria and the mucosal immune system of a genetically susceptible host are important in the development of inflammatory bowel disease (IBD) such as Crohn's disease (CD). There is evidence that an abnormal mucosal immune response to common intestinal bacteria underlies the mucosal inflammation in this disease in humans (Strober *et al.*, 2007).

Both human clinical studies and animal studies have shown differences in the bacterial communities of the intestine between disease and control subjects, which suggest the involvement of luminal bacteria and bacterial products in the initiation and progression of intestinal inflammation (Mangin *et al.*, 2004; Bibiloni *et al.*, 2005). Antibiotic

treatment decreased concentrations of intestinal bacteria and attenuated colitis in CD patients (Colombel *et al.*, 1997; Gui *et al.*, 1997) and in the *Il10*^{-/-} mouse model of IBD (Madsen *et al.*, 2000). Colonization studies with germ-free rodent models have also indicated that enteric bacteria vary in their ability to induce colitis and interact specifically with genetically susceptible hosts (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 in healthy humans and animals (Jett *et al.*, 1994; Sellon *et al.*, 1998), but *Enterococcus* spp., especially *Enterococcus faecalis*, have been shown to induce intestinal inflammation in germ-free *Il10*^{-/-} mice (Balish & Warner, 2002; Kim *et al.*, 2005, 2007). *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 (loss of tolerance) and develop intestinal inflammation, unlike their wild-type 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 (SPF) conditions (McCarthy *et al.*, 2003). These findings are 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 in a few human studies investigating faecal bacteria (Cummings *et al.*, 1978; Endo *et al.*, 1991; Eastwood & Allgood, 1995; Farnworth *et al.*, 2007). To our knowledge there have been no studies on the potential effects of dietary lipids such as polyunsaturated fatty acids (PUFA) on the bacterial profiles of the caeca of inflamed *Il10*^{-/-} mice. Anti-inflammatory actions of the n-3 PUFA eicosapentaenoic acid (EPA) have mainly been attributed to the substitution of arachidonic acid (AA) in cell membranes, which results in the decreased production of AA-derived pro-inflammatory eicosanoids (Calder, 2006). Long-chain PUFA also show antibiotic-like and growth inhibitory actions against pathogenic bacteria and can enhance the beneficial actions of probiotics, e.g. *Lactobacillus* spp., on the mucosal surface and aid probiotics in colonizing the gastrointestinal tract (Das, 2002).

Since the lack of IL10 protein leads to significant changes in metabolic and signalling processes in the colon of *Il10*^{-/-} mice (Knoch *et al.*, 2010a), the first aim of this study was to examine changes in the bacterial community of the caecum at 7 weeks of age (preclinical colitis) and 12 weeks of age (when clinical signs of colitis are present) that may be associated with the mouse genotype. As we previously showed that dietary n-3 and n-6 PUFA exerted anti-inflammatory effects and affected colonic gene expression profiles in *Il10*^{-/-} mice (Knoch *et al.*, 2009, 2010b), we aimed to test the effect of dietary n-3 EPA and n-6 AA on

the bacterial community of the caecum as a function of the host genotype.

METHODS

Animal experiments. The studies were approved by the AgResearch Ruakura Animal Ethics Committee in Hamilton, New Zealand, according to the Animal Protection Act (1960) and the Animal Protection Regulations (1987) and Amendments; animal ethics application nos. 10642 and 10587. The experimental design of study 1 (time-course; Knoch *et al.*, 2010a) and study 2 (PUFA intervention; Knoch *et al.*, 2009, 2010b) has been previously described. Briefly, male *Il10*^{-/-} mice (C57BL/6J background, formal designation B6.129P2-*Il10*^{-/-} <tm1Cgn>/J) raised under SPF conditions and wild-type C57 mice (C57BL/6J) were received from the Jackson Laboratory (Bar Harbor, Maine, USA) at approximately 5 weeks of age. Mice were individually housed under conventional conditions and had *ad libitum* access to water. *Il10*^{-/-} and C57 mice were randomly assigned to one of five sampling groups (7, 8.5, 10, 12 or 14-week-old mice fed a standard AIN-76A diet; study 1) or to one of the experimental diets [AIN-76A (control), AIN-76A enriched with oleic acid (OA, fatty acid control), EPA or AA; study 2]. To induce a consistent and increased intestinal inflammation, *Il10*^{-/-} and C57 mice were orally inoculated with a mixture of 12 *Enterococcus faecalis* and *Enterococcus faecium* strains, and complex intestinal microflora from healthy, age-matched C57 mice as described previously (Roy *et al.*, 2007; Barnett *et al.*, 2010).

Before sampling, mice were fasted overnight (14 h), after which food was returned the following morning for 2 h. Food was again removed for the 2 h prior to sampling to minimize the effect of the time variation between the last food intake and sampling on gene expression profiles (Park *et al.*, 1997). In study 1, five or six mice were euthanized at 7, 8.5, 10, 12 and 14 weeks of age. Histological signs of colitis were assessed at all time points as described by Knoch *et al.* (2010a). The two time points used for gene and protein expression profiling (Knoch *et al.*, 2010a) were also selected for profiling the bacterial community of the caeca; these were based on histopathological assessment to obtain groups of individuals with no (7 weeks of age) or severe (12 weeks of age) colitis: *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). One 7-week-old *Il10*^{-/-} mouse died of unknown causes and two others showed moderate colitis; these mice were excluded from the analysis. In study 2, four to six *Il10*^{-/-} and C57 mice were euthanized at 11 weeks of age. This time was selected based on study 1, where all bacterially inoculated *Il10*^{-/-} mice developed colitis. One *Il10*^{-/-} mouse in the OA diet group and two *Il10*^{-/-} mice in the EPA diet group died of 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 bacterial DNA. From each group, caeca including the digesta were snap-frozen in liquid nitrogen and stored at -80 °C until analyses were carried out.

Molecular analysis of caecal bacteria. Bacterial genomic DNA from frozen caecal samples (caecum and digesta) was isolated using a QIAamp DNA Stool mini kit (Qiagen) according to manufacturer's instructions. The extracted DNA was used as a template in PCRs, using the HDA1-GC (GC-clamp) and HDA2 universal primers to amplify the variable V2-V3 regions of the bacterial 16S rRNA genes (Tannock *et al.*, 2000). PCR amplifications were performed using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems). The PCR products, 200 bp in length, were checked on 2% agarose gels before performing denaturing gradient gel electrophoresis (DGGE), which has been described previously (Nones *et al.*, 2009).

Digital images of the DGGE gels were analysed to detect DNA bands using the band-searching algorithm of BioNumerics software (Applied Maths). After normalization of the gels relative to the control sample, differences and similarities between the profiles within the same gel were analysed for the presence and absence of 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.

For sequence analysis, DGGE bands were excised from the gel matrix and eluted in sterile water at 4 °C overnight. After reamplification with the HDA1/2 primers, the amplicons were purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions, cloned into the plasmid vector pCR 2.1 and transformed into *Escherichia coli* TOP10 competent cells using the TOPO-TA cloning system (Invitrogen). The transformed cells were grown on selective Luria–Bertani (LB) agar plates and incubated overnight at 37 °C. Random white colonies were selected, incubated overnight at 37 °C in LB medium broth, and the plasmid DNA was extracted using the Zippy Plasmid Miniprep kit (ZYMO Research) according to manufacturer's instructions. After reamplification with the HDA1-GC/HDA2 primers, the position of the clone-band in a DGGE gel was compared with the original gel band. Clones that migrated to the same position as the original band were sequenced using the vector-specific M13 forward primer (5'-CCCAGTCAC-GACGTTGTAAAACG-3') at the Allan Wilson Centre Genome Service at Massey University, Palmerston North, New Zealand. The sequences were compared against 16S rRNA gene sequences available from the online database of the NCBI to identify the most closely related bacterial species.

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* spp., *Enterococcus* spp., *Clostridium perfringens*, *E. coli* and *Bacteroides vulgatus*, using the caecal DNA from *Il10*^{-/-} and C57 mice, are listed in Table 1. The bacterial strains used for standard curves were as follows: *E. coli* ATCC 35150, *Bacteroides fragilis* NZRM 964, *Ent. faecalis* AGR 991, *C. perfringens* ATCC 13124 and *Lactobacillus 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) was used for growth of *E. coli*, de Man–Rogosa–Sharpe (MRS) broth (Oxoid) for *L. reuteri*, Todd–Hewitt broth (Oxoid) for *Ent. faecalis*, 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 were prepared using a 10-fold dilution series (1×10^4 to 1×10^9 c.f.u. ml⁻¹) of genomic DNA extracted from each bacterial reference strain. The real-time quantitative PCR (qPCR) analysis was carried out in triplicate on a LightCycler 480 instrument (Roche Diagnostics), as described by Nones *et al.* (2009), using LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics). PCR amplicons were checked on 1% agarose gels. The interpretations of the bacterial quantifications were based on the assumption that the analysed community represented a similar mean number of 16S rRNA gene copies per bacterial cell. The qPCR results (number of cells per g 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). $P < 0.05$ was considered significant. All caecal samples were also tested for the presence of *Helicobacter* spp. by conventional PCR using primers H267F/H676R (5'-CTATG-ACGGGTATCCGGC-3'/5'-ATTCCACCTACCTCTCCCA-3'; Riley *et al.*, 1996) and HotStarTaq DNA polymerase (Qiagen), as described by Nones *et al.* (2009). PCR amplicons were checked on 2% agarose gels.

RESULTS

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 using DGGE. 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, a similarity matrix analysis was conducted, using the D_{sc} for the DGGE profiles of individual mice at 7 and 12 weeks of age (Fig. 1, Table 2). This analysis showed similarities (81.4–91.0%) in DGGE profiles between individual mice within each age group and also within each mouse genotype. The profiles of the 7- and 12-week-old *Il10*^{-/-} mice were more similar (81.4% and 82%, respectively) than the profiles of the 7- and 12-week-old C57 mice (86% and 91%, respectively). At 7 weeks of age, the profiles of the *Il10*^{-/-} mice were more

Table 1. Primers used for bacterial quantification by real-time PCR

Bacterial group	Primers (5'–3')	Reference
Total bacteria	ACTCCTACGGGAGGCAGCAGT GTATTACCGCGGCTGCTGGCAC	Tannock <i>et al.</i> (2000)
<i>Bacteroides–Prevotella– Porphyromonas</i> spp.	GGTGTCTGGCTTAAGTGCCAT CGGA(C/T)GTAAGGGCCGTGC	Rinttilä <i>et al.</i> (2004)
<i>Enterococcus</i> spp.	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	Rinttilä <i>et al.</i> (2004)
<i>Clostridium perfringens</i>	ATGCAAGTCGAGCGA(G/T)G TATGCGGTATTAATCT(C/T)CCTTT	Rinttilä <i>et al.</i> (2004)
<i>Escherichia coli</i>	CATGCCGCGTGTATGAAGAA CGGGTAACGTCAATGAGCAAA	Huijsdens <i>et al.</i> (2002)
<i>Bacteroides vulgatus</i>	GCATCATGAGTCCGCATGTTT TCCATACCCGACTTTATTCCTT	Wang <i>et al.</i> (1996)

Fig. 1. DGGE profiles of caecal bacteria from individual *Il10*^{-/-} and C57 mice at 7 and 12 weeks of age. C, control sample, used only to compare profiles between gels. Arrows indicate the positions in the gel where bands were excised for DNA recovery and sequencing. Numbers match the sequencing results in Table 3.

similar to those of the C57 mice (81.4 % and 86 %, respectively) than when they were at 12 weeks of age (82 % and 91 %, respectively).

Table 2. Similarity analysis of DGGE profiles between mice in different experimental groups (weeks of age or diets)

Experimental group	No. of mice per group	<i>D</i> _{sc} (%) between mice within experimental groups
Study 1		
C57 7 weeks of age	4	86.0
C57 12 weeks of age	4	91.0
<i>Il10</i> ^{-/-} 7 weeks of age	3	81.4
<i>Il10</i> ^{-/-} 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
<i>Il10</i> ^{-/-} AIN-76A	6	79.3
<i>Il10</i> ^{-/-} OA diet	5	82.8
<i>Il10</i> ^{-/-} EPA diet	4	67.5
<i>Il10</i> ^{-/-} AA diet	5	81.9

When excised, purified and sequenced, the closest sequence match in BLAST of bands 1–6 and 9–16 from the DGGE profiles of 7- and 12-week-old *Il10*^{-/-} and C57 mice was to uncultured strains (Fig. 1, Table 3). Bands 7 and 8 from the caecal profiles of 7- and 12-week-old immunocompetent non-inflamed C57 mice were identified as *Helicobacter pullorum* (GenBank accession FJ236465, 99–100 % identity). This particular species appeared to be mostly absent in the *Il10*^{-/-} mice. This suggests that this species could belong to the resident murine microflora in control mice, as observed in the stools of healthy subjects (Ceelen *et al.*, 2006). As it has been shown that *Helicobacter* spp. can induce intestinal inflammation (Burnens *et al.*, 1994; Fox *et al.*, 1999; Varon *et al.*, 2009) the bacterial DNA isolated from the caecum of all *Il10*^{-/-} and C57 mice was tested for the presence of *Helicobacter* spp. by genus-specific conventional PCR (data not shown). As expected, the result was positive for all mice. Whether *H. pullorum* or other *Helicobacter* spp. play a role in inducing colitis in the conventional *Il10*^{-/-} mice requires further investigation.

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

Cell numbers of total bacteria, *Bacteroides*–*Prevotella*–*Porphyromonas* and *Enterococcus* spp. were determined by real-time PCR in 7- and 12-week-old *Il10*^{-/-} and C57 mice (Fig. 2). 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 ($P=0.02$) (Fig. 2a). The bacterial cell number of the *Bacteroides*–*Prevotella*–*Porphyromonas* spp. was higher in 7-week-old C57 mice compared to *Il10*^{-/-} mice at the same age ($P=0.003$). An increase in the cell number of *Bacteroides*–*Prevotella*–*Porphyromonas* spp. was also observed from 7 weeks to 12 weeks of age in the caecum of *Il10*^{-/-} mice ($P=0.05$) (Fig. 2b). While there was an increase in the total bacterial cell number from 7 weeks to 12 weeks of age in *Il10*^{-/-} mice, the cell number of *Enterococcus* spp. decreased from 7 weeks 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. 2c).

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 diets monitored with DGGE are shown in Fig. 3, and the *D*_{sc} values from the similarity analysis of the DGGE profiles within dietary groups are listed in Table 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 were the profiles between individual mice

Table 3. Species identified from excised bands in study 1

Band excised*	No. of clones sequenced per band	Species identified by sequencing	GenBank ID (Identity)
1, 2	3	Uncultured bacterium clone H83N4_89h02, isolated from mouse caecum	EU458036 (99–100 %)
		Closest known cultured relative – <i>Bacteroides fragilis</i>	ABZX01000086 (95 %)
3, 4	3	Uncultured bacterium clone R-8325, isolated from faeces of rats fed wheat bran	FJ881325 (100 %)
		Closest known cultured relative – <i>Bacteroides uniformis</i> 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 – <i>Verminephrobacter eiseniae</i> EF01-2	NC_008786 (87–88 %)
7, 8	3	<i>Helicobacter pullorum</i> NCTC 12824	FJ236465 (99–100 %)
9, 10	3	Uncultured bacterium clone OF2A10, isolated from stool sample	GQ285980 (99–100 %)
		Closest known cultured relative – <i>Bacteroides capillosus</i> 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 – <i>Robiginitalea biformata</i> HTCC2501	NZ_AA0I01000001 (85 %)
13, 14	3	Uncultured bacterium clone YO00363F09, isolated from <i>Salmonella</i> -infected mouse caecum	FJ834844 (100 %)
		Closest known cultured relative – <i>Bryantella formatexigens</i> DSM 14469	ACCL02000018 (93 %)
15, 16	2	Uncultured bacterium clone YO00278D01, isolated from mouse caecum	FJ837861 (100 %)
		Closest known cultured relative – <i>Bryantella formatexigens</i> DSM 14469	ACCL02000018 (92 %)

*Numbers correspond to bands labelled in Fig. 1.

in the other dietary groups (79.3–87.0 %). Changes were observed in the individual DGGE profiles that seem to be associated not only with the mouse genotype 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 mouse genotype. In contrast, the AA diet caused a significant shift in the bacterial pattern in C57 mice, whose profiles appeared of similar complexity to those of the AA-fed *Il10*^{-/-} mice.

B. vulgatus (99–100 % identity) was identified from bands A and B in the DGGE profiles of *Il10*^{-/-} mice fed PUFA or control diets, but not in the profiles of C57 mice (Table 4). 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- or AIN-76A-fed *Il10*^{-/-} mice. This band was not observed 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. (bands 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 and O–R) were identified as uncultured bacteria and their closest relatives are listed in Table 4.

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

The quantitative analyses revealing the numbers of cells of total bacteria, *Bacteroides*–*Prevotella*–*Porphyromonas* spp.,

E. coli, *C. perfringens*, *Enterococcus* spp. and *B. vulgatus* are shown in Fig. 4. The qPCR data suggest a significant effect of diet on cell numbers of total bacteria, *E. coli* and *B. vulgatus* ($P < 0.05$); a significant effect of mouse genotype on cell numbers of total bacteria, *C. perfringens* and *B. vulgatus* ($P < 0.01$); and a significant interaction between mouse genotype and diet affecting *E. coli* ($P < 0.01$); all based on the copy numbers of caecal bacteria in *Il10*^{-/-} and C57 mice. There was a lower cell number of total bacteria in the OA-fed *Il10*^{-/-} mice compared to the 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. 4a) tends to support the apparent reduced abundance of bands in DGGE profiles in response to dietary AA.

Cell numbers of *C. perfringens* 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* was observed in AA-fed compared to OA-fed C57 mice (Fig. 4b). 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, when compared to the OA diet. The number of *E. coli* cells was lower in EPA-fed C57 mice compared to AA-fed C57 mice (Fig. 4c). There was no significant effect of diet or mouse genotype on the number of cells of *Enterococcus* spp. in the caecum of *Il10*^{-/-} or C57 mice (Fig. 4d).

Fig. 2. qPCR 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 lines represent the mean bacterial cell numbers of the individual mice; cell numbers of bacteria that

differ significantly between groups ($P < 0.05$), when comparing between different mouse genotypes at the same age or between the same mouse genotypes at different age, are labelled with different letters. Mouse genotype/age groups are represented along the x-axis.

A significant effect of mouse genotype ($P < 0.001$) and diet ($P = 0.04$) was observed for *B. vulgatus*. Cell numbers of *B. vulgatus* (a member of the *Bacteroides-Prevotella-Porphyromonas* spp. group) were significantly higher in the caecum of *Il10*^{-/-} mice fed the AIN-76A ($P = 0.05$) and OA ($P = 0.003$) control diets or the AA ($P = 0.02$) diet, compared with C57 mice on the same diets. The EPA diet increased the number of *B. vulgatus* in the caecum of C57 mice compared with OA-fed C57 mice (Fig. 4f).

DISCUSSION

This study describes the use of DGGE and qPCR analysis of 16S rRNA genes to investigate differences in the bacterial community in the caeca of *Il10*^{-/-} and C57 mice, and the impact of the onset of colitis and dietary PUFA. The findings show shifts in bacterial profiles of the caecum intestine upon progression from preclinical colitis to clinical colitis in *Il10*^{-/-} mice. Some of these shifts were shown to be affected by dietary PUFA, especially AA, and were also influenced by the mouse genotype. A limitation of this type of study is that only a limited number of DGGE band-derived amplicons (in this case 34 bands) can be sequenced, providing an incomplete view of the complex, and bacteria-dense, caecal community. Next-generation sequencing technologies allow vast numbers of partial 16S rRNA genes from uncultured bacteria to be sequenced (Claesson *et al.*, 2009). While DGGE analysis misses these shifts in bacterial community composition, it is a method that, when combined with quantification, gives reproducible results revealing the most abundant bacterial changes.

Effect of host genotype on caecal bacteria

Studies with mice (Vahtovuo *et al.*, 2001, 2003; Zoetendal *et al.*, 2001; Nones *et al.*, 2009), human IBD patients and healthy subjects (Frank *et al.*, 2007) have shown that the host genotype affects the composition of the bacterial community of the intestine. The reduced number of caecal bacteria observed in *Il10*^{-/-} compared to C57 mice, before clinical signs of colitis, is likely to be associated with the genotype. A reduction in bacterial number and diversity was reported with inflamed *Il10*^{-/-} mice on the 129/SvEv background compared to the wild-type counterpart (Wohlgemuth *et al.*, 2009). From 7 to 12 weeks of age there was an increase in the number of caecal bacteria in *Il10*^{-/-} mice, suggesting that inflammation changes the intestinal environment. As two out of five *Il10*^{-/-} mice had already developed histological signs of moderate colitis at 7

Fig. 3. DGGE profiles of caecal bacteria from individual *Il10*^{-/-} and C57 mice fed PUFA diets. C, control sample, used only to compare profiles between gels. Arrows indicate the positions in the gel where bands were excised for DNA recovery and sequencing. Letters match the sequencing results in Table 4.

weeks of age (Knoch *et al.*, 2010a), it seems likely that non-clinical inflammation was present in 7-week-old *Il10*^{-/-} mice, implying an increased leakiness of the intestinal epithelium, an impaired barrier function, and a consequent accumulation of bacteria in the mucosa. It remains unclear whether the bacteria are responding to inflammation, or are a causative agent when accumulating due to the leaky epithelial barrier membrane; it is also unclear which bacterial species might be involved.

The only known bacterial species in the inoculum used here that is proven to be associated with IBD development in *Il10*^{-/-} mice was *Ent. faecalis* (Balish & Warner, 2002). Whether the enterococci quantified here were the specific inoculated strains was not investigated. Nevertheless, the decreased cell number of enterococci in the caecum of 12-week-old inflamed *Il10*^{-/-} mice suggests that other bacteria in the inflamed large intestine might have affected the number of *Enterococcus* spp. by competing with and displacing them. No enterococci could be detected in the caecum or colon of conventionally housed, inflamed *Il10*^{-/-} mice (129/SvEv background) or the respective wild-type mice (Wohlgemuth *et al.*, 2009), indicating that bacteria other than enterococci trigger colitis in the 129/SvEv *Il10*^{-/-} mice. Similarly, the differences in caecal *E. coli* cell numbers observed between *Il10*^{-/-} and C57 mice (measured in study 2 only) might not be related to colitis development. Wohlgemuth *et al.* (2009) found no

correlation between increasing *E. coli* cell numbers in *Il10*^{-/-} mice and colitis severity. Another study showed no increase in *E. coli* numbers in germ-free *Il10*^{-/-} mice colonized with faecal bacteria from healthy wild-type mice (Bibiloni *et al.*, 2005).

The increase in total cell numbers of *Bacteroides-Prevotella-Porphyromonas* spp. in caeca from 7- to 12-week-old *Il10*^{-/-} mice indicates the potential involvement of these bacteria in colitis development. These species have been detected in colitis models (Rath *et al.*, 1999) and in human IBD patients (Swidsinski *et al.*, 2005). Although *B. vulgatus* is known to be associated with colitis (Rath *et al.*, 1999; Kishi *et al.*, 2000), mono-colonization of germ-free *Il10*^{-/-} mice with *B. vulgatus* did not lead to colitis (Kim *et al.*, 2007), indicating the importance of bacterial community composition in the induction of colitis in a genetically susceptible host.

Associated effects of dietary PUFA and genotype on caecal bacteria

Changes in bacterial profiles can be attributed to factors other than genotype, such as age, diet or the composition and interaction of the bacteria in a particular community. The present results show, we believe for the first time, that changes in dietary fatty acid composition, i.e. diet enrichment with pure ethyl esters of EPA and in particular

Table 4. Species identified from excised bands in study 2

Band excised*	No. of clones sequenced per band	Species identified by sequencing	GenBank ID (identity)
A, B	3	Uncultured <i>Bacteroidales</i> bacterium clone Fhc31, isolated from human and animal faeces	EU913590 (99–100 %)
C, D	3	Closest known cultured relative – <i>Bacteroides vulgatus</i> ATCC 8482	NC_009614 (99–100 %)
		Uncultured bacterium clone 2.77F, isolated from mouse faeces	EU655985 (100 %)
		Closest known cultured relative – <i>Porphyromonas uenonis</i> 60-3 deg1118640599623	NZ_ACLR01000152 (89 %)
E, F	2	Uncultured bacterium clone YO00272E02, isolated from streptomycin-treated <i>Salmonella</i> -infected murine ileum	FJ838603 (100 %)
G, H	3	Closest known cultured relative – <i>Denitrovibrio acetiphilus</i> DSM 12809	NZ_ABTN01000006 (87 %)
I, J	3	<i>Escherichia coli</i> strain CNM 477-02	EU555536 (100 %)
		Uncultured bacterium clone OF2A10, isolated from stool of healthy human	GQ285980 (100 %)
K, L	2	Closest known cultured relative – <i>Bacteroides capillosus</i> ATCC 29799	NZ_AAAXG02000037 (96 %)
		<i>Clostridium perfringens</i> partial, isolate colony 3, isolated from canine faeces	FN356962 (100 %)
M, N	2	Uncultured <i>Clostridium</i> sp. clone MS213A1_H11, isolated after gastrointestinal resection of IBD patient	EF710221 (98 and 100 %)
		Closest known cultured relative – <i>Clostridium</i> 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 – <i>Desulfovibrio desulfuricans</i> subsp. <i>desulfuricans</i> ATCC 27774	NC_011883 (95–96 %)
Q, R	3	Uncultured bacterium clone YO00360E03, isolated from streptomycin-treated <i>Salmonella</i> -infected murine ileum	FJ837456 (100 %)
		Closest known cultured relative – <i>Bryantella formatexigens</i> DSM 14469	ACCL02000018 (93 %)

*Numbers correspond to bands labelled in Fig. 3.

AA, can alter the number and diversity of bacteria in the caecum of healthy C57 and inflamed *Il10*^{−/−} mice. Previously, we reported that dietary AA tended to decrease colonic and systemic inflammation levels, and reduced the expression levels of some genes associated with cellular stress, inflammatory and immune responses in *Il10*^{−/−} mice (Knoch *et al.*, 2010b). These effects are likely to occur via PUFA uptake from circulating blood into colonocytes and incorporation into membrane phospholipids. Das (2002) suggested a direct toxic effect of the PUFA on harmful bacteria. Approximately 98 % of dietary lipids are digested and absorbed mainly in the small intestine, leaving only 2 % to reach the colon (Carey *et al.*, 1983; Phan & Tso, 2001). Whether the small amount of PUFA that reached the large bowel via the luminal side is sufficient to cause a direct toxic effect on particular bacterial species is unknown.

The potential change in membrane fatty acid composition of bacterial adhesion sites after AA supplementation might favour the presence of commensal *E. coli*, which could be protective in colitis development in the *Il10*^{−/−} mice. The dietary OA control tended to increase colitis in *Il10*^{−/−} mice compared to the AA- or EPA-fed *Il10*^{−/−} mice (Knoch *et al.*, 2009, 2010b), and lower *E. coli* cell numbers were found in the caecum of OA-fed compared to PUFA-fed *Il10*^{−/−} mice.

Hudault *et al.* (2001) reported that non-virulent EM0 (a human faecal strain) and virulent JM105 K-12 *E. coli* strains protected germ-free C3H/He/Oujco mice against *Salmonella* infection. Accumulation of *E. coli* in inflamed ileal tissue, as a result of epithelial barrier damage, has been reported in C57BL/6 mice susceptible to oral infection with *Toxoplasma gondii* (Heimesaat *et al.*, 2006) and in human IBD patients (Swidsinski *et al.*, 2005). The protective effect of some *E. coli* against other *Enterobacteriaceae* occurs via biofilm formation that limits the ability of pathogens to bind to the epithelium (Hudault *et al.*, 2001). Similarly, Kankaanpää *et al.* (2001) also suggested a beneficial effect of PUFA on the adherence of probiotics.

In conclusion, the differences in bacterial composition of the caeca between the mouse strains might be, at least partly, associated with the genotype. An association between particular bacterial species and colitis could not be made due to the complexity and heterogeneity of the bacteria present in the large intestine. Colonization studies of germ-free *Il10*^{−/−} mice with an individual bacterium, or a defined mix of commensal bacteria, will be required to make clear associations between bacterial species and colitis development. A possible protective effect of *E. coli* strains mediated by dietary PUFA and the associated changes in the bacterial environment could be a subject for further

Fig. 4. qPCR quantification of cells of (a) total bacteria, (b) *C. 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 PUFA (AA, EPA) or control (AIN-76A, OA) diets. The horizontal lines represent the mean bacterial cell numbers of the individual mice; cell numbers of bacteria that differ significantly between groups ($P < 0.05$), when comparing between different mouse genotypes on the same diet or between the same mouse genotypes on different diets, are labelled with different letters. The AIN-76A diet has only been compared with the OA diet, and the PUFA diets only with the OA fatty acid control.

investigation to define the mode of action of dietary PUFA during colitis.

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