Imbalance in the composition of the duodenal microbiota of children with coeliac disease

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Coeliac disease (CD) is the most common immune-mediated enteropathy characterized by chronic inflammation of the small intestinal mucosa. The ingestion of gluten is responsible for the symptoms of CD, but other environmental factors are also thought to play a role in this disorder. In this study, the composition of the duodenal microbiota of coeliac children with active disease, symptom-free CD patients on a gluten-free diet and control children was determined. Bacteriological analyses of duodenal biopsy specimens were carried out by fluorescent *in situ* hybridization coupled with flow cytometry. The proportions of total bacteria and Gram-negative bacteria were significantly higher in CD patients with active disease than in symptom-free CD patients and controls. *Bacteroides* and *Escherichia coli* groups were significantly more abundant in CD patients with active disease than in controls, whilst these bacterial deviations were normalized in symptom-free CD patients. The ratio of *Lactobacillus*–*Bifidobacterium* to *Bacteroides*–*E. coli* was significantly reduced in coeliac patients with either active or inactive disease compared with controls. The differences in *Atopobium*, *Eubacterium rectale*–*Clostridium coccoides*, *Clostridium histolyticum*, *Clostridium lituseburense*, sulphate-reducing bacteria and *Faecalibacterium prausnitzii* populations among the three groups of children were less relevant. Overall, the higher incidence of Gram-negative and potentially pro-inflammatory bacteria in the duodenal microbiota of coeliac children was linked to the symptomatic presentation of the disease and could favour the pathological process of the disorder.

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INTRODUCTION

Coeliac disease (CD) is a chronic inflammatory disorder of the small intestine characterized by a permanent intolerance to dietary gluten. Affecting as many as 1–3 % of the European and American population, CD is considered to be the most common lifelong disorder (Mulder & Bartelsman, 2005; Rewers, 2005). This disease can manifest at any age and present a variety of clinical features, but often does so in early childhood with small intestinal villous atrophy and signs of malabsorption (Fasano & Catassi, 2005). In coeliac patients, exposure to gluten leads to activation of both an adaptive immune response dominated by Th₁ pro-inflammatory cytokines, such as gamma interferon, within the mucosa, and an innate immune response mediated by interleukin-15 (Koning *et al.*, 2005; Londei *et al.*, 2005). Alterations in epithelial permeability and tight junction integrity, which favour

access of antigens to the submucosa, are also considered to be key features of the active phase of the disease (Schulzke *et al.*, 1998; Drago *et al.*, 2006).

The ingestion of gluten is responsible for the signs and symptoms of CD, and its removal from the diet is currently the only available treatment. Moreover, environmental factors, such as microbial infections and imbalances in the composition of the gut microbiota, have been suggested to be associated with the presentation of this disorder (Forsberg *et al.*, 2004; Stene *et al.*, 2006; Collado *et al.*, 2007). In non-infectious, chronic immunological processes, such as those characteristic of inflammatory bowel disease (IBD) (Crohn's disease and ulcerative colitis), the microbial communities that inhabit the human gut appear to be an important source of antigens that trigger and perpetuate inflammation (Bibiloni *et al.*, 2006). In patients with IBD, the outgrowth of certain bacterial populations combined with the absence of specific protective commensals is thought to contribute to the impairment of intestinal immune homeostasis (Swidsinski *et al.*, 2002; Sartor, 2004; Lucke *et al.*, 2006). This theory is supported by the benefits

Abbreviations: CD, coeliac disease; DGGE, denaturing gradient gel electrophoresis; FISH, fluorescent *in situ* hybridization; IBD, inflammatory bowel disease.

obtained by the administration of antibiotics, as well as by nutritional intervention strategies based on the use of probiotic bacteria and prebiotics (Guarner *et al.*, 2006). The existing information on the composition and metabolic activity of the gut microbiota in coeliac patients mainly comes from analyses of faecal samples (Tjellstrom *et al.*, 2005; Collado *et al.*, 2007; Sanz *et al.*, 2007). It is known, however, that jejunal, ileal and colonic mucosa-associated bacteria differ from faecal bacteria (Wang *et al.*, 2005). It might also be expected that mucosal microbiota exert a major impact on human health due to their closer interaction with the host epithelia and immune system (Macfarlane *et al.*, 2004). Nonetheless, there appears to be only one microbial study of biopsy specimens from adult coeliac patients, which indicated that rod-shaped bacteria were often associated with the mucosa of these patients (Forsberg *et al.*, 2004).

The aim of this study was to characterize the specific composition of the duodenal microbiota of coeliac patients (with active and inactive disease) and controls in order to identify possible links between specific bacterial populations, and the presentation and evolution of the disease.

METHODS

Subjects. Three groups of children were included in this study: 20 coeliac patients on a normal gluten-containing diet, showing clinical symptoms of the disease, positive coeliac serology markers and signs of severe enteropathy by duodenal biopsy examination (mean age 5.1 years, range 1.6–12.0 years); 10 symptom-free coeliac patients, who had been on a gluten-free diet for 1–2 years (mean age 5.6 years, range 2.0–7.8 years); and 8 control children without known food intolerance (mean age 4.1 years, range 1.9–9.0 years). None of the children included in the study was treated with antibiotics for at least 1 month before the sampling time. The study protocol was approved by the committee on ethical practice of the corresponding hospitals and Consejo Superior de Investigaciones Científicas. Children were

enrolled in the study after written informed consent was obtained from their parents.

Sample preparation. Duodenal biopsy specimens were obtained by upper intestinal or capsule endoscopy. Each specimen was diluted 1:10 (w/v) in PBS (pH 7.2) and homogenized by thorough agitation in a vortex. Bacterial cells were fixed with 4% paraformaldehyde (Sigma) overnight at 4 °C. After fixation, bacteria were washed twice in PBS with centrifugation (12 000 *g* for 5 min). Finally, cell pellets were suspended in a 1:1 PBS:ethanol mixture and stored at –80 °C until analysed, as described previously (Collado *et al.*, 2007).

Fluorescent *in situ* hybridization (FISH) and flow cytometry detection. The oligonucleotide probes used in this study are summarized in Table 1. All were commercially synthesized by Molbiol. The group-specific probes were labelled at the 5' end with FITC (green fluorescence). The EUB 338 probe, targeting conserved sequences within the bacterial domain, was used as a positive control (Amann *et al.*, 1990). The NON-EUB 338 probe was used as a negative control to eliminate background fluorescence (Wallner *et al.*, 1993). Both control probes were labelled at the 5' end with either Cy3 (red fluorescence) or FITC. Aliquots of 45 µl fixed samples were incubated with 5 µl each fluorescent probe (50 ng µl^{–1}) in hybridization solution [10 mM Tris/HCl (pH 8.0), 0.9 M NaCl, 10% (w/v) SDS] at an appropriate temperature (45–50 °C) overnight. The bacterial cells were then incubated with 500 µl washing solution [10 mM Tris/HCl (pH 8.0), 0.9 M NaCl] at 50 °C for 30 min to remove non-specifically bound probe. Hybridized cells were pelleted by centrifugation (12 000 *g* for 5 min) and resuspended in 500 µl PBS for flow cytometry detection. Bacterial groups were enumerated by combining each FITC-labelled group-specific probe with the EUB 338 Cy3-labelled probe, and expressed as the ratio of cells hybridizing with the FITC-labelled specific probe to cells hybridizing with the EUB 338–Cy3 probe. This proportion was corrected by subtracting the background fluorescence obtained with the negative control probe NON-EUB 338 (Sokol *et al.*, 2006; Collado & Sanz, 2007).

Flow cytometry was performed using an EPICS XL-MCL flow cytometer (Beckman Coulter) as described previously (Collado & Sanz, 2007). This instrument is equipped with two light scatter detectors that measure forward and side scatter, and fluorescence detectors that detect appropriately filtered light at green (FL1,

Table 1. Oligonucleotide probes used in FISH/flow cytometry analysis of intestinal bacteria

Probe	Target bacterial group	Sequence (5'→3')	Reference
EUB 338	Domain bacteria	GCTGCCTCCCGTAGGAGT	Amann <i>et al.</i> (1990)
NON-EUB 338	Negative control	ACATCCTACGGGAGGC	Wallner <i>et al.</i> (1993)
Ato291	<i>Atopobium</i> group	GGTCGGTCTCTCAACCC	Harmsen <i>et al.</i> (2000)
Bif164	<i>Bifidobacterium</i> genus	CATCCGGCATTACCACCC	Langendijk <i>et al.</i> (1995)
Lab158	<i>Lactobacillus</i> and other lactic acid bacteria genera	GGTATTAGCA(C/T)C TGTTCCTCA	Harmsen <i>et al.</i> (1999)
Strc493	<i>Streptococcus</i> – <i>Lactococcus</i> group	GTTAGCCGTCCTTTCTGG	Franks <i>et al.</i> (1998)
Bac303	<i>Bacteroides</i> – <i>Prevotella</i> group	CCAATGTGGGGGACCTT	Manz <i>et al.</i> (1996)
Ecol1513	<i>Escherichia coli</i>	CACCGTAGTGCTCGTCATCA	Poulsen <i>et al.</i> (1994)
Erec0482	<i>Eubacterium rectale</i> – <i>Clostridium coccooides</i> group	GCTTCTTAGTCAGGTACCG	Franks <i>et al.</i> (1998)
CHis150	<i>Clostridium histolyticum</i> group	TTATGCGGTATTAATCT(C/T)CCTTT	Harmsen <i>et al.</i> (2000)
CLis135	<i>Clostridium lituseburense</i> group	GTTATCCGTGTGTACAGGG	Hold <i>et al.</i> (2003)
Fprau645	<i>Faecalibacterium prausnitzii</i> group	CCTCTGCACTACTCAAGAAAAAC	Suau <i>et al.</i> (2001)
SBR	Sulphate-reducing bacteria	TACGGATTTCACTCCT	Bullock <i>et al.</i> (2004)

525 nm) and red/orange (FL3, 620 nm) wavelengths. The event rate was kept at the lowest setting (200–300 events s^{-1}) to avoid cell coincidence. A total of 15 000 events was recorded in a list mode file and analysed using System II v.3 software (Beckman Coulter).

Statistical analyses. All statistical analyses were carried out using StatGraphics software (Manugistics). The differences in bacterial populations among coeliac children with active and inactive disease and controls were determined by applying Student's *t*-test for normally distributed data, and the Mann–Whitney (Wilcoxon) *W*-test for non-parametric data. Significant differences were established at a value of $P < 0.05$. Results were expressed as mean or median values of the proportion of each bacterial group determined in duplicate. The numbers of total, Gram-negative and Gram-positive bacteria were calculated by addition of the proportions of the corresponding groups detected by specific non-overlapping probes.

RESULTS AND DISCUSSION

General comparison of the duodenal microbiota of coeliac children and controls

The duodenal microbiota of coeliac children with active and inactive disease and control, children was characterized using oligonucleotide probes targeting the main bacterial groups colonizing the human gut (Fig. 1, Table 2). There were large variations in the community structure between different regions of the gastrointestinal tract and, to our knowledge, this is the first report on the composition of the human duodenal microbiota. Total and Gram-negative bacterial populations of duodenal biopsies calculated by adding the proportions of corresponding groups targeted

by the probes used reached significantly higher ($P = 0.005–0.007$) levels in CD patients with active disease than in symptom-free CD patients and controls (Fig. 1). The levels of Gram-positive bacteria calculated by adding the proportions of corresponding groups targeted by the probes used were lower in duodenal biopsies of coeliac patients with active disease, and even lower ($P = 0.005–0.007$) in those of symptom-free CD patients than in controls. Therefore, the overgrowth of total and Gram-negative bacteria detected by the probes used was reduced in duodenal samples of CD patients on a long-term gluten-free diet. In addition, environmental factors (e.g. dietary) and intrinsic genetic factors may have influenced the composition of the gut microbiota in symptom-free CD patients, leading to lower levels of bacterial populations of the studied groups, including Gram-positive bacteria, compared with the controls ($P = 0.05–0.029$). Increased numbers of mucosa-associated bacteria have been found in patients with IBD (Swidsinski *et al.*, 2002). In addition, cellular components of Gram-negative bacteria such as flagellins and lipopolysaccharides have been linked to systemic inflammatory responses and the pathogenesis of Crohn's disease (Erridge *et al.*, 2004; Lodes *et al.*, 2004; Gewirtz *et al.*, 2006). In what appears to be the only report on the mucosal microbiota of adult CD patients published so far, rod-shaped bacteria were often found to be associated with the mucosa of CD patients, but the identity of the bacteria was not reported (Forsberg *et al.*, 2004). The differences found in glycosylation between CD patients and controls were also suggested as a factor favouring bacterial adhesion in these subjects (Forsberg *et al.*, 2004). The changes detected in the overall composition of the duodenal microbiota of coeliac patients could be either a consequence or a cause of the disease. In the first case, the damaged mucosa covered by immature enterocytes in disease patients could create conditions favouring Gram-negative bacterial colonization to the detriment of Gram-positive colonization due to specific receptors/glycoproteins present on the surface of immature cells but not on the brush border of mature enterocytes. In the second case, it could be that predominant colonization of Gram-negative bacteria in genetically predisposed individuals contributes to induce loss of tolerance to gluten.

Bacterial group and species specificities of the duodenal microbiota of coeliac children and controls

The specific group and species composition of the duodenal microbiota of the control and coeliac children is shown in Table 2. Approximately 80–91% of the bacterial population was detected by the ten non-overlapping probes used in this study in biopsy specimens from coeliac patients and controls, whilst only 48% was detected in samples from symptom-free CD patients (Fig. 1, Table 2). The relative abundance of different bacterial groups varied among the three groups of children except for the *Streptococcus* group ($P = 0.306–0.525$). This group

Fig. 1. Composition of the duodenal microbiota of coeliac and control children as assessed by FISH and flow cytometry. Levels of total, Gram-negative and Gram-positive bacteria were calculated by adding the relative proportions of the corresponding groups, excluding overlapping probes. Mean values (percentage of total number of bacteria detected by the corresponding probes $\pm$ SD) are given, and columns having different letters (a–c) showed significant differences ($P < 0.05$) using Student's *t*-test. Black bars, coeliacs with active disease; white bars, coeliacs with non-active disease; grey bars, controls.

Table 2. Group and species composition of the duodenal microbiota of coeliac children with active and inactive disease (on a gluten-free diet) and controls, as assessed by FISH and flow cytometry

Median values (%) within the same row having different letters (*a*, *b*) were significantly different ($P < 0.05$) using the Mann–Whitney (Wilcoxon) *W*-test.

Bacterial group	Coeliac patients with active disease (<i>n</i> =20)		Coeliac patients with non-active disease (<i>n</i> =10)		Control children (<i>n</i> =8)	
	Median	Range	Median	Range	Median	Range
<i>Atopobium</i>	4.78 ^a	2.45–22.01	3.65 ^b	0.50–6.26	7.06 ^a	4.66–13.17
<i>Bifidobacterium</i>	9.24 ^a	4.69–33.37	4.32 ^b	0.63–22.28	10.55 ^a	8.66–17.91
<i>Lactobacillus</i> group	4.84 ^a	2.06–19.11	1.99 ^b	0.76–5.55	7.88 ^a	5.53–11.74
<i>Streptococcus–Lactococcus</i>	10.88 ^a	1.99–19.39	9.44 ^a	1.76–9.73	7.18 ^a	2.74–15.69
<i>Bacteroides–Prevotella</i>	12.98 ^a	5.28–32.08	4.52 ^b	2.88–10.83	6.07 ^b	1.21–11.62
<i>Escherichia coli</i>	10.98 ^a	5.52–35.13	4.10 ^b	2.14–10.46	5.04 ^b	1.16–11.56
<i>Eubacterium rectale–Clostridium coccoides</i>	5.71 ^a	0.60–22.13	2.62 ^b	0.88–11.68	7.72 ^a	2.83–12.04
<i>Clostridium histolyticum</i>	7.64 ^{ab}	1.15–12.22	7.58 ^a	0.50–8.06	11.43 ^b	3.73–11.73
<i>Clostridium lituseburense</i>	6.50 ^a	1.73–3.12	3.59 ^b	0.67–14.64	7.29 ^a	4.81–13.14
<i>Faecalibacterium prausnitzii</i>	9.14 ^{ab}	3.11–23.72	5.98 ^a	1.84–20.90	10.52 ^b	8.80–17.73
Sulphate-reducing bacteria	8.37 ^a	3.18–33.83	5.35 ^b	3.82–11.88	6.49 ^{ab}	1.75–10.93

was quantitatively important but was not the predominant group in duodenal biopsies, in contrast to findings in jejunal biopsies of healthy subjects by direct cloning and sequencing of 16S rRNA genes (Wang *et al.*, 2005). The *Bacteroides–Prevotella* group and *Escherichia coli* populations were significantly more abundant ($P=0.027$ and $P=0.013$, respectively) in biopsy specimens of CD patients with active disease than in those of controls (Table 2). However, the proportions of these bacterial groups were not significantly different between controls and symptom-free CD patients ($P=0.444$ and $P=0.452$, respectively), indicating that these bacterial deviations had been normalized after a long adherence to a strict gluten-free diet. In patients suffering from IBD, the concentration of the *Bacteroides–Prevotella* group associated with the mucosa has been shown to be higher and to increase with the severity of the disease. Some of these bacteria are located within the epithelial layer and even intracellularly (Swidsinski *et al.*, 2002; Lucke *et al.*, 2006). Comparative bacteriological analysis of biopsy specimens of patients with Crohn's disease and ulcerative colitis by denaturing gradient gel electrophoresis (DGGE) has recently indicated that members of the phylum *Bacteroidetes* are more prevalent in the Crohn's disease group (Bibiloni *et al.*, 2006). Although *Bacteroides* constitute an important fraction of human gut microbiota, some species, such as *Bacteroides vulgatus* and *Bacteroides fragilis*, have been found to exhibit pro-inflammatory properties in animal models of IBD and are thought to be involved in their pathogenesis (Setoyama *et al.*, 2003; Medina *et al.*, 2005). Analyses of ileocolonic mucosal samples by DGGE, as well as by FISH, have also revealed a higher prevalence of *E. coli* in both Crohn's disease and ulcerative colitis patients (Mylonaki *et al.*, 2005; Martinez-Medina *et al.*, 2006). An adherent invasive *E. coli* pathovar was identified in the ileal mucosa of patients with Crohn's disease, which was able to

replicate and induce tumour necrosis factor alpha (TNF- α) production in macrophages (Darfeuille-Michaud *et al.*, 2004). In addition, high levels of the *E. coli* outer-membrane protein C antibodies were detected in these patients, and the reactivity to these antibodies was associated with the severity of the disease (Barnich & Darfeuille-Michaud, 2007). Increased levels of *Bacteroides* have also been detected by FISH analysis in the faeces of coeliac children compared with controls, in the absence of increased levels of *E. coli* (Collado *et al.*, 2007). In the case of CD, infections as well as the overgrowth of pro-inflammatory opportunistic pathogens may initiate or contribute to the pathological process by increasing the production of inflammatory mediators, such as TNF- α and gamma interferon, which are known to increase permeability (Stene *et al.*, 2006). This in turn could favour the access of higher antigen loads (gliadin and microbial) to the submucosa and contribute towards perpetuating inflammation.

The ratios of beneficial bacterial groups (*Lactobacillus* plus *Bifidobacterium*) to potentially harmful Gram-negative bacteria (*Bacteroides–Prevotella* plus *E. coli* groups) detected in biopsy specimens were significantly different ($P=0.001–0.034$) among the three groups of children. This ratio reached its highest value in samples from control children (mean $\pm$ SD 1.57 ± 0.13), an intermediate value in samples from symptom-free CD patients (1.08 ± 0.23) and the lowest value in samples from CD patients with active disease (0.51 ± 0.15). *Bifidobacterium* numbers in the mucosa of IBD patients have also been found to be reduced in some studies (Macfarlane *et al.*, 2005; Mylonaki *et al.*, 2005). Total *Bifidobacterium* and *Lactobacillus* counts did not differ in the faeces of coeliac and healthy children, although changes in species composition have been identified by DGGE-PCR analysis (Collado *et al.*, 2007;

Sanz *et al.*, 2007). In symptom-free CD patients, the results of analysis of biopsy specimens could reflect the long-term benefits of a gluten-free diet in normalizing the intestinal milieu and improving the ratio of beneficial to potentially harmful Gram-negative bacteria. However, this ratio was not completely normalized, which might be an effect of dietary factors or a characteristic of the disease, regardless of the inflammatory and autoimmune status of the patients. The involvement of particular bacterial species and the relative abundance of specific bacterial groups in the manifestation of this disorder remain to be determined.

The proportions of *Atopobium*, *Eubacterium rectale*–*Clostridium coccoides*, *Clostridium histolyticum*, *Clostridium lituseburense*, sulphate-reducing bacteria and *Faecalibacterium prausnitzii* groups detected in biopsy specimens were not significantly different ($P=0.104$ – 0.526 ; Table 2) between controls and coeliac patients with active disease. Sulphate-reducing bacterial levels were higher in biopsy specimens of coeliac children compared with healthy children, as previously detected in faeces, but the differences did not reach statistical significance ($P=0.386$) in biopsies. *C. histolyticum* and *F. prausnitzii* levels were not significantly different in biopsy specimens of coeliac patients, regardless of the phase of the disease. Proportions of both bacterial groups were higher in biopsy specimens of control subjects, although the differences were not significant. The *Faecalibacterium* group has also been found more frequently in healthy biopsy specimens when compared with those of Crohn's disease patients (Martinez-Medina *et al.*, 2006). *F. prausnitzii* is a butyrate-producing bacterium that could exert a protective role in the mucosa of healthy controls, whilst its role could be limited in coeliac children (Hold *et al.*, 2003). Nonetheless, the possible role of these bacterial groups showing minor differences between CD patients and controls requires further investigation. In addition, further studies should be carried out to investigate whether the major changes detected in this study are secondary to or the cause of the disease, as well as to determine the mechanism that might link specific bacterial groups with the alterations in mucosal immunology and intestinal permeability characteristic of this disease.

Conclusions

The higher incidence of Gram-negative and potentially pro-inflammatory bacteria in the duodenal microbiota of coeliac children was found to be linked to the symptomatic presentation of the disease and could favour its pathological process during the active phase of this disorder. Although in symptom-free CD patients an adherence to a gluten-free diet was associated with reductions in Gram-negative bacterial populations, the relative abundance of beneficial to potentially harmful bacteria was not completely normalized when compared with controls. This work thus provides bacterial targets for further studies on a possible dysfunctional interaction between the gut

microbiota and the host, particularly during the symptomatic phase of CD.

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REFERENCES

- Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. & Stahl, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**, 1919–1925.
- Barnich, N. & Darfeuille-Michaud, A. (2007). Adherent-invasive *Escherichia coli* and Crohn's disease. *Curr Opin Gastroenterol* **23**, 16–20.
- Bibiloni, R., Mangold, M., Madsen, K. L., Fedorak, R. N. & Tannock, G. W. (2006). The bacteriology of biopsies differs between newly diagnosed, untreated, Crohn's disease and ulcerative colitis patients. *J Med Microbiol* **55**, 1141–1149.
- Bullock, N. R., Booth, J. C. & Gibson, G. R. (2004). Comparative composition of bacteria in the human intestinal microflora during remission and active ulcerative colitis. *Curr Issues Intest Microbiol* **5**, 59–64.
- Collado, M. C. & Sanz, Y. (2007). Quantification of mucosa-adhered microbiota of lambs and calves by the use of culture methods and fluorescent *in situ* hybridization coupled with flow cytometry techniques. *Vet Microbiol* **121**, 299–306.
- Collado, M. C., Calabuig, M. & Sanz, Y. (2007). Differences between the faecal microbiota of coeliac children and healthy controls. *Curr Issues Intest Microbiol* **8**, 9–14.
- Darfeuille-Michaud, A., Boudeau, J., Bulois, P., Neut, C., Glasser, A. L., Barnich, N., Bringer, M. A., Swidsinski, A., Beaugerie, L. & Colombel, J. F. (2004). High prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease. *Gastroenterology* **127**, 412–421.
- Drago, S., El Asmar, R., Di Pierro, M., Grazia-Clemente, M., Tripathi, A., Sapone, A., Thakar, M., Iacono, G., Carroccio, A. & other authors (2006). Gliadin, zonulin and gut permeability: effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scand J Gastroenterol* **41**, 408–419.
- Erridge, C., Pridmore, A., Eley, A., Stewart, J. & Poxton, I. R. (2004). Lipopolysaccharides of *Bacteroides fragilis*, *Chlamydia trachomatis* and *Pseudomonas aeruginosa* signal via toll-like receptor 2. *J Med Microbiol* **53**, 735–740.
- Fasano, A. & Catassi, C. (2005). Coeliac disease in children. *Best Pract Res Clin Gastroenterol* **19**, 467–478.
- Forsberg, G., Fahlgren, A., Horstedt, P., Hammarstrom, S., Hernell, O. & Hammarstrom, M. L. (2004). Presence of bacteria and innate immunity of intestinal epithelium in childhood coeliac disease. *Am J Gastroenterol* **99**, 894–904.
- Franks, A. H., Harmsen, H. J., Raangs, G. C., Jansen, G. J., Schut, F. & Welling, G. W. (1998). Variations of bacterial populations in human feces measured by fluorescent *in situ* hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl Environ Microbiol* **64**, 3336–3345.
- Gewirtz, A. T., Vijay-Kumar, M., Brant, S. R., Duerr, R. H., Nicolae, D. L. & Cho, J. H. (2006). Dominant-negative TLR5 polymorphism reduces adaptive immune response to flagellin and negatively associates with

- Crohn's disease. *Am J Physiol Gastrointest Liver Physiol* **290**, G1157–G1163.
- Guarner, F., Bourdet-Sicard, R., Brandtzaeg, P., Gill, H. S., McGuirk, P., van Eden, W., Versalovic, J., Weinstock, J. V. & Rook, G. A. W. (2006). Mechanisms of disease: the hygiene hypothesis revisited. *Nat Clin Pract Gastroenterol Hepatol* **3**, 275–284.
- Harmsen, H. J. M., Elfferich, P., Schut, F. & Welling, G. W. (1999). A 16S rRNA-targeted probe for detection of lactobacilli and enterococci in faecal samples by fluorescent *in situ* hybridization. *Microb Ecol Health Dis* **11**, 3–12.
- Harmsen, H. J. M., Wildeboer-Veloo, A. C., Grijpstra, J., Knol, J., Degener, J. E. & Welling, G. W. (2000). Development of 16S rRNA-based probes for the *Coriobacterium* group and the *Atopobium* cluster and their application for enumeration of *Coriobacteriaceae* in human feces from volunteers of different age groups. *Appl Environ Microbiol* **66**, 4523–4527.
- Hold, G. L., Schwartz, A., Aminov, R. I., Blaut, M. & Flint, H. J. (2003). Oligonucleotide probes that detect quantitatively significant groups of butyrate-producing bacteria in human feces. *Appl Environ Microbiol* **69**, 4320–4324.
- Koning, F., Schuppan, D., Cerf-Bensussan, N. & Sollid, L. M. (2005). Pathomechanisms in coeliac disease. *Best Pract Res Clin Gastroenterol* **19**, 373–387.
- Langendijk, P. S., Schut, F., Jansen, G. J., Raangs, G. C., Kamphuis, G. R., Wilkinson, M. H. F. & Welling, G. W. (1995). Quantitative fluorescence *in situ* hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Appl Environ Microbiol* **61**, 3069–3075.
- Lodes, M. J., Cong, Y., Elson, C. O., Mohamath, R., Landers, C. J., Targan, S. R., Fort, M. & Hershberg, R. M. (2004). Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest* **113**, 1296–1306.
- Londei, M., Ciacci, C., Ricciardelli, I., Vacca, L., Quarantino, S. & Maiuri, L. (2005). Gliadin as a stimulator of innate responses in celiac disease. *Mol Immunol* **42**, 913–918.
- Lucke, K., Miehke, S., Jacobs, E. & Schuppler, M. (2006). Prevalence of *Bacteroides* and *Prevotella* spp. in ulcerative colitis. *J Med Microbiol* **55**, 617–624.
- Macfarlane, G. T., Furrie, E. & Macfarlane, S. (2004). Bacterial milieu and mucosal bacteria in ulcerative colitis. *Novartis Found Symp* **263**, 57–64.
- Macfarlane, S., Furrie, E., Kennedy, A., Cummings, J. H. & Macfarlane, G. T. (2005). Mucosal bacteria in ulcerative colitis. *Br J Nutr* **93**, S67–S72.
- Manz, W., Amann, R., Ludwig, W., Vancanneyt, M. & Schleifer, K. H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-Flavobacter-Bacteroides* in the natural environment. *Microbiology* **142**, 1097–1106.
- Martinez-Medina, M., Aldegue, X., Gonzalez-Huix, F., Acero, D. & Garcia-Gil, L. J. (2006). Abnormal microbiota composition in the ileocolonic mucosa of Crohn's disease patients as revealed by polymerase chain reaction-denaturing gradient gel electrophoresis. *Inflamm Bowel Dis* **12**, 1136–1145.
- Medina, C., Santana, A., Llopis, M., Paz-Cabrera, M. C., Antolin, M., Mourelle, M., Guarner, F., Vilaseca, J., Gonzalez, C. & other authors (2005). Induction of colonic transmural inflammation by *Bacteroides fragilis*: implication of matrix metalloproteinases. *Inflamm Bowel Dis* **11**, 99–105.
- Mulder, C. J. & Bartelsman, J. F. (2005). Case-finding in coeliac disease should be intensified. *Best Pract Res Clin Gastroenterol* **19**, 479–486.
- Mylonaki, M., Rayment, N. B., Rampton, D. S., Hudspith, B. N. & Brostoff, J. (2005). Molecular characterization of rectal mucosa-associated bacterial flora in inflammatory bowel disease. *Inflamm Bowel Dis* **11**, 481–487.
- Poulsen, L. K., Lan, F., Kristensen, C. S., Hobolth, P., Molin, S. & Krogfelt, K. A. (1994). Spatial distribution of *Escherichia coli* in the mouse large intestine inferred from rRNA *in situ* hybridization. *Infect Immun* **62**, 5191–5194.
- Rewers, M. (2005). Epidemiology of celiac disease: what are the prevalence, incidence, and progression of celiac disease? *Gastroenterology* **128**, S47–S51.
- Sanz, Y., Sánchez, E., Marzotto, M., Calabuig, M., Torriani, S. & Dellaglio, F. (2007). Diversity of faecal bacterial communities in coeliac and healthy children detected by PCR and denaturing gradient gel electrophoresis. *FEMS Immunol Med Microbiol* (in press).
- Sartor, R. B. (2004). Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology* **126**, 1620–1633.
- Schulzke, J. D., Bentzel, C. J., Schulzke, I., Riecken, E. O. & Fromm, M. (1998). Epithelial tight junction structure in the jejunum of children with acute and treated celiac sprue. *Pediatr Res* **43**, 435–441.
- Setoyama, H., Imaoka, A., Ishikawa, H. & Umesaki, Y. (2003). Prevention of gut inflammation by *Bifidobacterium* in dextran sulfate-treated gnotobiotic mice associated with *Bacteroides* strains isolated from ulcerative colitis patients. *Microbes Infect* **5**, 115–122.
- Sokol, H., Seksik, P., Rigottier-Gois, L., Lay, C., Lepage, P., Podglajen, I., Marteau, P. & Dore, J. (2006). Specificities of the fecal microbiota in inflammatory bowel disease. *Inflamm Bowel Dis* **12**, 106–111.
- Stene, L. C., Honeyman, M. C., Hoffenberg, E. J., Haas, J. E., Sokol, R. J., Emery, L., Taki, I., Norris, J. M., Erlich, H. A. & other authors (2006). Rotavirus infection frequency and risk of celiac disease autoimmunity in early childhood: a longitudinal study. *Am J Gastroenterol* **101**, 2333–2340.
- Suau, A., Rochet, V., Sghir, A., Gramet, G., Brewaeys, S., Sutren, M., Rigottier-Gois, L. & Dore, J. (2001). *Fusobacterium prausnitzii* and related species represent a dominant group within the human fecal flora. *Syst Appl Microbiol* **24**, 139–145.
- Swidsinski, A., Ladhoff, A., Pernthaler, A., Hale, L. P. & Lochs, H. (2002). Mucosal flora in inflammatory bowel disease. *Gastroenterology* **122**, 44–54.
- Tjellstrom, B., Stenhammar, L., Hogberg, L., Falth-Magnusson, K., Magnusson, K. E., Midtvedt, T., Sundqvist, T. & Norin, E. (2005). Gut microflora associated characteristics in children with coeliac disease. *Am J Gastroenterol* **100**, 2784–2788.
- Wallner, G., Amann, R. & Beisker, W. (1993). Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14**, 136–143.
- Wang, M., Ahrne, S., Jeppsson, B. & Molin, G. (2005). Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. *FEMS Microbiol Ecol* **54**, 219–231.