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.

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 \textit{II10}\textsuperscript{–/–} 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 \textit{Lactobacillus} spp., were able to prevent colitis in \textit{II10}\textsuperscript{–/–} mice (Madsen et al., 2000). \textit{Enterococcus} spp. are common intestinal bacteria in healthy humans and animals (Jett et al., 1994; Sellon et al., 1998), but \textit{Enterococcus} spp., especially \textit{Enterococcus. faecalis}, have been shown to induce intestinal inflammation in germ-free \textit{II10}\textsuperscript{–/–} mice (Balish & Warner, 2002; Kim et al., 2005, 2007). \textit{II10}\textsuperscript{–/–} 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 \textit{II10}\textsuperscript{–/–} mice are born and maintained in germ-free conditions (Kim et al., 2005), but develops from 20 weeks of age in \textit{II10}\textsuperscript{–/–} 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 \textit{II10}\textsuperscript{–/–} 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. \textit{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 \textit{II10}\textsuperscript{–/–} 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 \textit{II10}\textsuperscript{–/–} 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 \textit{II10}\textsuperscript{–/–} mice (C57BL/6J background, formal designation B6.129P2-\textit{Il10}\textsuperscript{–/–}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. \textit{II10}\textsuperscript{–/–} 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, \textit{II10}\textsuperscript{–/–} and C57 mice were orally inoculated with a mixture of 12 \textit{Enterococcus faecalis} and \textit{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 caecae; these were based on histopathological assessment to obtain groups of individuals with no (7 weeks of age) or severe (12 weeks of age) colitis: \textit{II10}\textsuperscript{–/–} mice 7 weeks of age (three mice), \textit{II10}\textsuperscript{–/–} 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 \textit{II10}\textsuperscript{–/–} mouse died of unknown causes and two others showed moderate colitis; these mice were excluded from the analysis. In study 2, four to six \textit{II10}\textsuperscript{–/–} and C57 mice were euthanized at 11 weeks of age. This time was selected based on study 1, where all bacterially inoculated \textit{II10}\textsuperscript{–/–} mice developed colitis. One \textit{II10}\textsuperscript{–/–} mouse in the OA diet group and two \textit{II10}\textsuperscript{–/–} 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 \textit{II10}\textsuperscript{–/–} 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 HD1A-GC (GC-clamp) and HD2A 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).
Bacterial cells were counted using a Neubauer haemocytometer after 18 h incubation.

Microbiology 3308

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

<table>
<thead>
<tr>
<th>Bacterial group</th>
<th>Primers (5′–3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>ACTCCTACCGGGAGGCAAGCAGT</td>
<td>Tannock et al.</td>
</tr>
<tr>
<td></td>
<td>GTATTACCGGCCGCTGCGGAC</td>
<td></td>
</tr>
<tr>
<td>Bacteroides–Prevotella–Porphyromonas spp.</td>
<td>GGGTGGCGCTTAAGTGCCAT</td>
<td>Rinttilä et al.</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>CCCTATTGTTGATGCTCCATATT</td>
<td>Rinttilä et al.</td>
</tr>
<tr>
<td></td>
<td>ACTCGTTGTAATCTCCATATTG</td>
<td></td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>ATGCAGTCTGGACGAGCAGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TATGCGGTATTATCTTCCATATTCA</td>
<td>Rinttilä et al.</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>CATGCCGCGTGATGAGAA</td>
<td>Huijsdens et al.</td>
</tr>
<tr>
<td></td>
<td>CCGGTAACTGCTCAATGCGAAA</td>
<td></td>
</tr>
<tr>
<td>Bacteroides vulgatus</td>
<td>GCATCAGTATGGCAGTCATGCCC</td>
<td>Wang et al.</td>
</tr>
<tr>
<td></td>
<td>TCCATACCCGACTTTATCCATT</td>
<td></td>
</tr>
</tbody>
</table>

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 amplifications 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′-CTATGGACGGGTATCCCGGC-3′; Riley et al., 1996) and HotStarTaq DNA polymerase (Qiagen), as described by Nones et al. (2009). PCR amplifications 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∞ 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...
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).

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 Dsc 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

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

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. of mice per group</th>
<th>Dsc (%) between mice within experimental groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57 7 weeks of age</td>
<td>4</td>
<td>86.0</td>
</tr>
<tr>
<td>C57 12 weeks of age</td>
<td>4</td>
<td>91.0</td>
</tr>
<tr>
<td>Il10–/– 7 weeks of age</td>
<td>3</td>
<td>81.4</td>
</tr>
<tr>
<td>Il10–/– 12 weeks of age</td>
<td>4</td>
<td>82.0</td>
</tr>
<tr>
<td><strong>Study 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57 AIN-76A diet</td>
<td>5</td>
<td>82.5</td>
</tr>
<tr>
<td>C57 OA diet</td>
<td>5</td>
<td>87.0</td>
</tr>
<tr>
<td>C57 EPA diet</td>
<td>5</td>
<td>86.7</td>
</tr>
<tr>
<td>C57 AA diet</td>
<td>5</td>
<td>81.0</td>
</tr>
<tr>
<td>Il10–/– AIN-76A</td>
<td>6</td>
<td>79.3</td>
</tr>
<tr>
<td>Il10–/– OA diet</td>
<td>5</td>
<td>82.8</td>
</tr>
<tr>
<td>Il10–/– EPA diet</td>
<td>4</td>
<td>67.5</td>
</tr>
<tr>
<td>Il10–/– AA diet</td>
<td>5</td>
<td>81.9</td>
</tr>
</tbody>
</table>
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 \(11\text{O}^{14+}\) 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 \(11\text{O}^{14+}\) mice.

\(B\). \(vulgatus\) (99–100 % identity) was identified from bands A and B in the DGGE profiles of \(11\text{O}^{14+}\) 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 \(11\text{O}^{14+}\) mice. This band was not observed in the profile of \(11\text{O}^{14+}\) mice fed the OA diet. DNA fragments (bands K and L) identified as \(C.\ perfringens\) (FN356962, 100 % identity) were present in DGGE profiles of \(11\text{O}^{14+}\) mice fed the control diets, whereas an uncultured \(Clostridium\) spp. (bands M and N, EF710221, 98 and 100 % identities) was identified in profiles of \(11\text{O}^{14+}\) 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 \(11\text{O}^{14+}\) and C57 mice. There was a lower cell number of total bacteria in the OA-fed \(11\text{O}^{14+}\) 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 \(11\text{O}^{14+}\) 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 \(11\text{O}^{14+}\) mice. The number of \(E.\ coli\) cells was higher in AA-fed compared to OA-fed \(11\text{O}^{14+}\) 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 \(11\text{O}^{14+}\) 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 \(11\text{O}^{14+}\) or C57 mice (Fig. 4d).

### Table 3. Species identified from excised bands in study 1

<table>
<thead>
<tr>
<th>Band excised*</th>
<th>No. of clones sequenced per band</th>
<th>Species identified by sequencing</th>
<th>GenBank ID (Identity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2</td>
<td>3</td>
<td>Uncultured bacterium clone H83N4_89h02, isolated from mouse caecum</td>
<td>EU458036 (99–100 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Closest known cultured relative – (Bacteroides fragilis)</td>
<td>ABZX01000086 (95 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Closest known cultured relative – (Bacteroides uniformis) ATCC 8492</td>
<td>FJ881325 (100 %)</td>
</tr>
<tr>
<td>3, 4</td>
<td>3</td>
<td>Uncultured bacterium clone R-8325, isolated from faeces of rats fed wheat bran</td>
<td>NZ_AAYH020000029 (95 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Closest known cultured relative – (Bacteroides uniformis) ATCC 8492</td>
<td>EU791198 (98–100 %)</td>
</tr>
<tr>
<td>5, 6</td>
<td>3</td>
<td>Uncultured bacterium clone MMP7 + + 82, isolated from MMP7 + + mouse distal small intestine</td>
<td>NC_008786 (87–88 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Closest known cultured relative – (Verminephrobacter eiseniae) EF01-2</td>
<td>FJ36465 (99–100 %)</td>
</tr>
<tr>
<td>7, 8</td>
<td>3</td>
<td>(Helicobacter pullorum) NCTC 12824</td>
<td>GCQ285980 (99–100 %)</td>
</tr>
<tr>
<td>9, 10</td>
<td>3</td>
<td>Uncultured bacterium clone OF2A10, isolated from stool sample</td>
<td>NZ_AAXG020000037 (96 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Closest known cultured relative – (Bacteroides capillosus) ATCC 29799</td>
<td>EU566022 (100 %)</td>
</tr>
<tr>
<td>11, 12</td>
<td>3</td>
<td>Uncultured bacterium clone 5.14F, isolated from faeces of BALB/c mice</td>
<td>NZ_AAOI01000001 (85 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Closest known cultured relative – (Robuginitalea biformata) HTCC2501</td>
<td>FJ834844 (100 %)</td>
</tr>
<tr>
<td>13, 14</td>
<td>3</td>
<td>Uncultured bacterium clone Y000363F09, isolated from (Salmonella)-infected mouse caecum</td>
<td>ACCL02000018 (93 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Closest known cultured relative – (Bryantella formatexogens) DSM 14469</td>
<td>FI387861 (100 %)</td>
</tr>
<tr>
<td>15, 16</td>
<td>2</td>
<td>Uncultured bacterium clone Y000278D01, isolated from mouse caecum</td>
<td>ACCL02000018 (92 %)</td>
</tr>
</tbody>
</table>

*Numbers correspond to bands labelled in Fig. 1.*
Effect of mouse genotype and diet on B. vulgatus

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 caecal 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
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 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

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**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.
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 gut epithelium (Knoch et al., 2001). Whether the small amount of PUFA that reached the colon (Carey et al., 2003) made clear associations between bacterial species and colitis development. A possible protective effect of PUFA on the adherence of probiotics.

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 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 gut epithelium (Knoch et al., 2001). Whether the small amount of PUFA that reached the colon (Carey et al., 2003) made 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 study.

Table 4. Species identified from excised bands in study 2

<table>
<thead>
<tr>
<th>Band</th>
<th>No. of clones sequenced per band</th>
<th>Species identified by sequencing</th>
<th>GenBank ID (identity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, B</td>
<td>3</td>
<td>Uncultured Bacteroides bacterium clone Fhc31, isolated from human and animal faeces</td>
<td>EU913590 (99–100 %)</td>
</tr>
<tr>
<td>C, D</td>
<td>3</td>
<td>Uncultured bacterium clone 2.77F, isolated from mouse faeces</td>
<td>NC_009614 (99–100 %)</td>
</tr>
<tr>
<td>E, F</td>
<td>2</td>
<td>Uncultured bacterium clone YO00272E02, isolated from streptomycin-treated Salmonella-infected murine ileum</td>
<td>FJ838603 (100 %)</td>
</tr>
<tr>
<td>G, H</td>
<td>3</td>
<td>Escherichia coli strain CNM 477-02</td>
<td>NZ_ABTN010000066 (87 %)</td>
</tr>
<tr>
<td>I, J</td>
<td>3</td>
<td>Uncultured bacterium clone OF2A10, isolated from stool of healthy human</td>
<td>EU555356 (100 %)</td>
</tr>
<tr>
<td>K, L</td>
<td>2</td>
<td>Clostridium perfringens partial, isolate colony 3, isolated from canine faeces</td>
<td>NZ_AAXG020000037 (96 %)</td>
</tr>
<tr>
<td>M, N</td>
<td>2</td>
<td>Uncultured Clostridium sp. clone MS213A1_H11, isolated after gastrointestinal resection of IBD patient</td>
<td>FN356962 (100 %)</td>
</tr>
<tr>
<td>O, P</td>
<td>3</td>
<td>Uncultured bacterium clone mcbc120, isolated from caecum of TLR2-deficient mice</td>
<td>ACDK010000101 (98 and 100 %)</td>
</tr>
<tr>
<td>Q, R</td>
<td>3</td>
<td>Uncultured bacterium clone YO00360E03, isolated from streptomycin-treated Salmonella-infected murine ileum</td>
<td>P837456 (100 %)</td>
</tr>
</tbody>
</table>

*Numbers correspond to bands labelled in Fig. 3.
investigation to define the mode of action of dietary PUFA during colitis.

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