Coeliac disease (CD) is an immune-mediated enteropathy with a multifactorial aetiology, characterized by chronic inflammation of the small intestinal mucosa. Although evidence suggests that the gut microbiota contributes to other chronic inflammatory disorders, its possible role in CD has not been determined. In this study, the composition of the fecal microbiota of coeliac children and age-matched controls was investigated by culture-dependent and -independent methodologies, using fluorescent in situ hybridization (FISH). The levels of Bacteroides, Clostridium and Staphylococcus were significantly higher \((p<0.05)\) in fecal samples from coeliac patients than in healthy subjects when analysed by culture methods. The numbers of Bacteroides-Prevotella, Clostridium histolyticum, Eubacterium rectale-C. coccoides, Atopobium, and sulfate reducing bacterial groups were also significantly higher \((p<0.05)\) in fecal samples from coeliac infants when analysed by FISH. The counts of Bifidobacterium tended to be higher in healthy controls by the two type of analysis but the differences were not significant. This is the first report on the identification of the specific bacterial groups responsible for alterations in the intestinal microecology of children with active CD. The bacterial pattern detected in coeliac patients, correlates with the epidemiological data and metabolic deviations associated with CD, and involve bacterial groups link to other chronic inflammatory disorders.

**Introduction**

Coeliac disease (CD) is an autoimmune enteropathy characterized by a permanent intolerance to wheat-gluten proteins and related prolamines of rye and barley. In coeliac patients, the ingestion of gluten proteins triggers an abnormal T cell-mediated immune response that leads to inflammation of the small intestinal mucosa. CD can present at any age with a varied of clinical features but typical cases often manifest in early childhood with small intestinal mucosal injury and signs of malabsorption (Pasano and Catassi, 2005). The aetiology of CD involves a combination of genetic and environmental factors that lead to alterations in the gut-barrier function with loss of gluten tolerance (Kagnoff, 2005). The human major histocompatibility complex (MHC) molecules DQ2 and DQ8 are important genetic factors for CD, but only account for 40% of the genetic risk (Louka and Sollid, 2003). The ingestion of gluten is regarded as the key environmental factor involved in the presentation of CD. Moreover, epidemiological and clinical evidence suggest that other environmental factors, which are involved in shaping the composition of the gut microbiota, could be additional trigger elements or predispose to CD. These include the milk-feeding practices, the incidence of early microbial infections, and the use of antibiotics (Ivarsson et al., 2002; 2003).

The commensal microbiota provides a natural defence against pathogenic microorganisms and plays a pivotal role in numerous physiological functions, including regulation of epithelial permeability and immune tolerance (Majamaa and Isolauri, 1996; Isolauri et al., 2001; Sanz and Collado, 2005). It has been demonstrated that the intestinal microbiota is implicated in the pathogenesis of chronic inflammatory bowel diseases, and other immune-related disorders (Cummings et al., 2003; Bullock et al., 2004; Sartor, 2004; Swidsinski et al., 2005). In contrast, specific strains of the gut microbiota and the use of probiotics have been found to reduce intestinal inflammation and normalize gut mucosal dysfunction (Sartor, 2004). At present, there are not data on the composition of the intestinal microbiota of coeliac patients and only alterations in the composition of fecal short-chain fatty acids (SCFA) have been reported (Tjellström et al., 2005). In addition, rod-shaped bacteria were often found to be associated with the mucosa of CD patients (Forsberg et al., 2004).

The aim of this study was to compare the composition of the fecal microbiota of coeliac children and healthy controls in order to define the specific microbial changes associated with CD and, thereby, contribute to the understanding of the possible role of the microbiota in this disorder.

**Results**

The results of the analysis of the fecal microbiota of coeliac and control children by conventional culture techniques are shown in Table 1. Significantly higher counts \((p < 0.05)\) of strict anaerobic bacteria of the genera Bacteroides and Clostridium were detected in fecal samples of coeliac children than in those of controls. The aerobic genus Staphylococcus was also present in significantly higher levels \((p < 0.05)\) in fecal samples of coeliac patients. Bacteroides, Clostridium, and Enterobacteriaceae were the dominant bacterial groups in coeliac samples while Bifidobacterium, and Enterobacteriaceae were dominant in controls. The levels of Bifidobacterium were slightly higher in controls than in coeliac infants but the differences were not significant.

The results obtained by FISH using 16S rRNA probes targeting the main intestinal bacterial groups are shown in Table 2. Overall, these results confirmed the trends and

**Table 2. Overall, these results confirmed the trends and**

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Significant differences between coeliac and control children (p < 0.05) in coeliac samples than in controls. The numbers of Clostridium histolyticum and Eubacterium rectale-C. coccoides groups were also significantly higher (p < 0.05) in coeliac samples, indicating that both clostridial groups contributed to the differences detected in total clostridial levels by culture techniques. The C. histolyticum probe allows the detection of Clostridium clusters I and II (Collins et al., 1994), including a representative number of the known clostridial species commonly found in the infant gut such as C. paraputrificum, C. butyricum, and C. perfringens as well as Eubacterium multiforme (Stark et al., 1982; Franks et al., 1998). The E. rectale-C. coccoides probe allows the detection of Clostridium clusters XIVa and b (Collins et al., 1994), which also includes other species of the genera Eubacteria, Clostridium and Ruminococcus (Franks et al., 1998). Therefore, other bacterial groups besides clostridia could contribute to the differences found by FISH analysis using these two probes. The numbers of sulphate-reducing bacteria (SRB) were significantly higher (p<0.05) in coeliac infants than in controls. The counts of the Atopobium group (which includes the Coriobacterium group and comprises the family Coriobacteriaceae without the genera Slakia and Denitrobacterium; Harmsen et al., 2000b) were also significantly higher (p<0.05) in coeliac infants than in healthy controls while significant differences were not observed for the Coriobacterium group.

**Discussion**

This study has demonstrated for the first time the specific bacterial groups that define the alterations of the intestinal microecology of coeliac children at the presentation of the disease. Increased levels of Bacteroides, Clostridium, and SRB were detected in fecal samples of coeliac children as reported for inflammatory bowel diseases, which are also characterized by T-helper 1 polarized responses (Starkor, 2004). In these cases, mucosal cytokine profiles are associated with up-regulation of inflammatory cytokines (mainly IFN gamma), with concurrent induction of nitric oxide synthase, which are known to alter regulation of cell junction proteins and thus barrier integrity (De Stefano et al., 2006; Tagkalidis et al., 2006). This inflammatory milieu could lead to modifications of the intestinal microbiota in coeliac infants. Otherwise, the alterations detected in the intestinal microbiota could contribute to the pathological process by increasing the permeability of the mucosa to

Table 1. Microbial counts (log CFU/g feces) determined by using selective culture media.

<table>
<thead>
<tr>
<th>Microbial group</th>
<th>Coeliac children (n=26)</th>
<th>Control children (n=23)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>Total anaerobs</td>
<td>10.0</td>
<td>10.9–9.2</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>8.4*</td>
<td>8.6–6.0</td>
</tr>
<tr>
<td>Clostridium</td>
<td>8.5*</td>
<td>9.0–5.3</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>8.3</td>
<td>8.6–5.3</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>6.2</td>
<td>7.4–5.2</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>7.7</td>
<td>9.4–5.8</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>7.9</td>
<td>9.2–6.8</td>
</tr>
<tr>
<td>Staphylococcus</td>
<td>5.7*</td>
<td>7.7–3.7</td>
</tr>
<tr>
<td>Yeast</td>
<td>3.5</td>
<td>5.9–1.7</td>
</tr>
</tbody>
</table>

* Significant differences between coeliac and control subjects (p < 0.05).

Table 2. Microbial counts (log cells/g feces) determined by fluorescent in situ hybridization (FISH) using group-specific fluorescent probes.

<table>
<thead>
<tr>
<th>Microbial group</th>
<th>Coeliac children (n=26)</th>
<th>Control children (n=23)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>Total microbial counts*</td>
<td>11.1</td>
<td>11.7–10.3</td>
</tr>
<tr>
<td>Bacteroides/Prevotella</td>
<td>8.7*</td>
<td>10.4–7.4</td>
</tr>
<tr>
<td>Clostridium histolyticum</td>
<td>6.9*</td>
<td>10.1–5.6</td>
</tr>
<tr>
<td>Clostridium lituseburens</td>
<td>6.4</td>
<td>9.1–3.5</td>
</tr>
<tr>
<td>E. rectale-C. coccoides</td>
<td>9.1*</td>
<td>10.5–7.1</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>7.6</td>
<td>10.3–5.0</td>
</tr>
<tr>
<td>Lactobacillus-Enterococcus</td>
<td>7.2</td>
<td>10.5–3.0</td>
</tr>
<tr>
<td>E. coli</td>
<td>7.3</td>
<td>10.8–4.8</td>
</tr>
<tr>
<td>Atopobium</td>
<td>9.3*</td>
<td>10.4–8.3</td>
</tr>
<tr>
<td>Coriobacterium</td>
<td>8.6</td>
<td>9.7–6.2</td>
</tr>
<tr>
<td>Sulphate-reducing bacteria</td>
<td>8.4*</td>
<td>11.3–6.4</td>
</tr>
</tbody>
</table>

* Assessed by 4', 6-diamidino-2-phenylindole (DAPI) staining.
* Significant differences between coeliac and control children (p<0.05).
gluten antigens and promoting gut inflammation through the activation of macrophages and dendritic cells with production of pro-inflammatory cytokines (Kagnoff, 2005). In patients suffering from ulcerative colitis and Crohn’s disease the concentration of Bacteroides associated with the mucosa has been shown to be higher and increased with the severity of the disease (Swidsinski et al., 2002). The species Bacteroides vulgatus and B. fragilis are thought to be implicated in the pathogenesis of these diseases by triggering inflammatory responses (Setoymama et al., 2003; Medina et al., 2005). In addition, induction of intestinal inflammation has been detected in rodent models of colitis mono-associated with Bacteroides vulgatus (Setoymama et al., 2003). Although the present study was only focused on the fecal microbiota, a similar role of species of the Bacteroides genus in CD cannot be disregarded. In contrast to what we detected in coeliac infants, a higher presence of Clostridium groups has not been reported in patients with inflammatory bowel diseases (Sokol et al., 2006). Nevertheless, the clostridium toxins were found to exacerbate inflammation in patients with chronic colitis (Staror et al., 2004). The presence of higher levels of SRB in coeliac patients could be of clinical significance for their recognized contribution to the generation of hydrogen sulphide as end metabolic product, which is a highly cytotoxic compound. Hydrogen sulphide can inhibit butyrate oxidation, which is the primary energy source of colonocytes leading to cell death, epithelia atrophy, and chronic inflammation (Babidge et al., 1998). As a consequence, SRB have been proposed to play a role in inflammatory bowel diseases and cancer (Pitcher and Cummings, 1996). The high prevalence of other bacterial groups such as Clostridium in coeliac infants could also favour the growth of SRB by supplying the hydrogen necessary for their particular mode of energy generation. In addition, a higher cell turnover of the small intestine is expected to occur in coeliac patients as a reflection of the crypt hyperplasia that develops with villous atrophy. This could lead to an increase in the availability of endogenous substrates such as chondroitin sulfate, mucins, and endogenous amino acids derived from the expelled cells, which could promote the growth of SRB. The abundance of the integrants of the Coriobacterium group and the Atopobium clusters has been associated with the type of feeding and infant age, but only some species of the Coriobacterium group have been isolated from patients with colon cancer and inflammatory bowel diseases (Harmsen et al., 2000b). The relevance of the higher prevalence of the Atopobium group in coeliac patients remains to be investigated. The aerobic genus Staphylococcus was also present in higher levels in fecal samples of coeliac patients. A specific higher prevalence of Saphylococcus has only been found in fecal samples of allergic infants (Bjorksten et al., 2001).

The information about the relationships between coeliac disease and the gut microbiota is scarce. Moreover, epidemiological and clinical evidence have suggested a role for the microbiota in CD. In the present study, the bacterial pattern of coeliac children at the presentation of the disease was also in agreement with the differences found between the fecal microbiota of Swedish and Estonian infants, two population groups with high and low incidence of CD, respectively. In that study, the former population group also showed increased levels of Clostridium and Bacteroides (Sepp et al., 1997). Recent studies also indicated that levels of total SCFA, as well as acetic, valeric and butyric acids were higher in fecal samples of coeliac patients than in those of healthy controls (Tjellström et al., 2005). In this study, the higher prevalence of bacterial groups (Bacteroides, and Clostridium) that are important producers of SCFA and particularly of butyric acid (E. rectale-C. coccoides group; MacFarlane and Gibson, 1997; Hold et al., 2003) found in coeliac children could account for the alteration of SCFA previously reported by other authors (Tjellström et al., 2005).

In summary, this study has provided the first evidence of the bacterial groups that are responsible for microecological changes in the intestinal tract of coeliac infants. The bacterial groups overrepresented in these patients could be either a consequence of the pro-inflammatory status of individuals with symptomatic coeliac disease or involved in its presentation and evolution. Further studies should be carried out to determine whether the microbial unbalance persists after remission under a gluten-free diet in order to progress in the understanding of the role of the microbiota in this pathology.

Experimental procedures

Subjects and sampling
The studied population comprised a total of 49 children, 26 coeliac patients (medium age 26.3 months, range 12–48 months) and 23 age-matched controls (medium age 23.0 months, range 11–45 months). The diagnosis of CD was based on clinical symptoms, positive detection of coeliac serology markers (anti-gliadin, antiendomysial and antitransglutaminase antibodies), and small duodenal biopsy showing severe enteropathy.

Fecal samples from both population groups were immediately kept at 4ºC, under anaerobiosis using AnaeroGen sachets (Oxoid, Hampshire, UK), and analysed in less than 12 h. Samples from coeliac children were collected at the presentation of the disease and still following a normal-gluten diet. The children included in the study were not treated with antibiotics for at least one month before the sampling time.

The study protocol was approved by the Committee on Ethical Practice of General University Hospital and CSIC, and infants were enrolled in the study after written informed consent obtained from their parents.

Analysis of cultivable fecal bacteria using plate culture media
Fecal samples (2 g wet weight) were 10-fold diluted in phosphate-buffered saline (PBS, 130 mM sodium chloride, 10 mM sodium phosphate, [pH 7.2]), and homogenized in a Lab Blender 400 stomacher (Seward Medical London, UK). Appropriate serial decimal dilutions were made in PBS and aliquots were plated on each agar media in duplicated. Total anaerobes were enumerated on Wilkins-Chalgren agar (Oxoid, Hampshire, England), Bifidobacterium on BFM agar (Nebra and Blanch, 1999),
**Clostridium** on Reinforced Clostridial Medium (Oxoid, Hampshire, England) supplemented with novobiocin (8 mg/L) and colistin (8 mg/L), *Bacteroides* on Schaedler agar (Scharlau, Barcelona, Spain) supplemented with kanamycin (100 mg/L), vancomycin (7.5 mg/L) and vitamin K (0.5 mg/L). In all these cases, plates were incubated in anaerobic conditions at 37°C for 72 h. *Enterobacteriaceae* were enumerated on VRBD agar (Scharlau, Barcelona, Spain) and *Staphylococcus* on Baird Parker agar (BP, Scharlau, Barcelona, Spain) after incubation in anaerobic conditions at 37°C for 48 h. *Lactobacillus* were enumerated on Rogosa Agar (Scharlau, Barcelona, Spain) after incubation in anaerobiosis at 30°C for 72 h. *Enterococcus* were enumerated on kanamyacin-esculin agar (KAA, Scharlau, Barcelona, Spain) and yeast in Bengal Pink agar (Scharlau, Barcelona, Spain), after incubation under aerobic conditions at 30°C for 72 h. The identity of representative colonies recovered from each selective medium was confirmed by conventional microbiological methods including colony and cellular morphology, Gram staining, biochemical test using the API systems (BioMerieux, Lyon, France) and antibiotic susceptibility assays (Simpson et al., 2004). Bacterial counts were expressed as the log of the number of colony forming units (CFU) per gram of wet-weight feces.

**Analysis of fecal bacteria by fluorescent in situ hybridization (FISH)**

One volume of the first decimal dilution of the fecal samples was added to three volumes of 4% paraformaldehyde (PFA) in PBS and fixed at 4°C overnight. After fixation, bacteria were washed twice in PBS by centrifugation (12000 rpm for 5 min). The bacterial pellets were suspended and stored in 50% ethanol–PBS at –80°C until use. The enumeration of cells present in fecal samples was carried out by fluorescent in situ hybridization (FISH) using fluorescein isothiocyanate (FITC)-labelled oligonucleotide probes as described elsewhere (MOLBIOL, Berlin, Germany; Collado et al., 2006). The group- and genus-specific probes and hybridization conditions used in this study are summarized in Table 3. Total cell numbers were enumerated using the nucleic acid stain 4, 6-diamidino-2-phenylindole (DAPI). Briefly, fixed cell suspensions were hybridized with the corresponding probe in hybridization buffer (10 mM Tris–HCl, 0.9 m NaCl and 10% SDS) overnight. Then, cells were washed with the same buffer but without SDS, applied to a 0.2 μm polycarbonate filter (Millipore Corporation, Bedford, USA) and mounted on a glass slide. Slides were visualized in a Nikon Eclipse E800 microscope (Nikon corporation, Tokyo, Japan). Results were the average of 20 fields counted per sample and probe, and were expressed as the log of cells per gram of wet-weight feces.

**Statistical analysis**

The differences in bacterial counts between control and coeliac children were determined by applying the Mann-Whitney U-test using StatGraphics software (Manugistics, Rockville, MD, USA). Significant differences were established at a confidence level of 95% (p value less than 0.05).

**Acknowledgements**

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**References**


### Table 3. Oligonucleotide probes and hybridization conditions used in FISH analysis of fecal bacteria.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target bacterial group</th>
<th>Sequence (5′–3′)</th>
<th>Hybridization conditions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bf164</td>
<td>Bifidobacterium</td>
<td>CATCCGGCATATCCACCC</td>
<td>50</td>
<td>Langendijk et al. (1995)</td>
</tr>
<tr>
<td>Bac303</td>
<td>Bacteroides/Prevotella</td>
<td>CCATGTGGG/GGACCTTT</td>
<td>45</td>
<td>Manz et al. (1996)</td>
</tr>
<tr>
<td>Erec0482</td>
<td>Eubacterium rectale/Clostridium coccoides</td>
<td>GCT TCT TAG TCA GGTACCG</td>
<td>50</td>
<td>Franks et al. (1998)</td>
</tr>
<tr>
<td>CLis135</td>
<td>Clostridium lituseburensense</td>
<td>GGT ATC CGT GTG TAC AGG G</td>
<td>50</td>
<td>Hold et al. (2003)</td>
</tr>
<tr>
<td>CHis150</td>
<td>Clostridium histoyticum</td>
<td>TTA TGC GGT ATT ATC CT/C/T CCT TT</td>
<td>50</td>
<td>Harmsen et al. (2000a)</td>
</tr>
<tr>
<td>Lac168</td>
<td>Lactobacillus/Enterococcus</td>
<td>GGT ATT AGC A/C/T/C TGT TTC CA</td>
<td>45</td>
<td>Harmsen et al. (2000a)</td>
</tr>
<tr>
<td>Ecol1513</td>
<td>Escherichia coli</td>
<td>CAC CGT AGT GCC TCG TCA TCA</td>
<td>37</td>
<td>Poulsen et al. (1994)</td>
</tr>
<tr>
<td>Ato291</td>
<td>Atopobium</td>
<td>GGTGCGGTCTCTCAACCC</td>
<td>50</td>
<td>Harmsen et al. (2000b)</td>
</tr>
<tr>
<td>Cor653</td>
<td>Coriobacterium</td>
<td>CCC TCC C(A/C)/CC GAC CC</td>
<td>50</td>
<td>Harmsen et al. (2000b)</td>
</tr>
</tbody>
</table>
during fermented milk administration. Food Res. Int. 39, 530–535.


medium for the enumeration of bifidobacteria from probiotic animal feed. J. Microbiol. Methods, 57, 9–16.